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Investigation of protecting group for sialic acid carboxy moiety toward sialylglycopeptide synthesis by the TFA-labile protection strategy

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ABSTRACT

The sialylation using sialyl donor with 2,6-dimethylbenzyl (DMBn) ester at C-1 carboxy group rapidly proceeded with good α-selectivity to yield sialyl-*N*-acetylgalactosaminylated 9-fluorenylmethoxycarbonyl (Fmoc) serine unit. The unit was used for the solid-phase peptide synthesis (SPPS) of the sialyl glycopeptide. The DMBn group was removed during the final deprotection by the trifluoroacetic acid (TFA) treatment keeping the sialyl bond intact and the desired sialyl glycopeptide was successfully obtained. This protecting group strategy provided easier access to sialyl glycoamino acids for sialyl glycopeptides synthesis.

1. Introduction

A glycoprotein often bears a sialic acid at the reducing terminus of its sugar chain. Sialic acid plays numerous roles in biological processes [1], such as in the control of immune cell activation [2], cell attachment [3], and glycoprotein half-life determination [4]. To study the role of sialic acid in glycoproteins, synthetic efforts have been undertaken to prepare samples of homogeneous sialyl glycopeptide [5]. Sialic acid retains C-1 carboxy group, C-2 quaternary-substituted carbon, C-3 deoxy carbon, and C-7 to C-9 glycerol chain (Fig. 1). This unique structure leads to challenges in chemical sialylation, because 1) the electron-withdrawing carboxy group destabilizes the oxocarbenium ion, which makes the sialyl donor less reactive; 2) the bulky anomeric carbon and glycerol moiety block the approach of a glycosyl acceptor, which prevents an efficient sialylation reaction; and 3) the deoxy C-3, next to the anomeric position, cannot be used for a neighboring group participation. To overcome these problems, many methods have been established [6]. For example, modification of the C-5 acetamido group successfully improves sialylation efficiency and stereoselectivity [6b,d,e,f]. Notably, Ando's and Tanaka's group independently reported the synthesis of oligosialosides containing α-2,8 linkage, one of the most difficult glycosidic linkages, with complete α-selectivity using bicyclic sialyl donors [7]. These developments allow flexible access to a range of sialosides.

However, in the chemical synthesis of glycopeptides containing a sialic acid, acid instability of the sialyl linkage causes a problem. The sialyl linkage is generally unstable under acidic conditions compared with the glycosidic bond of a hexose/hexosamine [8]; it is easily cleaved by hydrolysis due to intramolecular protonation by the carboxy group [9]. This acid instability conflicts with the SPPS, because Fmoc-SPPS necessitates an acidic treatment using trifluoroacetic acid (TFA) for the final deprotection step. To avoid the unwanted cleavage of the sialyl linkage, protection of the carboxy group by ester i.e. methyl ester has been used to make the sialyl bond inert [5a–c]. However, saponification using strong bases are required for the deprotection of these esters, which can cause the β-elimination and racemization of the amino acid residues.

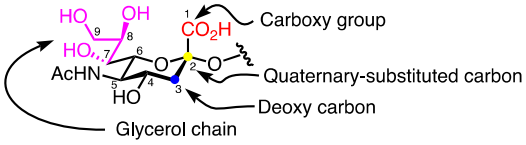

Fig. 1. Structure of sialic acid.

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Additionally, this synthetic route cannot be incorporated in preparation of a peptide thioester that is a key intermediate of the peptide condensation method, i.e., the thioester method [10], and the native chemical ligation method [11], to assemble a long polypeptide chain.

From this background, we developed a novel protection strategy using TFA-labile protecting groups, i.e., 4-methylbenzyl (MBn) group and 4-methoxybenzyl (MPM) group, for the synthesis of glycopeptides (Fig. 2) [12]. In this strategy, sugar hydroxy groups and the carboxy group were protected by TFA-labile protecting groups and the glycoamino acid derivative was then assembled during the carbohydrate synthesis. In the subsequent stage of peptide synthesis, the glycoamino acid derivative was applied in the SPPS based on Fmoc chemistry. At the end of SPPS, the glycopeptidyl resin was treated with a TFA cocktail to remove all peptide and sugar protecting groups. Thus, the fully deprotected glycopeptide was obtained in one step, avoiding the troublesome basic treatment. Surprisingly, in this protecting system, the sialic acid linkage almost withstood the TFA treatment [12b]. However, in this previous study, the yield of the α2,6-sialylation reaction and α-selectivity were moderate. The efficiency of this sialylation reaction should be improved to assemble more complex sialylglycoamino acids, containing not only the α2,6 linkage but also the more challenging α2,3 linkage for the synthesis of glycoproteins. In this study, we report the properties of sialyl donor bearing TFA-labile protecting group in sialylation and improvement of sialylation using this methodology.

2. Results and discussion

Prior to this study, the side reaction of the previously reported sialylation [12b] was re-investigated to understand the reason for the moderate yield (Fig. 3). The glycosylation reaction GalNAc **2** with sialyl donor **1** was performed using a combination of triflic acid (TfOH) and *N*-iodosuccinimide (NIS) as an activator in acetonitrile or propionitrile [13]. Disaccharide **3** was obtained as 49% α-isomer and 8% β-isomer. Additionally, the intermolecular migrated byproduct, 4-methoxybenzylated acceptor **4** (2%), and C-2 amidoglycoside **5** (13%) with free carboxy group were isolated. Therefore, the mechanism of the side reaction was proposed as shown in Fig. 3, wherein the sulfur atom should react with the iodonium ion to yield desired product **3**; however, if the sulfur atom attacks the benzylic position of the neighboring MPM group, the sulfonium ion leaves the anomer position and the nitrile solvent immediately

