

CHEMOENZYMATIC SYNTHESIS OF NeuAc α -(2 \rightarrow 3)-Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc α 1- O-(Z)-Serine (N-PROTECTED MUC II OLIGOSACCHARIDE-SERINE)

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CHEMOENZYMATIC SYNTHESIS OF NeuAc α -(2 \rightarrow 3)-Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]- GalNAc α 1-*O*-(Z)-Serine (N-PROTECTED MUC II OLIGOSACCHARIDE–SERINE)

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ABSTRACT

An efficient synthesis of NeuAc α -(2 \rightarrow 3)-Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc α 1-*O*-(Z)-Serine (N-protected MUC II oligosaccharide–serine, **14**) by a chemoenzymatic strategy is described. The enzymatic reaction of GalNAc α 1-*O*-(Z)-Ser-OAll **7** with *p*NP- β -Gal in the presence of recombinant β 1,3-galactosidase from *Bacillus circulans* gave Gal β -(1 \rightarrow 3)-GalNAc α 1-*O*-(Z)-Ser-OAll **3** in 68%. The introduction of two sialic acids into **3** was accomplished by a stepwise method. The branched Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc α 1-*O*-(Z)-Ser-OAll **11** was constructed by a chemical method. Sialylation at the C-3 position of the terminal Gal residue on Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc α 1-*O*-(Z)-Serine **2** using α 2,3-(*O*)-sialyltransferase from rat liver gave a target compound **14** in a practical yield.

Keywords: Mucin II oligosaccharide; Glycopeptide; Chemoenzymatic synthesis

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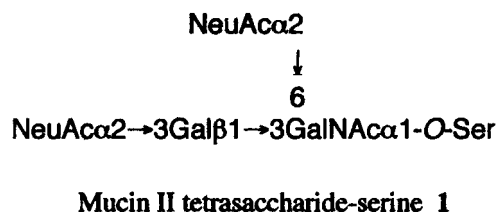


Figure 1.

INTRODUCTION

Natural type oligosaccharides are necessary for understanding various biological phenomena originated by sugar chains in glycoconjugates. To use oligosaccharides as tools for biological research, many chemical synthetic reactions of carbohydrates have been reported,^[1a,1b] but large-scale synthesis of oligosaccharides for glycoconjugates requires many steps due to the protection of hydroxyl groups for unambiguous glycosylations resulting in low yields. On the other hand, enzymatic synthesis using glycosyltransferases^[2a–2j] or glycosidases,^[3a–3c] which catalyzes glycosylation without protection of hydroxyl groups, can be an efficient method. For example, sialyl-T antigen was synthesized using a one-pot multi-enzyme system.^[4a–4c] Recently, various glycosidases have become readily available, and various oligosaccharide units have been synthesized by two types of reactions, reverse hydrolysis and transglycosylation.^[5a–5h] We have discovered a useful enzyme, a recombinant β -galactosidase, from *Bacillus circulans*^[6,7] for the synthesis of the Gal β -(1 \rightarrow 3)-GalNAc linkage, which is a partial structure of the mucin-type glycopeptide. Additionally, we have demonstrated the synthesis of the Gal β -(1 \rightarrow 3)-GalNAc α 1-O-Ser derivative in excellent yield.^[8] The appropriately protected Gal β -(1 \rightarrow 3)-GalNAc α 1-O-Ser derivative was expected to be a key compound for the synthesis of various mucin type oligosaccharides.

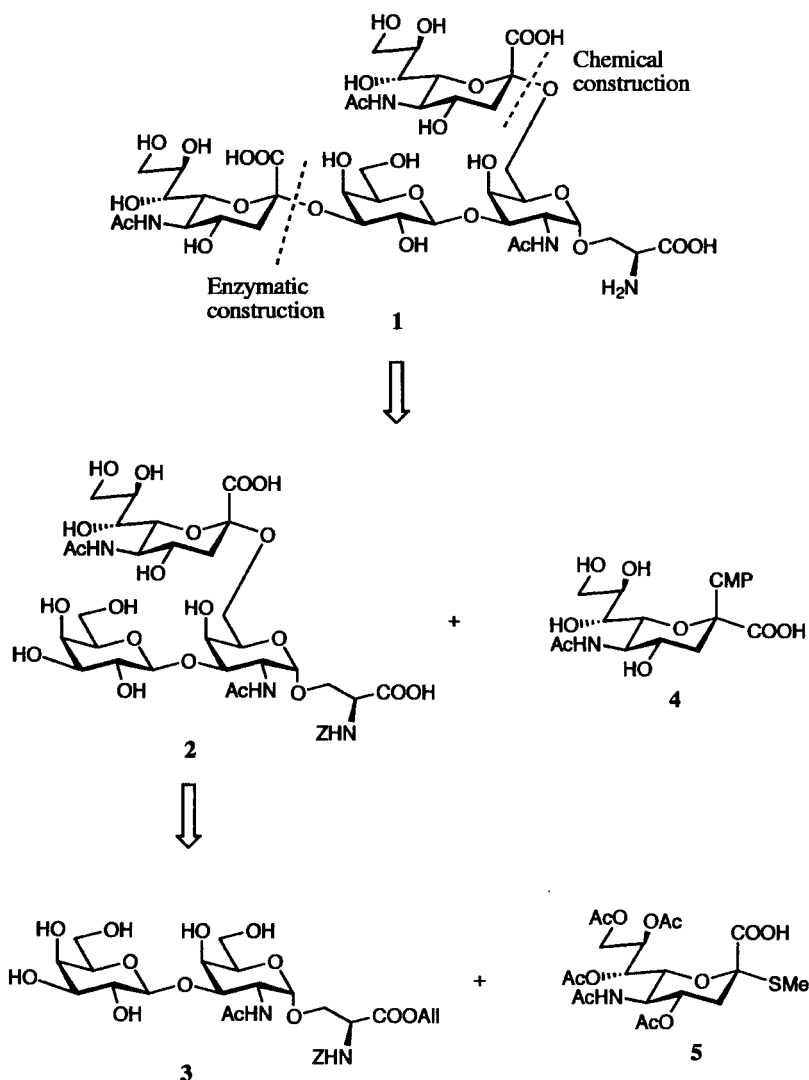
MUC II **1** is disialylated tetrasaccharide-L-serine isolated from the urine of a patient suffering from mucopolipidosis I.^[9a,9b] The MUC II oligosaccharide, linking two sialic acids on Gal β -(1 \rightarrow 3)-GalNAc α 1-O-Serine, has already been synthesized by a pure chemical approach.^[10] In the present report, we would like to show an efficient synthesis of MUC II as *N*-protected form by the combination of chemical and enzymatic methods (Figure 1).

