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Chemoenzymatic synthesis of diverse asparagine-linked α-(2,3)-sialyloligosaccharides

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Partial sialyl transfer reaction by α-(2,3)-sialyltransferase toward (Gal-β-1,4-GlcNAc-β-1,2-Man-α-1,6/1,3-)$_2$Man-β-1,4-GlcNAc-β-1,4-GlcNAc-β-1-asparagine-Fmoc 1 was examined to obtain mono-α-(2,3)-sialyloligosaccharides and then branch-specific exo-glycosidase digestion (β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase and α-D-mannosidase) toward the asialo-branch was performed to obtain diverse asparagine-linked complex type α-(2,3)-sialyloligosaccharides. In addition, two kinds of disialyloligosaccharides in which the sialyl linkage was a mixture of α-(2,3)- and α-(2,6)-types were also specifically prepared by an additional α-(2,6)-sialyltransferase reaction toward mono-α-(2,3)-sialyloligosaccharides thus obtained.
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Keywords: **asparagine linked oligosaccharide, complex type, sialyltransferase, glycosidase**

Introduction

Sialyloligosaccharides on the cell surface are implicated in several important biological events, such as cell-cell interaction, differentiation, immune response, and inflammation [1]. The sialyl linkages are divided into three types, α-(2,3), α-(2,6), and α-(2,8), and these sialyl epitopes are synthesized by the action of sialyltransferases at the Golgi apparatus. The sialyltransferase catalyzes a transfer of sialic acid to the corresponding acceptor to form sialyl linkage and is now specified into several groups dependent on its enzymatic nature. In order to reveal the role of such sialyloligosaccharides, many kinds of sialyloligosaccharides have been synthesized by chemical and enzymatic methods [2], and then used for biological assays. However, preparation of the oligosaccharides on a large scale has been problematic, since formation of the sialyl linkage is relatively difficult in a chemical method. On the other hand, chemoenzymatic methods in which the synthetic strategy adopts preparation of an oligosaccharyl acceptor by a chemical method and subsequent enzymatic transfer of sialic acid by sialyltransferase reaction have been developed. In addition, production of the sialyltransferase has succeeded by use of a baculovirus-infected insect cell system in order to apply a large-scale sialyloligosaccharide synthesis [3]. We have developed a semisynthetic method of asparagine-linked dibranched oligosaccharides in which sialyl linkage was α-(2,6) type [4]. Our synthetic method adopted branch-specific glycosidase digestion toward the asialo-branch of complex type dibranched oligosaccharide prepared from egg yolk in order to obtain diverse asparagine-linked oligosaccharides. Although α-(2,3) sialyl linkage is abundant in several tissues, our method have limited to preparation of diverse oligosaccharides having only α-(2,6) sialyl linkage. In this paper we report a convenient synthesis of diverse asparagine-linked oligosaccharides having α-(2,3) sialyl linkages.