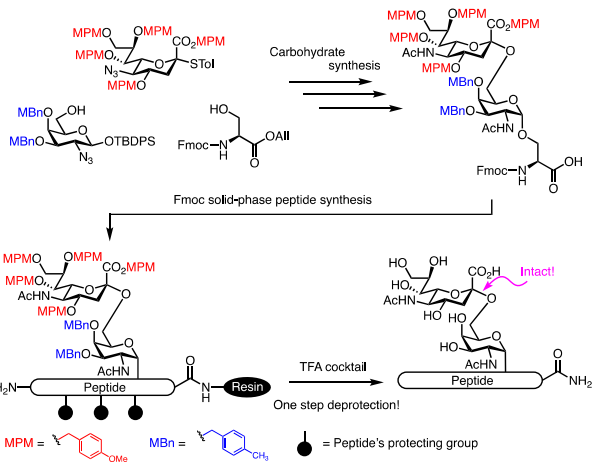


Fig. 2. Previous synthesis of sialyl glycoprotein using TFA-labile protection strategy [12b].

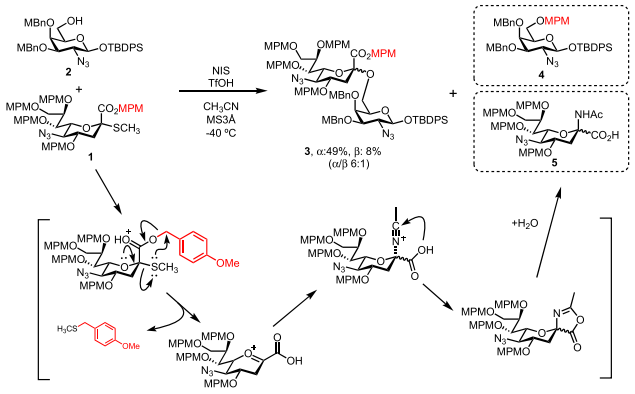
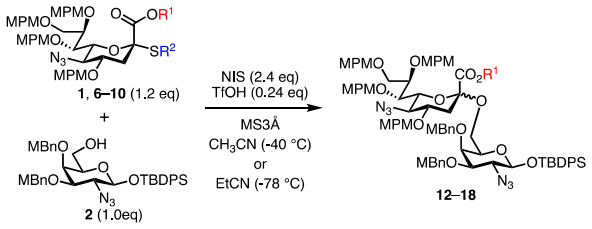


Fig. 3. Reaction mechanisms of the side reaction during previous sialylation.

traps the oxocarbenium cation, generating the amidated byproduct **5** via the formation of an intramolecular asymmetric anhydride followed by hydrolysis. Therefore, we concluded that the C-1 protecting group must be more stable under acidic conditions than MPM group, but still removable by TFA treatment at the final deprotection of the SPPS. Namely, accurate choice of the protecting group considering sialylation and TFA treatment is important. From this hypothesis, an alternative TFA-labile protecting group was examined for the carboxy group of the sialic acid.

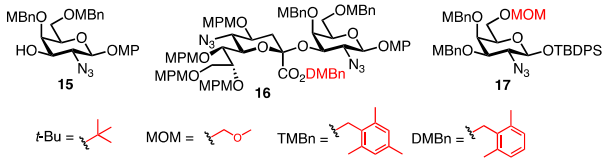
The followings were selected as candidates for the acid-labile protecting group of the carboxy group: *tert*-butyl, methoxymethyl (MOM), 2,4-dimethylbenzyl (DMBn), and 2,4,6-trimethylbenzyl (TMBn). The sialylation reaction using GalNAc acceptor **2** (1.0 eq), having a free 6-OH, with the donors (1.2 eq) was then performed using NIS-TfOH activation system (Table 1). The result obtained following the previous study is shown in entry 1 as a reference [12b]. For the *tert*-butyl group, the donor **6** could not be completely consumed during the reaction and consequently, the disaccharide yield was low (entry 2). The reason for incomplete activation seemed to be the high steric hindrance of the *tert*-butyl group at C-1, which prevented the approach of the iodonium ion. When the MOM group was employed (entry 3), the desired sialylation reaction proceeded partially to give product **12** (α: 38%, β: 5%), but the amidated byproduct **5** was obtained in 25% yield. Moreover, **17** bearing the MOM group on its C-6 alcohol was also obtained in 31% yield [14]. Employing the TMBn group resulted in a yield that was comparable to entry 1 (**13**, α: 40%, β: 8%, entry 4), but the amidation reaction could not be completely suppressed (**5**, 17%), which indicated that the TMBn group is also too acid-sensitive under glycosylation conditions. In the next trial using the DMBn group (entry 5), the product yields were comparable (**14**, α: 42%, β: 8%) to the those of the reference reaction. Notably, in this reaction, the formation of amidated byproduct **5** was reduced to 5% and the migration of the DMBn group to acceptor **2** was not detected. To improve the yield and α-selectivity, the glycosylation reaction was performed in propionitrile under lower temperature (entry 6, -78 °C), which gave a significantly better result (α: 74%, β: 9%) with 17% of unreacted donor **9** [15]. Further improvement for the yield was investigated by using more reactive sialyl donor **10**, in which the aglycone was substituted to isopropyl group from methyl group of **9** [16]. As a result, donor **10** was completely consumed and the desired disaccharide **14** was obtained in yields of 84% for the α-sialoside and 6% for the β-sialoside (entry 7). Therefore, the α-selectivity was also improved to 14:1 and amidation product **5** was reduced to a trace amount. α2,3 Sialylation was also demonstrated using 3-OH free Gal derivative **15** as a glycosyl acceptor (entry 8). As a result, the desired disaccharide **16** was obtained with complete

Table 1
Study of the protecting groups for the sialic acid carboxy moiety..