RESULTS AND DISCUSSION

Our synthetic plan for obtaining **1** is based on a stepwise introduction of two sialic acids into Gal β -(1 \rightarrow 3)-GalNAc α 1-O-Ser derivative (Scheme 1). The route in Scheme 1, including individual glycosylation at the C-3 position of Gal and the C-6 position of GalNAc, seems to be widely applicable for the synthesis of various mucin type oligosaccharides which have Gal β -(1 \rightarrow 3)-GalNAc α 1-O-Ser/Thr at the reducing end as a core structure. Sialylation at the terminal Gal residue on trisaccharide **2** might be carried out using α 2,3-(O)-sialyltransferase.^[11] However, the enzymatic construction

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Scheme 1.

of a branched structure at the GalNAc residue of Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc]-Ser/Thr derivative is still challenging even at present. In this study, we undertook construction of a branched NeuAc α -(2 \rightarrow 6)-GalNAc structure by a chemical method.

We first began synthesis of a Gal β -(1 \rightarrow 3)-GalNAc α 1-*O*-Ser derivative, which was essential for the total synthesis of mucin-type oligosaccharides of various structures. GalNAc α 1-*O*-Ser **6** was synthesized by a reverse hydrolysis reaction using GalNAc and serine in the presence of *N*-acetyl- α -D-galactosaminidase from *Aspergillus niger* [EC 3.2.1.49].^[12a,12b] The transglycosylation of the Gal residue of *p*NP- β -D-galactoside to GalNAc α 1-*O*-Ser **6** was performed by using recombinant β 1,3-galacto-

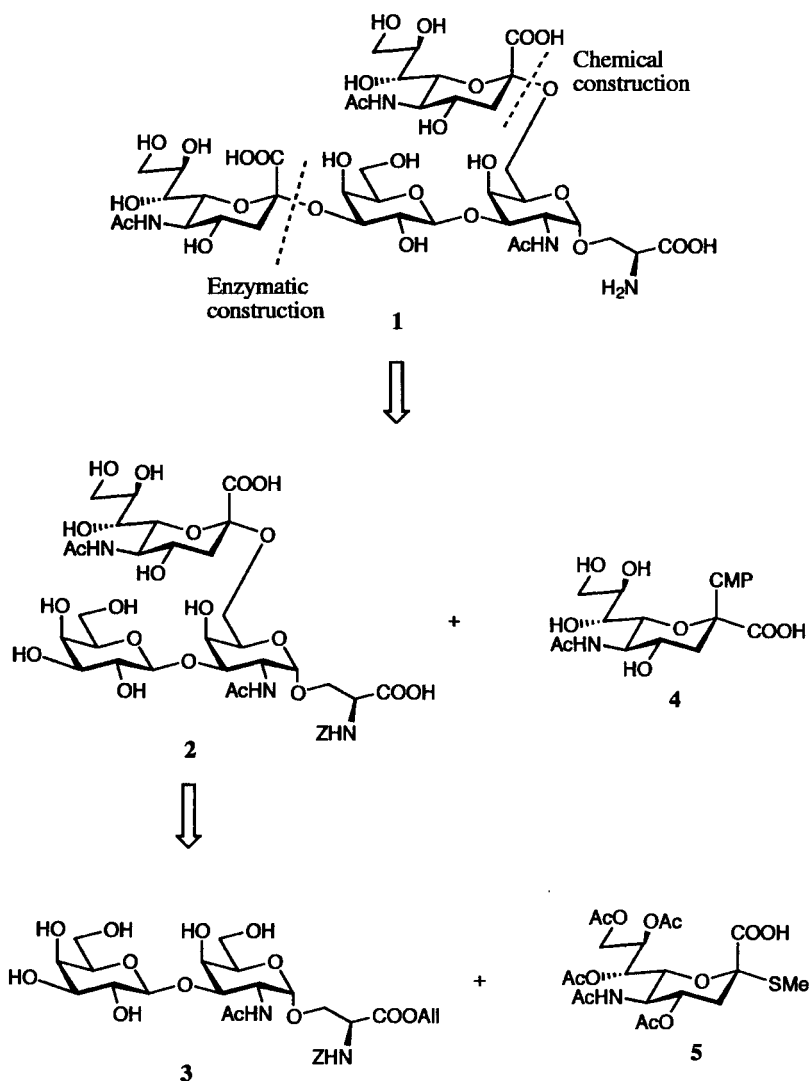
sidase from *B. circulans* [EC 3.2.0.23]. The mixture of GalNAc α 1-*O*-Ser **6** and *p*NP- β -Gal in 20% DMF–80% potassium phosphate buffer (KPB, pH 6.0, 0.1 M) was incubated at 37 °C in the presence of the β -galactosidase. The progress of the reaction was monitored by a HPLC fitted with a UV detector (215 nm) and a NH₂-column (CAPCELL PAK). Two small peaks appeared after 3 hours but thereafter the reaction proceeded no further. The yield of the transglycosylation product was very low. We considered that the low reactivity and regioselectivity of this transglycosylation reaction might be due to the carboxyl and/or amino group of the serine residue interacting with the enzyme.