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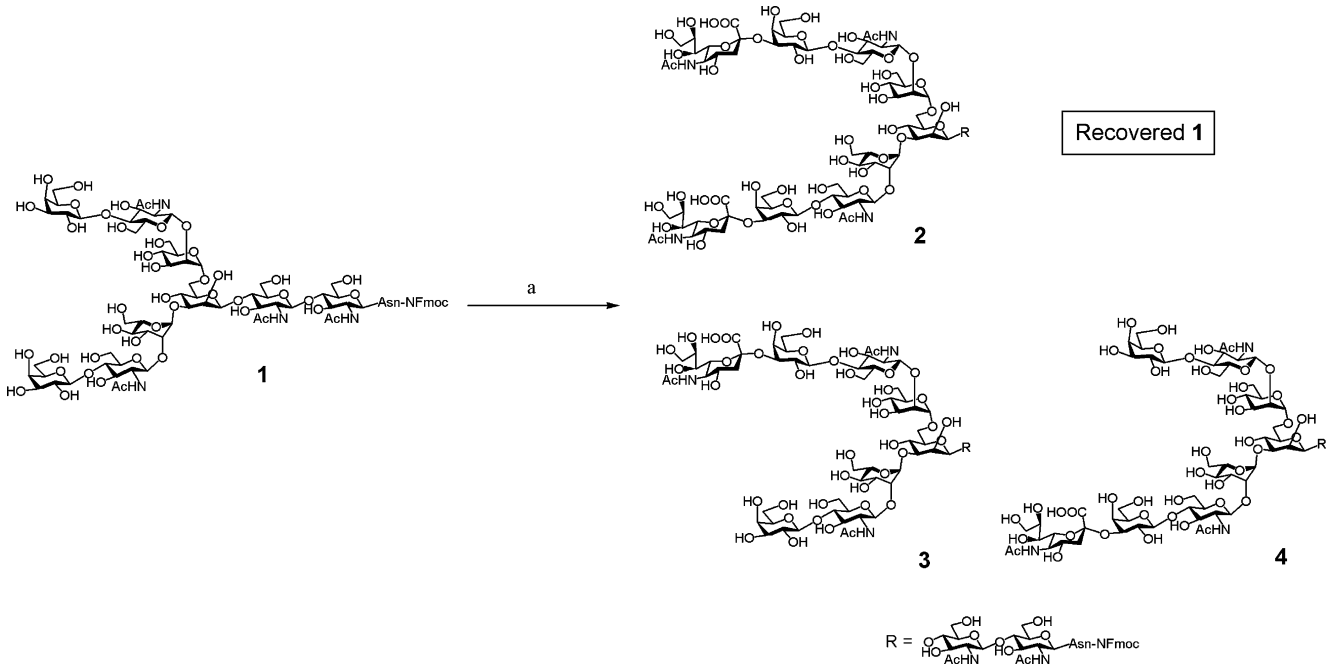
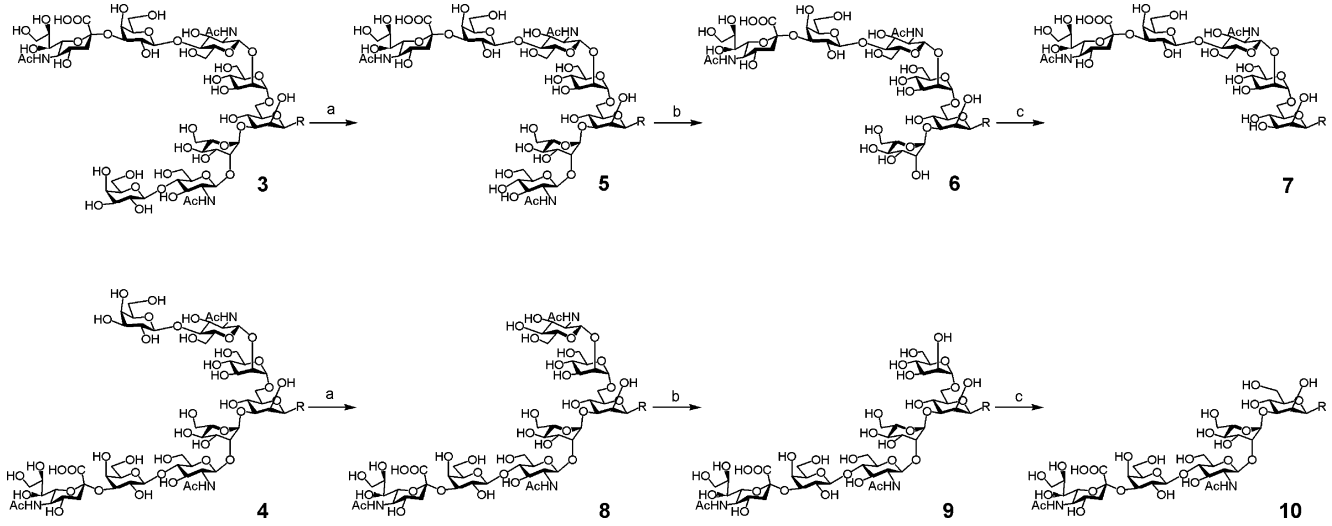
Results