Entry	Donor	R^1, R^2	Temp. (°C)	Time	Product	Yield α/β (%)
1	**1**	MPM, CH_3	-40	1 h	**3**	49/8
2	**6**	*t*-Bu, CH_3	-40	4.5 h	**11**	16/3
3	**7**	MOM, CH_3	-40	2 h	**12**	38/5
4	**8**	TMBn, CH_3	-40	0.5 h	**13**	40/8
5	**9**	DMBn, CH_3	-40	0.5 h	**14**	42/8
6	**9**	DMBn, CH_3	-78	10 min	**14**	74/8
7	**10**	DMBn, *i*-Pr	-78	10 min	**14**	84/6
8[a]	**10**	DMBn, *i*-Pr	-78	10 min	**16**	94, α only

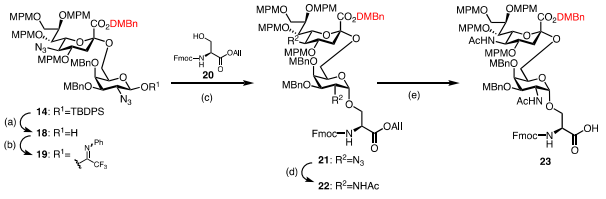
a) Glycosylation reaction using 3-OH free acceptor **19**. Its product is shown as **18**.



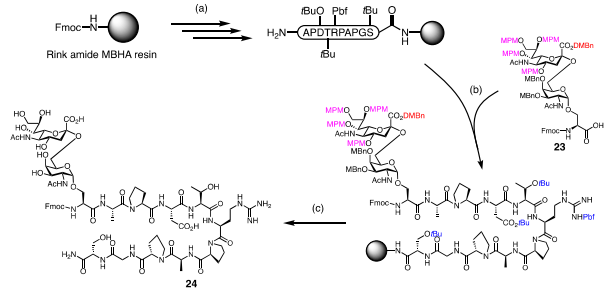
stereoselectivity and excellent yield (94%, α only). Based on these results, the DMBn group was selected as the best protecting group for the sialic acid carboxy group.

In the next stage, removal of the DMBn group in the final deprotection reaction was validated via the Fmoc SPPS and treatment with the TFA cocktail. Prior to the peptide synthesis, the Tn antigen disaccharide **14** containing the α2,6 sialyl linkage was derivatized to Fmoc-glycoamino acid **23** (Scheme 1). Disaccharide **14** was converted to the glycosyl imidate **19** by treating with tetra-*n*-butylammonium fluoride (TBAF), followed by 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride. The glycosylation reaction of the Fmoc-serine allyl ester **20** [17] with imidate **19** was performed using a catalytic amount of Lewis acid [18]. In the first trial, trimethylsilyl trifluoromethanesulfonate (TMSOTf), a general Lewis acid in the imidate method, was employed; however, cleavage of MPM groups on the sialic acid moiety and O-trimethylsilylation of serine derivative was partially observed. Thus, triisopropylsilyl trifluoromethanesulfonate (TIPSOTf), which is more bulky and milder Lewis acid, was used to suppress these side reactions. The diastereomeric mixture of crude disaccharide **21** was separated by silica gel chromatography. The desired α-isomer was obtained in 60% yield of α-isomer with an isomeric ratio of 6:1 α/β, minimizing the problematic side reactions. Subsequently, the azido group was reduced using zinc powder in the presence of acetic acid and the generated amine was reacted with acetic anhydride to obtain diacetamide **22**. After deprotection of the allyl ester using Pd(0) catalyst with phenylsilane as a hydride donor, glycoamino acid **23** was successfully obtained. Throughout this synthetic route, the DMBn group was completely stable.

In the next step, the SPPS using glycoamino acid was carried out according to Scheme 2. The model undecapeptide, the same sequence in the previous study [12b], was a part of the tandem repeat domain of mucin-1 (MUC1) SAPDTRPAPGS (underline shows the glycosylated serine). The peptide chain was elongated from a Rink amide resin by the Fmoc method using a microwave peptide synthesizer. At the introduction of the N-terminal serine, glycosyl serine **23** was manually loaded into the peptidyl resin using *N*,*N*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt)



Scheme 1. Synthetic route of sialylglycoamino acid 23. Reaction conditions: (a) TBAF, AcOH, THF, 0 °C to r.t., 24 h, 94%; (b) 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride, Et_3N, DMAP, CH_2Cl_2, 0 °C to r.t., 30 min, 95%; (c) TIPSOTf, MS4Å, CH_2Cl_2, -20 °C, 30 min, α-isomer 60%, β-isomer 10%; (d) (1) Zn, AcOH, THF, 0 °C to r.t., 30 min; (2) Ac_2O, CH_2Cl_2, r.t., 30 min, 83% (in two steps); (e) $Pd(PPh_3)_4$, $PhSiH_3$, THF, r.t., 10 min, 95%.



Scheme 2. Synthetic scheme of glycopeptide 24 using sialylglycoamino acid 23. Reaction condition: (a) Fmoc SPPS; (b) 23, DIC, HOBt, DMF, 50 °C, 1 h; (c) TFA/*i*-Pr_3SiH/H_2O/DODT 92.5:2.5:2.5:2.5, r.t., 2 h.