Therefore, we used a serine derivative as an acceptor whose amino and carboxyl groups were protected. Treatment of GalNAc α 1-*O*-Ser **6** with benzyl chloroformate in aqueous NaOH afforded GalNAc α 1-*O*-(*Z*)-Ser. Reaction of GalNAc α 1-*O*-(*Z*)-Ser with allyl bromide in the presence of triethylamine gave GalNAc α 1-*O*-(*Z*)-Ser-*O*All **7** in 77% yield. Transglycosylation using GalNAc α 1-*O*-(*Z*)-Ser-*O*All **7** and *p*NP- β -Gal in the presence of β 1,3-galactosidase from *B. circulans* gave Gal β -(1 \rightarrow 3)-GalNAc α 1-*O*-(*Z*)-Ser-*O*All **3** as a single product in 68% yield. The structure of the product was supported by ¹H and ¹³C NMR spectroscopy and the coupling constant $J_{1',2'}=7.8$ Hz proved the newly constructed β -structure. The Gal β -(1 \rightarrow 3)-linkage was also confirmed by an NOE interaction between the H-1' and H-3 protons.^[8]

For the chemical construction of the branched Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc-structure, protection of hydroxyl groups on **3** was performed. Isopropylidenation of disaccharide-serine **3** by use of 2,2-dimethoxypropane in the presence of (\pm)-camphorsulfonic acid gave a di-*O*-isopropylidene derivative **8** in good yield. After the toluoylation of remaining hydroxyl groups, the 4,6-*O*-isopropylidene group on the GalNAc residue was cleaved selectively to afford a diol derivative **10**. Sialylation of **10** using the *S*-methyl donor **5**^[13] promoted by NIS-TfOH^[14] at –40 °C gave mono-sialoside **11** as an anomeric mixture at the NeuAc residue (α : β =74:26). The α -(2 \rightarrow 6)-linkage of **11** was determined by conversion into the acetylated derivative **12**, which showed a signal for H-4 at δ 5.44 as a doublet with $J_{3,4}=2.6$ Hz in the ¹H NMR spectrum. The anomeric configuration of the NeuAc residue at C-2 of **11** was determined as α by NMR (NeuAc H-3e δ 2.57, NeuAc H-3a δ 1.89, NeuAc C-3 δ 37.33).^[15] The 3,4-*O*-Isopropylidene group of **11** was easily removed by 70% aq HOAc to give a triol **13**. Deallylation of **13** was cleanly achieved by use of Pd(PPh₃)₄ in EtOH–H₂O,^[16] and the product was deacylated to give OH-free trisaccharide **2**. Two methods (chemical and enzymatic) were considered to introduce a second NeuAc residue into the triol **13**, but we chose to use α 2,3-(*O*)-sialyltransferase [EC 2.4.99.4] from rat liver, which was known to be highly regio- and stereo-selective for the synthesis of NeuAc α -(2 \rightarrow 3)-Gal β -(1 \rightarrow 3)-GalNAc derivatives. A mixture of trisaccharide **2** and CMP-NeuAc **4** was incubated for 12 hours at 37 °C in the presence of α 2,3-(*O*)-sialyltransferase in 0.1 M cacodylate buffer (0.2 mL, pH 6.0) containing bovine serum albumin. The reaction was monitored with HPLC using an ODS column (Mightysil) connected to a UV monitor (215 nm). The target tetrasaccharide **14** was produced quantitatively. In the ¹H NMR spectrum of **14**, the H-3a and H-3e signals of α -(2 \rightarrow 6)-GalNAc-linked sialic acid appeared at δ 1.59 and δ 2.59, respectively, and those of α -(2 \rightarrow 3)-Gal-linked sialic acid appeared at δ 1.69 and δ 2.64, respectively. From the empirical rule of the chemical shifts in sialic acid linkage,^[17] the structure of **14** was confirmed to be NeuAc α -(2 \rightarrow 3)-Gal β -(1 \rightarrow 3)-[NeuAc α -(2 \rightarrow 6)]-GalNAc (Scheme 2).

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Scheme 2. a) 1. Benzyl chloroformate, 1M NaOH 2. Allyl bromide, Et₃N, DMF; b) *p*NP-β-galactoside, β(1,3)-galactosidase, DMF, 0.1M KPB; c) 2,2-Dimethoxypropane, (±)-Camphorsulfonic acid, acetone; d) Toluoyl chloride, pyridine; e) Me₃SiCl, ethylene glycol, MeOH; f) **5**, NIS, TfOH, MS3A, MeCN, -40 °C; g) Ac₂O, Pyridine; h) 70% HOAc; i) 1. Pd(PPh₃)₄, EtOH, H₂O 2. 1 M NaOH, MeOH; j) **4**, α(2,3)-*O*-Sialyltransferase, BSA, cacodylate buffer (0.1 M, pH 6.0).