The strategy of a chemoenzymatic method for the preparation of diverse α-(2,3)-sialyloligosaccharides adopts monosialylation by α-(2,3)-sialyltransferase toward asialooligosaccharide **1** [4] and subsequent branch-specific glycosidase digestions (β-D-galactosidase, *N*-Acetyl-β-D-glucosamindase and α-D-mannosidase) toward the asialo-branch. Preparation of an asialooligosaccharide **1** has been previously reported and the method can supply asialooligosaccharide **1** in over gram scale [4]. The critical point in this strategy is that non-specific α-(2,3)-mono-sialyltransfer reaction should occur toward an asialooligosaccharide in order to have sufficient quantity of both mono-α-(2,3)-sialyloligosaccharides **3** and **4** (Scheme 1). The α-(2,3)-sialyltransferases used in this examination were commercially available enzyme and a bacterial enzyme [5].



Scheme 1. (a) CMP-Neu5Ac, *Neisseria meningitidits* α-2,3-sialyltransferase.



Scheme 2. (a) Jack Beans β-D-galactosidase, (b) Jack Beans *N*-acetyl-β-D-glucosaminidase, (c) Jack Beans α-D-mannosidase.

Treatment of asialooligosaccharide by *Rat recombinant* α-(2,3)-sialyltransferase and 2 equivalents of CMP-NeuAc afforded new three components. Detecting of the UV signal of the Fmoc group at the reducing end easily monitored the reaction. Purification of these products by HPLC (ODS column chromatography) afforded three kinds of sialyloligosaccharides **2**–**4** and an asialooligosaccharide **1** (Scheme 1). Structural analysis using ^{1}H NMR and mass spectra revealed that a peak A was a disialyloligosaccharide. Peaks B and C corresponded with mono-sialyloligosaccharides and peak D was an asialooligosaccharide **1** (Figure 1). The structure of **3** and **4** was identified by ^{1}H NMR after conversion into **7** and **10**, respectively (Scheme 2), comparing a database of a reporter group [6]. However, a monosialooligosaccharide of which sialic acid was linked to the terminal of the Man-α-1,3-Man-β-arm, was not obtained in sufficient yield (3.6%). On the other hand, transfer of sialic acid to the terminal of the Man-α-1,6-Man-β-arm was predominant. In the case of bacterial sialyltransferase (*Neisseria meningitidis*), the transfer reaction did not show such branch specificity, resulting in desired both monosialyloligosaccharides. Fortunately, since HPLC profiles of each sialyl- and asialo-oligosaccharides were well separated, isolation of these oligosaccharides was easily performed (Figure 1).

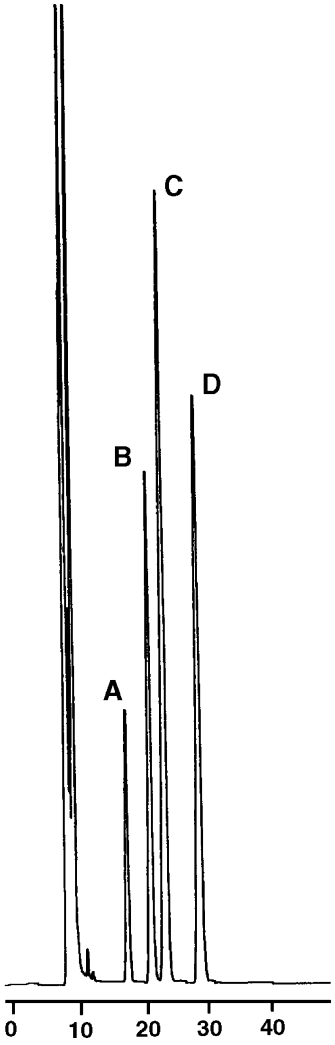


Figure 1. HPLC profile of a bacterial α-(2,3)-sialyltransferase reaction toward **1**. Peak A is a disialooligosaccharide. Peaks B and C are correspond with **3** and **4**, respectively. Peak D is a asialooligosaccharide **1**.

Toward these two mono-sialyloligosaccharides **3** and **4**, practical branch-specific digestions were individually performed as shown in Scheme 2. All enzymatic reactions smoothly proceeded and afforded the desired diverse oligosaccharides.