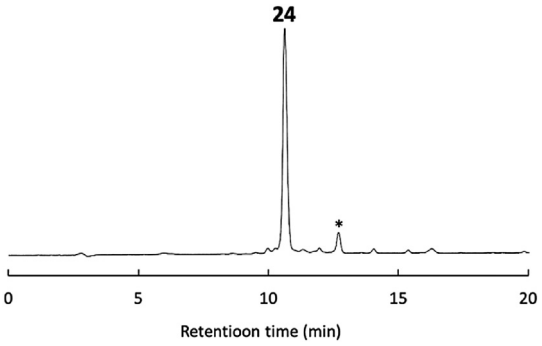


Fig. 4. HPLC profile of deprotection reaction for glycopeptidyl resin. Asterisk is desialyl glycopeptide. A linear gradient starting from 20% CH_3CN at 0 min to 40% at 20 min was applied.

at 50 °C. The reaction was monitored by the ninhydrin test and completed within 1 h. The peptidyl resin was then treated by a TFA cocktail containing 2.5% water, 2.5% triisopropylsilane, and 2.5% 3,6-dioxa-1,8-octanedithiol (DODT) [19] for 2 h at room temperature to remove all protecting groups from the peptide and the carbohydrate moiety. The deprotection profile of this peptide was analyzed by reverse-phase (RP) HPLC followed by mass spectrometry. The chromatogram is shown in Fig. 4. The fully deprotected glycopeptide **24** was observed as the highest peak. Additionally, a product with the DMBn group was not detected. Moreover, hydrolysis of the α2,6 sialyl linkage during the TFA treatment was minimized, which was comparable with previous reactions employing the MPM protecting group. These results proved that the DMBn group is a convenient, compatible and stable protecting group for not only the carbohydrate synthesis but also the TFA treatment in the final deprotection step of glycopeptide synthesis.

3. Conclusion

In conclusion, we developed a novel TFA-labile protecting group, DMBn group, for the sialic acid carboxy moiety in the synthesis of glycopeptides. During carbohydrate synthesis, the DMBn group almost suppressed the problematic side reactions, such as the intermolecular migration reaction of the protecting group to the glycosyl acceptor and the amidation reaction of the sialyl donor. Additionally, the DMBn protection of the carboxy moiety significantly improved the yield of the chemical sialylation reactions (α2,6: 84%, α/β 14:1 and α2,3: 94%, α only) compared with that achieved in a previous study using the MPM ester. In the peptide synthesis, the DMBn group behaved as a stable protecting group for the carboxylic acid. After the final deprotection using the TFA cocktail, the DMBn group was removed completely, minimizing the undesired cleavage of the sialic acid. These accomplishments significantly increased the efficiency and the productivity of sialylglycopeptide synthesis. Currently, the synthesis of more complex sialyl sugar chains is ongoing in our laboratory.

4. Experimental section

4.1. General

Analytical thin-layer chromatography (TLC) was performed on glass plates using TLC Silica gel 60 F_{254}. Flash column chromatography was performed on silica gel (40–50 μm). CH_2Cl_2 and DMF were dried with molecular sieves (MS) 3Å or 4Å before use. EtCN was used for the glycosylation after two step purifications described as follows: (1) reflux with diphosphorus pentoxide for 6 h followed by distillation; (2) reflux with CaH_2 for 6 h followed by distillation. THF was used for the reactions after distilling from sodium dispersion and benzophenone [20]. Unless otherwise noted, materials purchased from distributors were used without any purification. The specific rotation values were determined at 20–25 °C using a SEPA-300 polarimeter (Horiba Ltd., Kyoto). The NMR spectra were recorded using a AV400 spectrometer at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C{^{1}H} NMR) or AV500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C{^{1}H} NMR) (Bruker corporation, MA). The chemical shifts are expressed in ppm downfield from the signal for the internal trimethylsilane for the solution in the deuterated solvents. Multiplicities are given as "s" (singlet), "d" (doublet), "t" (triplet), and "m" (multiplet). Broad peaks in NMR spectra are indicated as "br". The SPPS was performed using a Liberty Blue peptide synthesizer (CEM corporation, NC). RP-HPLC was carried out on RP-18 GP II (4.6 × 150 mm) (Kanto Chemical Co., Inc., Tokyo) using a linear increasing gradient of MeCN in 0.1% TFA/H_2O. Detection was by an absorbance measurement at 220 nm. ESI-HRMS spectrum was measured using an ESI-LTQ- Orbitrap XL (Thermo Fisher Scientific, Inc., MA). Experimental procedures for the preparation of monosaccharide derivatives were shown in the supplementary data.

4.2. *tert*-Butyldiphenylsilyl {2,6-dimethylbenzyl [5-azido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate}-α-(2 → 6)-2-azido-2-deoxy-3,4-di-O-(4-methylbenzyl)-β-D-galactopyranoside (**14**)