In conclusion, the mucin type oligosaccharide NeuAcα-(2 → 3)-Galβ-(1 → 3)-[NeuAcα-(2 → 6)]-GalNAcα1-*O*-Ser was synthesized by the stepwise introduction of two sialic acids into a Galβ-(1 → 3)-GalNAcα1-*O*-(Z)-Ser-*O*AlI. The branched Galβ-(1 → 3)-NeuAcα-(2 → 6)-GalNAc derivative was constructed by use of a chemical method. Sialylation on the terminal Gal residue was accomplished by use of sialyltransferase from rat liver. The present method, including individual glycosylation at the C-3 position of Gal and the C-6 position of a GalNAc of Galβ-(1 → 3)-GalNAcα1-*O*-Ser/

The derivative by a chemoenzymatic strategy, was shown to be effective for the synthesis of various mucin type oligosaccharides.

EXPERIMENTAL

General Methods. Optical rotations were measured at 25 °C with a HORIBA polarimeter SEPA-300. NMR spectra were measured on a UNITY INOVA 500 spectrometer (500 MHz) or JEOL JNM-ECP 400 spectrometer (400 MHz). ¹H NMR were recorded in CDCl₃ or D₂O using Me₄Si (δ 0.00) or DOH (δ 4.65) as the internal standard. ¹³C NMR spectra were recorded in CDCl₃ or D₂O using CDCl₃ (δ 77.0) or 1,4-dioxane (δ 67.6) as the internal standard. Coupling constants were measured in Hz. TOF-MS spectra were recorded on Finnigan mat LASERMAT 2000 with 2,5-dihydroxybenzoic acid as the matrix. High resolution mass spectra were obtained on JMS-HX110 (Jeol) spectrometer with diethanolamine as the matrix. Silica gel column chromatography was performed using Wakogel C-300 (Wako Pure Chemical Industries). Analytical TLC was performed on aluminium plates coated with silica gel 60 F254 (Merck). Preparative TLC was performed on glass plates coated with silica gel 60 F254 (Merck). Molecular sieves (3 Å) were purchased from Aldrich Chemical Company, Inc., and activated at 180 °C under vacuum immediately prior to use. α2,3-(*O*)-Sialyltransferase (EC 2.4.99.4) was purchased from Carbiochem–Novabiochem Corporation.

***N*-(Benzyloxycarbonyl)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-L-serine Allyl Ester (7).** To a 1 M NaOH solution (50 mL) of GalNAcα1-*O*-Ser **6** (4.0 g, 13.0 mmol) was added slowly benzyl chloroformate (6.6 g, 39.0 mmol), and the mixture was stirred for 10 hours at 0 °C. After the reaction mixture was washed with CHCl₃, an aqueous layer was neutralized with ion-exchange resin (Dowex 50W) and concentrated in vacuo. To the DMF solution (130 mL) of the residue containing Et₃N (3.95 g, 39.0 mmol) was added allyl bromide (4.72 g, 39.0 mmol) at 0 °C. The mixture was stirred for 20 hours at room temperature and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc ~ EtOAc:MeOH = 7:3) to afford GalNAcα1-*O*-(*Z*)-Ser *O*All **7** (4.83 g, 77%) as crystals: mp 185–187 °C (EtOH); [α]_D + 115.3° (*c* 2.08, MeOH); ¹H NMR (500 MHz, D₂O) δ 7.36–7.29 (m, 5H, Phenyl), 5.95–5.87 (m, 1H, CH₂–CH=CH₂), 5.33 (d, 1H, CH₂–CH=CH₂, *J* = 16.9 Hz), 5.23 (d, 1H, CH₂–CH=CH₂, *J* = 10.3 Hz), 5.13 (d, 1H, benzyl, *J* = 12.1 Hz), 5.09 (d, 1H, benzyl), 4.78 (d, 1H, H-1, *J* = 3.3 Hz), 4.62 (m, 2H, CH₂–CH=CH₂), 4.49 (br s, 1H, Ser-α), 4.22 (dd, 1H, H-2, *J* = 11.0 Hz), 3.94 (dd, 1H, Ser-β, *J* = 3.7, 11.0 Hz), 3.89 (dd, 1H, Ser-β, *J* = 4.4 Hz), 3.86 (d, 1H, H-4, *J* = 2.2 Hz), 3.79–3.65 (m, 4H, H-3,5,6,6'), 1.95 (s, 3H, Ac); ¹³C NMR (125 MHz, CDCl₃) δ 173.76, 171.53, 158.53, 138.01, 133.08, 129.49, 129.10, 128.96, 119.06, 99.96, 72.83, 70.20, 69.48, 68.90, 67.87, 67.07, 62.73, 56.00, 51.34, 22.90.

Anal. Calcd for C₂₂H₃₀N₂O₁₀: C, 54.77; H, 6.27; N, 5.81. Found: C, 54.56; H, 6.31; N, 5.82.