A stirred mixture of sialyl donor **10** (0.11 g, 0.12 mmol), *tert*-butyldiphenylsilyl 2-azido-2-deoxy-3,4-di-*O*-(4-methylbenzyl)-β-D-galactopyranoside (65 mg, 0.10 mmol) [12b], NIS (54 mg, 0.24 mmol) and dried MS3Å (0.36 g) in EtCN (3.6 mL) was cooled to −78 °C under an Ar atmosphere and then stirred for 30 min. After 1% TfOH/EtCN (0.21 mL, 24 μmol) was slowly added, the mixture was stirred for 10 min at the same temperature. Sat. $NaHCO_3$ aq. and 10% Na_2SO_3 aq. were added to quench the reaction. The mixture was diluted with EtOAc and then filtrated through Celite pad. The organic layer was successively washed with H_2O and brine, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The residue was purified by gel filtration chromatography (Biobeads SX-3 toluene/EtOAc 3:1) followed by silica gel column chromatography (toluene/EtOAc 19:1) to afford disaccharide **14** (0.13 g, 84%) and β-isomer (9.7 mg, 6%). $[\alpha]_D$ −26.6 (c 1.5, $CHCl_3$). Anal. Calcd for $C_{88}H_{100}N_6O_{16}Si$: C, 69.27; H, 6.61; N, 5.51, found: C, 69.00; H, 6.42; N, 5.62. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.71–7.67 (m, 4H, Ar*H*), 7.31–7.28 (m, 5H, Ar*H*), 7.25–7.22 (m, 5H, Ar*H*), 7.17–7.07 (m, 11H, Ar*H*), 7.03 (d, 2H, *J* = 7.9 Hz, Ar*H*), 6.97 (d, 2H, *J* = 7.6 Hz, Ar*H*), 6.87–6.82 (m, 6H, Ar*H*), 6.75 (d, 2H, *J* = 8.6 Hz, Ar*H*), 5.25 (d, 1H, *J* = 12.0 Hz, ArC*H*$_2$-), 5.08 (d, 1H, *J* = 12.0 Hz, ArC*H*$_2$-), 4.68 (d, 1H, *J* = 11.2 Hz, ArC*H*$_2$-), 4.58 (d, 1H, *J* = 10.9 Hz, ArC*H*$_2$-), 4.47–4.23 (m, 11H, H-1a, ArC*H*$_2$-), 3.85–3.75 (m, 11H, H-7b, H-8b, -OC*H*$_3$), 3.72–3.61 (m, 8H, H-2a, H-4a, H-6a, H-6b, H-9b, -OC*H*$_3$), 3.54–3.43 (m, 3H, H-6a, H-5b, H-9b), 3.38–3.32 (m, 1H, H-4b), 3.20 (brt, 1H, *J* = 6.7 Hz, H-5a), 3.00 (dd, 1H, *J* = 2.8 Hz, 10.4 Hz, H-3a), 2.55 (dd, 1H, *J* = 4.5 Hz, 12.9 Hz, H-3b$_{eq}$), 2.33 (s, 3H, ArC*H*$_3$), 2.28 (s, 9H, ArC*H*$_3$), 1.55 (brt, 1H, *J* = 9.3 Hz, H-3b$_{ax}$), 1.04 (s, 9H, C(C*H*$_3$)$_3$). ^{13}C{^{1}H} NMR ($CDCl_3$, 125 MHz): δ 167.4 (C-1b), 96.8 (C-1a), 37.4 (C-3b), ($^3J_{C1b\text{-}H3bax}$ = 7.0 Hz).

4.3. 4-Methoxyphenyl {2,6-dimethylbenzyl [5-azido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate}-α-(2 → 3)-2,4,6-tri-O-(4-methylbenzyl)-β-D-galactopyranoside (**16**)

A stirred mixture of sialyl donor **10** (0.14 g, 0.15 mmol), glycosyl

acceptor **15** (60 mg, 0.10 mmol), NIS (67 mg, 0.30 mmol) and dried MS3Å (0.45 g) in EtCN (4.5 mL) was cooled to −78 °C under an Ar atmosphere and then stirred for 30 min. After 1% TfOH/EtCN (0.54 mL, 60 μmol) was slowly added, the mixture was stirred for 30 min at the same temperature. Sat. $NaHCO_3$ aq. and 10% Na_2SO_3 aq. were added to quench the reaction. The mixture was diluted with EtOAc and then filtrated through Celite pad. The organic layer was successively washed with H_2O and brine, dried over Na_2SO_4, filetaraed, and concentrated under reduced pressure. The residue was purified by gel filtration chromatography (Biobeads SX-3 toluene/EtOAc 3:1) followed by silica gel column chromatography (hexane/EtOAc 3:1) to afford disaccharide **16** (0.14 g, 94%) α only. $[\alpha]_D$ −22.6 (c 1.0, $CHCl_3$). Anal. Calcd for $C_{87}H_{97}N_3O_{18}$: C, 70.95; H, 6.64; N, 2.85, found: C, 70.98; H, 6.82; N, 2.83. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.27 (d, 2H, J = 8.6 Hz, Ar*H*), 7.18−7.01 (m, 19H, Ar*H*), 6.95 (d, 2H, J = 9.1 Hz, Ar*H*), 6.91 (d, 2H, J = 7.8 Hz, Ar*H*), 6.81−6.76 (m, 10H, Ar*H*), 5.34 (d, 1H, J = 12.0 Hz, ArCH_2-), 5.30 (d, 1H, J = 12.0 Hz, ArCH_2-), 4.95 (d, 1H, J = 11.2 Hz, ArCH_2-), 4.81 (d, 1H, J = 10.4 Hz, ArCH_2-), 4.64 (d, 1H, J = 7.6 Hz, H-1a), 4.58 (d, 2H, J = 10.8 Hz, ArCH_2-), 4.52−4.33 (m, 7H, ArCH_2-), 4.28 (d, 1H, J = 11.6 Hz, ArCH_2-), 4.05 (d, 1H, J = 10.6 Hz, ArCH_2-), 4.02 (d, 1H, J = 10.6 Hz, ArCH_2-), 3.94−3.72 (m, 18H, H-2a, H-3a, H-4a, H-6b, H-7b, H-8b, -OCH_3), 3.65 (s, 3H, -OCH_3), 3.57 (brdd, 1H, J = 1.9 Hz, 10.7 Hz, H-9b), 3.50−3.35 (m, 5H, H-6a, H-6a′, H-4b, H-5b, H-9b'), 3.27 (t, 1H, J = 6.3 Hz, H-5a), 2.50 (dd, 1H, J = 3.9 Hz, 13.5 Hz, H-3b$_{eq}$), 2.37 (s, 6H, ArCH_3), 2.32 (s, 3H, ArCH_3), 2.30 (s, 3H, ArCH_3), 2.22 (s, 3H, ArCH_3), 1.84 (dd, 1H, J = 11.8 Hz, 13.2 Hz, H-3b$_{ax}$). ^{13}C{^{1}H} NMR ($CDCl_3$, 100 MHz): δ 168.2 (C-1b), 102.8 (C-1a), 34.4 (C-3b), ($^3J_{C1b\text{-}H3bax}$ = 7.1 Hz).