***N*-(Benzyloxycarbonyl)-*O*-(β-D-galactopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-L-serine Allyl Ester (3).** To the β1,3-galactosi-

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dase solution (recombinant, from *B. circulans*, 0.1 mL, 130 mU)^[6,7] was added 20% DMF–80% KPB (pH 6.0, 0.1 M) buffer (0.6 mL) including *p*NP- β -Gal (10.7 mg, 35.3×10^{-3} mmol) and GalNAc α 1-*O*-(*Z*)-Ser-OAll **7** (51.1 mg, 106×10^{-3} mmol) and the mixture was incubated for 4.5 hours at 37 °C. The reaction was stopped by heating at 100 °C for 5 min, then filtered and purified by HPLC [ODS (Waters AccQ TAG), eluent: 30% aq MeOH–50% aq MeOH] to afford Gal β 1-3GalNAc α 1-*O*-(*Z*)-Ser-OAll **3** (15.5 mg, 68%) as crystals: mp 196–198 °C (EtOH); $[\alpha]_D + 70.0^\circ$ (*c* 1.10, H₂O); ¹H NMR (500 MHz, D₂O) δ 7.42–7.34 (m, 5H, Phenyl), 5.88–5.85 (m, 2H, CH₂–CH=CH₂), 5.29 (d, 1H, CH₂–CH=CH₂, *J* = 17.1 Hz), 5.23 (d, 1H, CH₂–CH=CH₂, *J* = 10.5 Hz), 5.14 (d, 1H, benzyl, *J* = 12.5 Hz), 5.09 (d, 1H, benzyl), 4.81 (d, 1H, H-1, *J* = 3.2 Hz), 4.51 (br s, 1H, Ser- α), 4.40 (d, 1H, H-1', *J* = 7.8 Hz), 4.23 (dd, 1H, H-2, *J* = 11.0 Hz), 4.17 (d, 1H, H-4, *J* = 2.9 Hz), 3.94 (dd, 1H, H-3, *J* = 3.2 Hz), 3.85 (d, 1H, H-4', *J* = 2.9 Hz), 3.59 (m, 1H, H-5'), 3.56 (dd, 1H, H-3', *J* = 10.0 Hz), 3.46 (dd, 1H, H-2', *J* = 10.0 Hz), 1.93 (s, 3H, Ac); ¹³C NMR (125 MHz, CDCl₃) δ 103.57 (C-1'), 97.03 (C-1), 75.87 (C-3), 73.96 (C-5'), 71.49 (C-3'), 69.88 (C-5), 69.58 (C-2'), 67.55, 67.52 (C-4,4'), 60.06, 59.94 (C-6,6'), 47.51 (C-2).

Anal. Calcd for C₂₈H₄₀N₂O₁₅: C, 52.17; H, 6.25; N, 4.35. Found: C, 51.83; H, 6.27; N, 4.37.

***N*-(Benzyloxycarbonyl)-*O*-(3,4-*O*-isopropylidene- β -D-galactopyranosyl)-(1 \rightarrow 3)-*O*-(2-acetamido-2-deoxy-4,6-*O*-isopropylidene- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (**8**).** To an acetone solution (46 mL) of Gal β 1-3GalNAc α 1-*O*-(*Z*)-Ser-OAll **3** (1.16 g, 1.8 mmol) was added 2,2-dimethoxypropane (2.2 g, 21.6 mmol) containing (\pm)-camphorsulfonic acid (230 mg, 0.99 mmol) and the mixture was stirred for 24 hours at room temperature. After the reaction was stopped by addition of Et₃N (0.165 mL, 1.19 mmol), the solution was concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc:MeOH=98:2) to afford diacetone **8** (910 mg, 70%): $[\alpha]_D + 77.0^\circ$ (*c* 0.96, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.37–7.34 (m, 5H, Phenyl), 5.92–5.82 (m, 2H, CH₂–CH=CH₂ and NH), 5.70 (br d, 1H, NH, *J* = 5.7 Hz), 5.34 (d, 1H, CH₂–CH=CH₂, *J* = 17.3 Hz), 5.30 (d, 1H, CH₂–CH=CH₂, *J* = 10.5 Hz), 5.14 (s, 2H, benzyl), 4.87 (d, 1H, H-1, *J* = 3.7 Hz), 4.67 (d, 2H, CH₂–CH=CH₂, *J* = 5.1 Hz), 4.61 (ddd, 1H, H-2, *J* = 9.3, 10.7 Hz), 4.58 (br s, 1H, Ser- α), 4.30 (d, 1H, H-4, *J* = 2.9 Hz), 4.26 (d, 1H, H-1', *J* = 8.1 Hz), 4.15 (dd, 1H, H-4', *J* = 1.7, 5.5 Hz), 4.03 (dd, 1H, H-3', *J* = 7.7 Hz), 4.01–3.65 (m, 9H, H-3, 5, 6a, 6b, 5', 6'a, 6'b, Ser- β), 3.59 (dd, 1H, H-2'), 1.98 (s, 3H, Ac), 1.52, 1.49, 1.46, 1.34 (each s, 12H, acetone); ¹³C NMR (125 MHz, CDCl₃) δ 171.6, 170.2, 155.9, 135.9, 130.9, 128.6, 128.4, 128.3, 119.6, 110.4, 104.4, 99.1, 78.6, 77.2, 73.8, 73.4, 72.2, 69.0, 68.7, 67.3, 66.5, 63.3, 62.5, 54.5, 47.8, 29.3, 28.0, 26.3, 23.3, 18.4.

Anal. Calcd for C₃₄H₄₈N₂O₁₅: C, 56.35; H, 6.68; N, 3.87. Found: C, 56.00; H, 6.74; N, 3.68.

***N*-(Benzyloxycarbonyl)-*O*-(3,4-*O*-isopropylidene-2,6-di-*O*-toluoyl- β -D-galactopyranosyl)-(1 \rightarrow 3)-*O*-(2-acetamido-2-deoxy-4,6-*O*-isopropylidene- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (**9**).** To a pyridine solution (5 mL) of diacetone **8** (300 mg, 0.414 mmol) was added toluoyl chloride (320 mg, 2.07 mmol) and the mixture was stirred for 2 h at room temperature. After the reaction was stopped by addition of MeOH (5 mL), the solution was concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 1:1 ~ 1:2 ~ EtOAc) to afford ditoluoyl

derivative **9** (328 mg, 82%): $[\alpha]_D + 69.7^\circ$ (c 1.01, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 7.94–7.91 (m, 4H, Phenyl), 7.36–7.20 (m, 13H, Phenyl), 5.89–5.78 (m, 2H, $\text{CH}_2\text{--CH=CH}_2$ and NH), 5.59 (br d, 1H, NH , $J = 6.9$ Hz), 5.30 (d, 1H, $\text{CH}_2\text{--CH=CH}_2$, $J = 16.9$ Hz), 5.24 (d, 1H, $\text{CH}_2\text{--CH=CH}_2$, $J = 10.1$ Hz), 5.13–5.10 (m, 3H, H-2', benzyl, H-1), 4.88 (d, 1H, H-1', $J = 6.4$ Hz), 4.41 (ddd, 1H, H-2, $J = 11.1$ Hz), 4.18 (m, 1H, H-5'), 3.92 (dd, 1H, Ser- β , $J = 3.2$, 11.3 Hz), 3.85 (dd, 1H, Ser- β , $J = 2.7$ Hz), 3.75 (dd, 1H, H-3, $J = 2.5$ Hz), 2.41, 2.40 (each s, 6H, toluoyl), 1.62 (s, 3H, Ac), 1.47, 1.41, 1.36, 1.31 (12H, each s, acetamide); ^{13}C NMR (125 MHz, CDCl_3) δ 170.21, 169.83, 166.29, 165.45, 155.90, 144.36, 144.22, 135.99, 131.19, 129.92, 129.55, 129.22, 128.55, 128.29, 128.21, 126.62, 119.15, 110.93, 102.67, 99.30, 98.91, 76.22, 73.18, 72.94, 71.17, 69.28, 68.03, 67.18, 66.22, 63.78, 63.08, 62.54, 54.60, 48.27.

Anal. Calcd for $\text{C}_{50}\text{H}_{60}\text{N}_2\text{O}_{17}$: C, 62.49; H, 6.29; N, 2.91. Found: C, 62.08; H, 6.48; N, 2.88.

***N*-(Benzyloxycarbonyl)-*O*-(3,4-*O*-isopropylidene-2,6-di-*O*-toluoyl- β -D-galactopyranosyl)-(1 \rightarrow 3)-*O*-(2-acetamido-2-deoxy- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (**10**).** To a MeOH solution (10 mL) of ditoluoyl derivative **9** (338 mg, 0.352 mmol) containing ethylene glycol (39×10^{-3} mL, 0.704 mmol) was added Me_3SiCl (22×10^{-3} mL, 0.176 mmol) at 0 °C. After being stirred for 30 min, the reaction was stopped by addition of Et_3N (0.102 mL, 0.704 mmol) and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 1:2 \sim EtOAc) to afford monoacetamide **10** (243 mg, 75%): $[\alpha]_D + 203.7^\circ$ (c 0.35, MeOH); ^1H NMR (500 MHz, CDCl_3) δ 7.96–7.90 (m, 4H, Phenyl), 7.35–7.26 (m, 13H, Phenyl), 5.84–5.77 (m, 1H, $\text{CH}_2\text{--CH=CH}_2$), 5.73 (d, 1H, NH , $J = 8.4$ Hz), 5.29 (d, 1H, NH , $J = 7.6$ Hz), 5.26 (d, 1H, $\text{CH}_2\text{--CH=CH}_2$, $J = 15.5$ Hz), 5.21 (d, 1H, $\text{CH}_2\text{--CH=CH}_2$, $J = 10.6$ Hz), 5.17 (dd, 1H, H-2', $J = 6.6$ Hz), 5.11 (d, 1H, benzyl, $J = 12.2$ Hz), 5.07 (d, 1H, benzyl), 4.87 (d, 1H, H-1, $J = 3.7$ Hz), 4.74 (d, 1H, H-1', $J = 6.9$ Hz), 3.89 (dd, 1H, Ser- β , $J = 2.2$, 10.8 Hz), 3.83 (dd, 1H, Ser- β , $J = 3.2$ Hz), 2.42, 2.40 (each s, 6H, toluoyl), 1.63 (s, 3H, Ac), 1.45, 1.36 (each s, 6H, acetamide); ^{13}C NMR (125 MHz, CDCl_3) δ 169.79, 169.68, 166.16, 164.89, 155.90, 144.14, 144.10, 135.90, 130.87, 129.66, 129.48, 129.05, 128.33, 128.08, 127.98, 126.52, 126.44, 119.01, 110.89, 100.60, 98.67, 78.83, 75.86, 72.79, 72.56, 70.88, 69.57, 68.85, 68.25, 66.99, 63.43, 62.31, 54.26.

Anal. Calcd for $\text{C}_{47}\text{H}_{56}\text{N}_2\text{O}_{17}$: C, 61.30; H, 6.13; N, 3.04. Found: C, 60.97; H, 6.19; N, 3.08.