4.4. 2,6-Dimethylbenzyl [5-azido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate-α-(2 → 6)-2-azido-2-deoxy- 3,4-di-O-(4-methylbenzyl)-D-galactopyranoside (**18**)

Disaccharide **14** (1.4 g, 0.94 mmol) in THF (5.0 mL) containing AcOH (0.54 mL, 9.4 mmol) was stirred at 0 °C. After 1 M *n*-tetrabutylammonium fluoride (5.6 mL) was slowly added, the mixture was stirred for 19 h at room temperature. After concentration, the residue was purified by silica gel column chromatography (toluene/EtOAc 7:1) to afford product **18** (1.1 g, 94%). Anal. Calcd for $C_{72}H_{82}N_6O_{16}$: C, 67.17; H, 6.42; N, 6.53, found: C, 67.18; H, 6.62; N, 6.71. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.30−7.09 (m, 15H, Ar*H*), 7.03−6.98 (m, 4H, Ar*H*), 6.87−6.79 (m, 8H, Ar*H*), 5,28−5.18 (m, 2H, ArCH_2-), 5.08 (d, 0.67H, J = 3.4 Hz, H-1a-α isomer), 4.77−4.72 (m, 1H, ArCH_2-), 4.62−4.36 (m, 10H, ArCH_2-), 4.31 (d, 1H, J = 10.9 Hz, ArCH_2-), 4.18 (d, 0.33H, J = 8.0 Hz, H-1a-β isomer), 4.11 (dd, 0.67H, J = 5.0 Hz, 7.7 Hz, H-5a-α isomer), 3.88−3.71 (m, 19.34H, H-2a-α isomer, H-3a-α isomer, H-4a-α isomer, H-4a-β isomer, H-6a-α,β complex, H-6b, H-7b, H-8b, H-9b-α,β complex, -OCH_3), 3.65−3.56 (m, 2.33H, H-2a-β isomer, H-6a′-α,β complex, H-9b′-α,β complex), 3.49 (t, 1H, J = 9.6 Hz, H-5b), 3.45−3.36 (m, 1.33H, H-5a-β isomer, H-4b), 3.11 (dd, 0.33H, J = 2.7 Hz, 10.3 Hz, H-3a-β isomer), 2.66−2.60 (m, 1H, H-3b$_{eq}$), 2.34−2.33 (m, 9H, ArCH_3), 2.28 (s, 3H, ArCH_3), 1.64−1.56 (m, 1H, H-3b$_{ax}$). ^{13}C{^{1}H} NMR ($CDCl_3$, 100 MHz): δ 96.2 (C-1a, β isomer), 92.4 (C-1a, α isomer), 37.6 (C-3b).

4.5. 2,2,2-Trifluoro-N-phenylacetimidoyl {2,6-dimethylbenzyl [5-azido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate}-α-(2 → 6)-2-azido-2-deoxy-3,4-di-O-(4-methylbenzyl)-D-galactopyranoside (**19**)

Product **18** (0.84 g, 0.65 mmol) in CH_2Cl_2 (7.0 mL) was stirred at 0 °C under an Ar atmosphere. After 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (0.15 mL, 0.98 mmol), triethylamine (0.18 mL, 1.3 mmol) and DMAP (8.0 mg, 65 μmol) were successively added, the mixture was stirred for 30 min at room temperature. H_2O was added to quench the reaction. The mixture was then diluted with EtOAc. The organic layer was successively washed with H_2O and brine, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1) to afford product **19** (0.90 g, 95%). Anal. Calcd for $C_{80}H_{86}F_3N_7O_{16}$: C, 65.88; H, 5.94; N, 6.72, found: C, 65.75; H, 6.09; N, 6.68. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.24−7.09 (m, 17H, Ar*H*), 7.05−6.96 (m, 5H, Ar*H*), 6.86−6.74 (m, 10H, Ar*H*), 5.36 (brs, 1H, H-1a) 5.23 (d, 1H, J = 12.0 Hz, ArCH_2-), 5.14 (d, 1H, J = 12.0 Hz, ArCH_2-), 4.70 (d, 1H, J = 11.0 Hz, ArCH_2-), 4.60 (d, 1H, J = 10.7 Hz, ArCH_2-), 4.48−4.22 (m, 10H, ArCH_2-), 3.98−3.93 (m, 1H, H-2a), 3.88−3.84 (m, 2H, H-6a, H-7b), 3.80−3.33 (m, 22H, H-4a, H-5a, H-6a′, H-4b, H-5b, H-6b, H-8b, H-9b, H-9b', -OCH_3), 3.14 (brd, 1H, J = 9.4 Hz, H-3a), 2.69 (dd, 0.15H, J = 4.3 Hz, 12.6 Hz, H-3b$_{eq}$), 2.61 (dd, 0.85H, J = 4.4 Hz, 13.1 Hz, H-3b$_{eq}$), 2.33 (s, 3H, ArCH_3), 2.29 (s, 6H, ArCH_3), 2.27 (s, 3H, ArCH_3), 1.66 (brt, 0.15H, J = 12.1 Hz, H-3b$_{ax}$), 1.55 (brt, 0.85H, J = 11.4 Hz, H-3b$_{ax}$ isomerA). ^{13}C{^{1}H} NMR ($CDCl_3$, 100 MHz): 37.6 (C-3b, isomerA), 37.2 (C-3b, isomerB).