***N*-(Benzyloxycarbonyl)-*O*-(3,4-*O*-isopropylidene-2,6-di-*O*-toluoyl- β -D-galactopyranosyl)-(1 \rightarrow 3)-*O*-[methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate-(2 \rightarrow 6)]-*O*-(2-acetamido-2-deoxy- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (**11**).** The mixture of acceptor **10** (261 mg, 0.284 mmol), donor **5** (296 mg, 0.568 mmol) and molecular sieves 3 Å (600 mg) in MeCN (0.5 mL) was stirred for 1 h at room temperature under an argon atmosphere. To this mixture was added the MeCN solution (0.5 mL) of NIS (383 mg, 1.7 mmol) containing TfOH (50×10^{-3} mL, 0.568 mmol) at -40°C . After being stirred for 14 hours, the solution was diluted with CH_2Cl_2 , filtered and washed with saturated NaHSO_3 and brine, respectively. The organic layer was dried over MgSO_4 and concentrated in vacuo. The residue was purified by gel filtration (Sephadex LH-20, MeOH) to afford sialosides

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as an anomeric mixture ($\alpha:\beta=76:24$). These isomers were separated by preparative TLC ($\text{CH}_2\text{Cl}_2:\text{MeOH}=19:1$, 2 times) to give α isomer **11** (166 mg, 42%) and β isomer (50 mg, 13%) respectively. Compound **11**: $[\alpha]_D^{+34.5^\circ}$ (c 0.35, MeOH); ^1H NMR (500 MHz, CDCl_3) δ 7.94–7.90 (m, 4H, Phenyl), 7.37–7.23 (m, 13H, Phenyl), 4.87 (d, 1H, H-1', $J=6.1$ Hz), 4.78 (d, 1H, H-1, $J=3.7$ Hz), 3.77 (s, 3H, OMe), 2.57 (dd, 1H, NeuAc H-3e, $J=4.7$, 12.3 Hz), 2.42, 2.40 (each s, 6H, toluoyl), 2.11, 2.02, 1.98, 1.87, 1.63, 1.53, 1.36 (each s, 24H, Ac and acetonide), 1.89 (dd, 1H, NeuAc H-3a, $J=12.3$ Hz); ^{13}C NMR (125 MHz, CDCl_3) δ 170.63, 170.50, 169.97, 169.93, 167.75, 165.97, 164.99, 144.05, 143.86, 131.01, 129.71, 129.53, 129.05, 128.38, 128.11, 128.02, 126.81, 126.57, 119.16, 110.74, 101.04, 98.78, 98.70, 78.68, 75.40, 72.68, 72.49, 72.44, 70.22, 68.99, 68.92, 68.44, 67.50, 67.03, 66.00, 63.17, 63.04, 62.30, 52.59, 49.31, 47.77, 37.33, 29.48, 26.77, 25.55, 22.97, 22.40, 21.45, 20.79, 20.56, 20.51.

Anal. Calcd for $\text{C}_{67}\text{H}_{83}\text{N}_3\text{O}_{29}$: C, 57.71; H, 6.00; N, 3.01. Found: C, 57.27; H, 5.52; N, 3.05.

N-(Benzyloxycarbonyl)-O-(3,4-O-isopropylidene-2,6-di-O-toluoyl- β -D-galactopyranosyl)-(1 \rightarrow 3)-O-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate-(2 \rightarrow 6)]-O-(2-acetamido-4-O-acetyl-2-deoxy- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (12**).** To a pyridine solution (1 mL) of **11** (35 mg, 25.1×10^{-3} mmol) was added Ac_2O (0.5 mL) and the mixture was stirred for 30 min at 70 °C. The reaction was stopped by addition of MeOH (5 mL), and the solution was concentrated in vacuo. The residue was purified by silica gel column chromatography ($\text{CH}_2\text{Cl}_2:\text{MeOH}=15:1$) to afford acetate **12** (30 mg, 83%): ^1H NMR (400 MHz, CDCl_3) δ 7.96–7.90 (m, 4H, Phenyl), 7.36–7.16 (m, 13H, Phenyl), 5.82 (m, 1H, $\text{CH}_2\text{-CH=CH}_2$), 5.76 (d, 1H, NH, $J=8.1$ Hz), 5.66 (d, 1H, NH, $J=7.7$ Hz), 5.44 (d, 1H, H-4, $J=2.6$ Hz), 5.15 (dd, 1H, H-2', $J=6.1$, 6.4 Hz), 4.92 (d, 1H, H-1, $J=3.3$ Hz), 4.85 (m, 1H, NeuAc H-4), 4.73 (d, 1H, H-1', $J=6.4$ Hz), 4.39 (dd, 1H, H-3', $J=6.1$ Hz), 3.73 (s, 3H, OMe), 2.54 (dd, 1H, NeuAc H-3e, $J=4.8$, 12.8 Hz), 2.41, 2.38 (each s, 6H, toluoyl), 2.11, 2.09, 2.07, 2.02, 1.87, 1.64, 1.57, 1.33 (each s, 27H, Ac and acetonide); ^{13}C NMR (100 MHz, CDCl_3) δ 170.87, 170.64, 170.17, 170.12, 169.93, 169.68, 167.73, 166.29, 165.38, 155.83, 144.28, 143.91, 136.06, 131.21, 129.86, 129.69, 129.21, 129.14, 128.54, 128.28, 128.22, 119.28, 110.69, 100.62, 98.53, 98.49, 77.21, 76.21, 73.18, 72.81, 72.57, 71.00, 96.03, 68.44, 68.21, 67.30, 67.12, 66.17, 63.76, 63.01, 62.36, 54.48, 52.79, 49.28, 49.10, 37.57, 27.20, 25.92, 23.16, 21.64, 20.93, 20.81, 20.78, 20.70.

N-(Benzyloxycarbonyl)-O-(2,6-di-O-toluoyl- β -D-galactopyranosyl)-(1 \rightarrow 3)-O-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate-(2 \rightarrow 6)]-O-(2-acetamido-2-deoxy- α -D-galactopyranosyl)-(1 \rightarrow 3)-L-serine Allyl Ester (13**).** The aq HOAc solution (70%, 5 mL) of sialoside **11** (129 mg, 93×10^{-3} mmol) was stirred for 48 hours at room temperature. The solution was concentrated in vacuo to give a triol **13** (95 mg, 76%): $[\alpha]_D^{+38.0^\circ}$ (c 0.90, MeOH); ^1H NMR (400 MHz, CDCl_3) δ 7.92–7.88 (m, 4H, Phenyl), 7.37–7.18 (m, 13H, Phenyl), 6.00 (d, 1H, NH, $J=9.9$ Hz), 5.71 (d, 1H, NH, $J=8.1$ Hz), 5.56 (d, 1H, NH, $J=9.2$ Hz), 5.27 (d, 1H, $\text{CH}_2\text{-CH=CH}_2$, $J=17.2$ Hz), 5.21 (d, 1H, $\text{CH}_2\text{-CH=CH}_2$, $J=10.3$ Hz), 4.78 (d, 1H, H-1', $J=7.3$ Hz), 4.61 (d, 1H, H-1, $J=3.3$ Hz), 3.84 (s, 3H, OMe), 2.56 (dd,

¹H, NeuAc H-3e, *J* = 4.8, 12.8 Hz), 2.40, 2.36 (each s, 6H, toluoyl), 2.14, 2.12, 2.03, 1.97, 1.85, 1.19 (each s, 18H, Ac and acetamide).

Anal. Calcd for C₆₄H₇₉N₃O₂₉: C, 56.76; H, 5.88; N, 3.10. Found: C, 56.42; H, 5.83; N, 3.16.

***N*-(Benzyloxycarbonyl)-*O*-(β-D-galactopyranosyl)-(1→3)-*O*-[(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)]-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-L-serine (2).** To an EtOH–H₂O solution (2 mL, 1:1) of triol **13** (14.7 mg, 10.9 × 10^{−3} mmol) was added Pd(PPh₃)₄ (12.5 mg, 10.9 × 10^{−3} mmol), and the mixture was stirred for 18 hours at room temperature. The mixture was filtered through a celite pad and the filtrate was concentrated in vacuo. The residue was purified by preparative TLC (EtOAc:MeOH = 7:3) to afford an acid. To the MeOH solution (1 mL) of the acid was added 1 M NaOH (1 mL), and the mixture was stirred for 10 hours at room temperature. The solution was neutralized with ion-exchange resin (Dowex 50Wx8, H⁺ form) and concentrated in vacuo. The residue was purified by gel filtration (Sephadex G-15, H₂O) to give compound **2** (7.8 mg, 80%): [α]_D + 27.8° (*c* 0.44, H₂O); ¹H NMR (500 MHz, D₂O) δ 7.35–7.29 (m, 5H, Phenyl), 5.06 (s, 2H, benzyl), 4.33 (d, 1H, H-1', *J* = 7.9 Hz), 4.18 (dd, 1H, *J* = 3.7, 11.0 Hz), 4.06 (d, 1H, H-1, *J* = 3.0 Hz), 4.08 (m, 1H, Ser-α), 3.38 (dd, 1H, H-2', *J* = 9.8 Hz), 2.61 (dd, 1H, NeuAc H-3e, *J* = 4.4, 12.3 Hz), 1.92, 1.87 (each s, 6H, Ac), 1.55 (dd, 1H, NeuAc H-3a, *J* = 12.3 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 104.87, 100.54, 98.15. TOFMS *m/z*: 918.6 [M+Na]⁺. HRMS (FAB[−]) Calcd for C₃₆H₅₂O₂₃N₃ [M–H][−]: 894.2992; Found: 849.2957.

***N*-(Benzyloxycarbonyl)-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→3)-*O*-[(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)]-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-L-serine (14).** A mixture of compound **2** (13.2 mg, 14.7 × 10^{−3} mmol), CMP-NeuAc **4** (19.4 mg, 29.4 × 10^{−3} mmol), α2,3-(*O*)-sialyltransferase (EC 2.4.99.4) from rat liver (100 mU in 50 mM MES, pH 6.0, 250 mM NaCl, 0.5 mM β-mercaptoethanol, 50% glycerol) in 0.1 M cacodylate buffer (0.7 mL, pH 6.0) containing bovine serum albumin (2 mg) was incubated for 12 hours at 37 °C. The solution was purified by HPLC (ODS, 10% MeCN containing 0.1% TFA) and gel filtration (Sephadex G-15, H₂O) to afford tetrasaccharide-serine **14** (16 mg, 83%): [α]_D + 19.3° (*c* 0.29, H₂O); ¹H NMR (500 MHz, D₂O) δ 7.38–7.28 (m, 5H, Phenyl), 5.07 (s, 2H, benzyl), 4.40 (d, 1H, H-1', *J* = 7.8 Hz), 4.15 (dd, 1H, *J* = 3.7, 10.8 Hz), 4.12 (br s, 1H, H-1), 3.41 (dd, 1H, H-2', *J* = 9.8 Hz), 2.64 (dd, 1H, NeuAc H-3e, *J* = 4.4, 12.3 Hz), 2.59 (dd, 1H, NeuAc H-3e, *J* = 4.7, 12.5 Hz), 1.92, 1.86 (each s, 6H, Ac), 1.69 (dd, 1H, NeuAc H-3a, *J* = 12.3 Hz), 1.59 (dd, 1H, NeuAc H-3a, *J* = 12.3 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 104.87, 100.54, 98.15. TOFMS *m/z*: 1211.9 [M+Na]⁺. HRMS (FAB[−]) Calcd for C₄₇H₆₉O₃₁N₄ [M–H][−]: 1185.3946; Found: 1185.3993.

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