4.6. N-(9-Fluorenylmethoxycarbonyl)-O-{2,6-dimethylbenzyl [5-azido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate-α-(2 → 6)-2-azido-2-deoxy-3,4-di-O-(4-methylbenzyl)-α-D-galactopyranosyl}-L-serine allyl ester (**21**)

A stirred mixture of glycosyl donor **19** (0.38 g, 0.26 mmol), *N*-(9-fluorenylmethoxycarbonyl)-L-serine allyl ester (0.11 g, 0.31 mmol) [16], and dried MS4Å (0.78 g) in CH_2Cl_2 (7.8 mL) was cooled to −20 °C under an Ar atmosphere and then stirred for 30 min. After 1% TIPSOTf/CH_2Cl_2 (0.35 mL, 13 μmol) was slowly added, the reaction mixture was stirred for 30 min at the same temperature. Sat. $NaHCO_3$ aq. was added to quench the reaction. The mixture was diluted with EtOAc and then filtrated through Celite pad. The organic layer was successively washed with H_2O and brine, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The residue was purified by gel filtration chromatography (Biobeads SX-3 toluene/EtOAc 3:1) followed by silica gel column chromatography (toluene/EtOAc 19:1) to afford product **21** (0.25 g, 60%) and β-isomer (44 mg, 10%). $[\alpha]_D$ +20.2 (c 1.0, $CHCl_3$). Anal. Calcd for $C_{93}H_{101}N_7O_{20}$: C, 68.24; H, 6.22; N, 5.99. found: C, 68.24; H, 6.08; N, 6.01. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.73 (d, 2H, J = 7.4 Hz, Ar*H*), 7.58 (dd, 2H, J = 2.9 Hz, 7.1 Hz, Ar*H*), 7.38−7.34 (m, 2H, Ar*H*), 7.29−7.21 (m, 8H, Ar*H*), 7.16−7.09 (m, 8H, Ar*H*), 7.07 (d, 1H, J = 7.2 Hz, Ar*H*), 7.01 (d, 2H, J = 7.8 Hz, Ar*H*), 6.96 (d, 2H, J = 7.5 Hz, Ar*H*), 6.85−6.81 (m, 6H, Ar*H*), 6.77 (d, 2H, J = 8.5 Hz, Ar*H*), 5.93−5.83 (m, 1H, C*H*═CH$_2$), 5.79 (d, 1H, J = 8.5 Hz, FmocN*H*), 5.33−5.20 (m, 3H, CH═CH_2, ArCH_2-), 5.12 (d, 1H, J = 12.0 Hz, ArCH_2-), 4.79 (d, 1H, J = 3.2 Hz, H-1a), 4.72 (d, 1H, J = 10.8 Hz, ArCH_2-), 4.63−4.55 (m, 5H, ArCH_2-), 4.52−4.40 (m, 8H, Ser-αH, CH_2−CH═

CH_2, Ar_2CHCH_2-, $ArCH_2$-), 4.31 (d, 1H, J = 10.8 Hz, $ArCH_2$-), 4.28–4.18 (m, 3H, Ar_2CH-, $ArCH_2$-), 3.89–3.73 (m, 19H, Ser-βH, H-2a, H-3a, H-4a, H-5a, H-6a, H-7b, H-8b, H-9b, $-OCH_3$), 3.70–3.67 (m, 4H, H-6b, -OCH_3), 3.61 (dd, 1H, J = 4.7 Hz, 11.0 Hz, H-9b′), 3.54–3.46 (m, 2H, H-6a', H-5b), 3.39–3.32 (m, 1H, H-4b), 2.66 (dd, 1H, J = 4.2 Hz, 12.9 Hz, H-$3b_{eq}$), 2.33 (s, 3H, $ArCH_3$), 2.29 (s, 6H, $ArCH_3$), 2.26 (s, 3H, $ArCH_3$), 1.69–1.63 (m, 1H, H-$3b_{ax}$). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 100 MHz): δ 167.5 (C-1b) 99.4 (C-1a), 37.1 (C-3b).

4.7. N-(9-Fluorenylmethoxycarbonyl)-O-{2,6-dimethylbenzyl [5-acetamido-3,5-dideoxy-4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate-α-(2 → 6)-2-acetamido-2-deoxy 3,4-di-O-(4-methylbenzyl)-α-D-galactopyranosyl}-L-serine allyl ester (**22**)

Product **21** (0.20 g, 0.12 mmol) in THF (2.0 mL) containing AcOH (0.80 mL, 14 mmol) was stirred at room temperature. After powdered Zn (0.47 g, 7.2 mmol) was added, the mixture was stirred for 30 min at room temperature. Sat. $NaHCO_3$ aq. was added to quench the reaction. The mixture was diluted with EtOAc and then filtrated through Celite pad. The organic layer was successively washed with H_2O and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (2.0 mL) containing Ac_2O (0.23 mL, 2.4 mmol) and stirred for 30 min at room temperature. After the mixture was concentrated under reduced pressure, the residue was purified by silica gel column chromatography ($CHCl_3$:MeCN 9:1 containing 1% AcOH) to afford product **22** (0.17 g, 83%). $[\alpha]_D$ +38.3 (c 1.8, $CHCl_3$). ESI-HRMS: m/z 1690.7228, calcd for $[C_{97}H_{109}N_3O_{22}+Na]^+$ 1690.7395. ^1H NMR (CD_3OD, 500 MHz): δ 7.74 (d, 2H, J = 6.9 Hz, ArH), 7.61–7.57 (m, 2H, ArH), 7.34–6.96 (m, 23H, ArH), 6.86–6.73 (m, 8H, ArH), 5.85–5.79 (m, 1H, CH=CH_2), 5.33 (d, 1H, J = 12 Hz, $ArCH_2$-), 5.26–5.09 (m, 3H, CH=CH_2, $ArCH_2$-), 4.65–4.32 (m, 18H, Ser-αH, H-1a, H-2a, CH_2–CH=CH_2, Ar_2CHCH_2-), 4.20–4.10 (m, 4H, H-5b, H-8b, Ar_2CH-, $ArCH_2$-), 3.81–3.54 (m, 22H, Ser-βH, H-3a, H-4a, H-5a, H-6a, H-6b, H-7b, H-9b, H-9b′, -OCH_3), 3.49–3.44 (m, 1H, H-6a'), 3.31 (br, 1H, H-4b), 2.71 (dd, 1H, J = 4.3 Hz, 13 Hz, H-$3b_{eq}$), 2.34 (s, 6H, $ArCH_3$), 2.29 (s, 3H, $ArCH_3$), 2.21 (s, 3H, $ArCH_3$), 1.88 (s, 6H, Ac), 1.69 (t, 1H, J = 12 Hz, H-$3b_{ax}$). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 100 MHz): δ 100.5 (C-1a), 38.7 (C-3b).

4.8. N-(9-Fluorenylmethoxycarbonyl)-O-{2,6-dimethylbenzyl [5-acetamido-3,5-dideoxy- 4,7,8,9-tetra-O-(4-methoxybenzyl)-D-glycero-α-D-galacto-2-nonulopyranosyl]onate-α-(2 → 6)-2-acetamido-2-deoxy-3,4-di-O-(4-methylbenzyl)-α-D-galactopyranosyl}-L-serine (**23**)

A mixture of **22** (0.43 g, 0.26 mmol) and $PhSiH_3$ (38 μL, 0.31 mmol) in THF (3.0 mL) was stirred at room temperature under an Ar atmosphere. After $(PPh_3)_4Pd$ (15 mg, 13 μmol) was added, the mixture was stirred for 10 min and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$:MeOH 9:1 containing 1% AcOH) to afford product **23** (0.40 g, 95%). $[\alpha]_D$ +58.4 (c 1.0, $CHCl_3$). Anal. Calcd for $C_{94}H_{105}N_3O_{22}$: C, 69.31; H, 6.50; N, 2.58. found: C, 69.31; H, 6.64; N, 2.57. ^1H NMR (CD_3OD, 400 MHz): δ 7.76 (d, 2H, J = 7.6 Hz, ArH), 7.61 (t, 2H, J = 7.5 Hz, ArH), 7.37–7.32 (m, 4H, ArH), 7.27–7.19 (m, 6H, ArH), 7.12–7.06 (m, 9H, ArH), 6.99–6.96 (m, 4H, ArH), 6.86–6.81 (m, 6H, ArH), 6.74 (d, 2H, J = 8.3 Hz, ArH), 5.34 (d, 1H, J = 12.1 Hz, $ArCH_2$-), 5.24 (d, 1H, J = 12.1 Hz, $ArCH_2$-), 4.70 (d, 1H, J = 3.4 Hz, H-1a), 4.61 (d, 1H, J = 11.0 Hz, $ArCH_2$-), 4.58 (d, 1H, J = 10.0 Hz, $ArCH_2$-), 4.53 (d, 1H, J = 10.0 Hz, $ArCH_2$-), 4.48–4.28 (m, 12H, Ser-αH, H-2a, Ar_2CHCH_2-, $ArCH_2$-), 4.20 (d, 1H, J = 11.6 Hz, $ArCH_2$-), 4.16–4.09 (m, 3H, H-5b, H-6b, Ar_2CH-), 3.81–3.70 (m, 16H, Ser-βH, H-4a, H-6a, H-7b, H-8b, H-9b, -OCH_3), 3.62–3.57 (m, 6H, H-3a, H-5a, H-9b′, -OCH_3), 3.43 (dd, 1H, J = 8.7 Hz, 12.6 Hz, H-6a'), 3.33–3.27 (m, 1H, H-4b), 2,61 (dd, 1H, J = 3.7 Hz, 12.5 Hz, H-$3b_{eq}$), 2.36 (s, 3H, $ArCH_3$), 2.30 (s, 3H, $ArCH_3$), 2.22 (s, 3H, $ArCH_3$), 1.89 (s, 6H, $ArCH_3$), 1.69 (brt, 1H, J = 12.5 Hz, H-$3b_{ax}$). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 100 MHz): δ 98.7 (C-1a), 37.3 (C-3b).

4.9. Synthesis of the glycopeptide carrying Tn antigen (**24**)

Rink amide MBHA resin (60 mg, 20 μmol) was subjected to peptide elongation using automate peptide synthesizer (Liberty Blue, CEM) to give the resin, H-Ala-Pro-Asp(*t*Bu)-Thr(*t*Bu)-Arg(Pbf)-Pro-Ala-Pro-Gly-Ser(*t*Bu)-NH-resin. After compound **23** (65 mg, 40 μmol), 1 M HOBt/DMF (60 μL), 0.50 M DIC/DMF (0.12 mL) was successively added to the resin at room temperature, the mixture was vortexed for 1 h at 50 °C. After acetyl capping by treating with 10% Ac_2O and 5% DIEA in DMF for 3 min at room temperature, the resin was successively washed with DMF (x3), CH_2Cl_2 (x3), ether (x3), and dried under reduced pressure to give the resin, Fmoc-Ser(Neu5Ac-GalNAc)-Ala-Pro-Asp(*t*Bu)-Thr(*t*Bu)-Arg(Pbf)-Pro-Ala-Pro-Gly-Ser(*t*Bu)-NH-resin. A part of the resin (5.0 mg) was treated with TFA cocktail (TFA/TIS/H_2O/DODT 92.5:2.5:2.5:2.5) for 2 h at room temperature. The mixture was concentrated by N_2 stream and then precipitated with cold ether. The precipitate was washed with ether (x3) and then dried. After the crude peptide was dissolved in CH_3CN aq. and then filtered, the solution was analyzed by RPHPLC. The profile of the crude was shown in Fig. 4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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