It is known that disialyloligosaccharides in which sialyl linkages are a mixture of α-(2,3)- and α-(2,6) exist on the protein backbone. We also examined the transfer of a second sialic acid to the mono-α-(2,3)-sialyloligosaccharides **3** and **4** by use of α-(2,6)-sialyltransferase in order to obtain such disialyloligosaccharides **11** and **12**. The reaction proceeded smoothly and afforded the desired disialooligosaccharide as shown in Scheme 3. However, since α-(2,6)-sialyl linkage originally exits in the substrate prepared from an egg yolk, this strategy was wasteful due to twice α-(2,3)- and α-(2,6)-sialyltransferase reactions.

Therefore, we examined the α-(2,3)-sialyltransferase reaction toward a mixture of mono-α-(2,6)-sialyloligosaccharide,

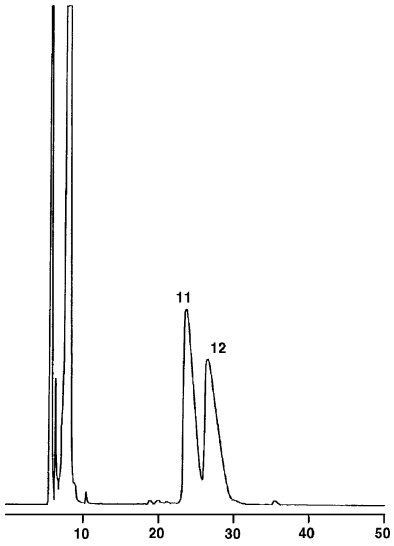


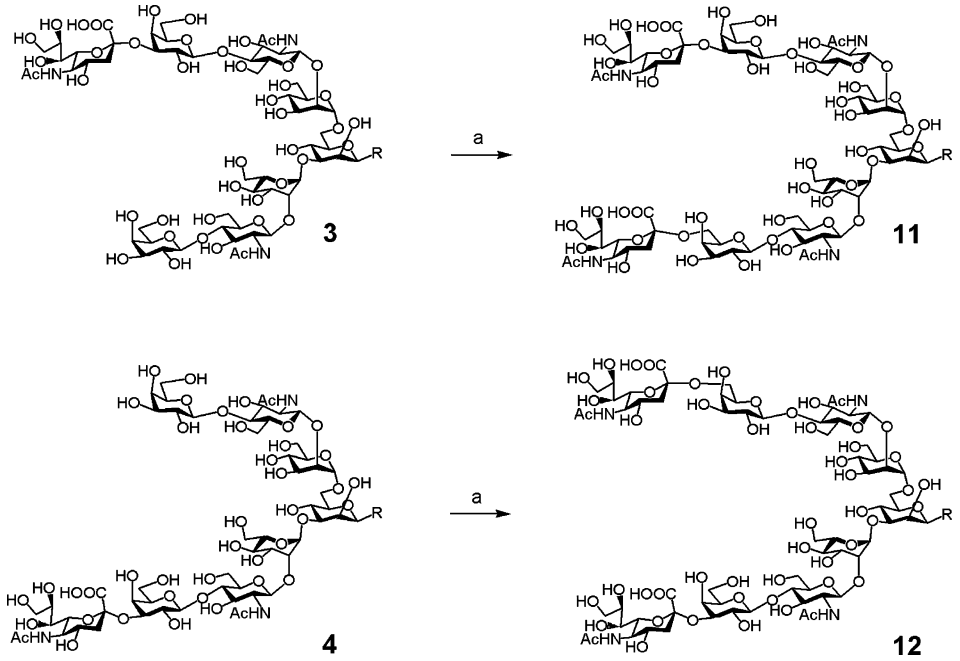
Figure 2. HPLC profile of disialooligosaccharide **11** and **12**.

13 and **14**, which were prepared from di-α-(2,6)-sialyloligosaccharide by partial acid hydrolysis [4]. As expected, the reaction smoothly proceeded and the products were sufficiently separated for isolation of the desired disialooligosaccharides (Figure 2). Purification of this oligosaccharide mixture afforded the desired two kinds of disialooligosaccharides having both α-(2,3)- and α-(2,6)-sialyl linkages at the two terminals.

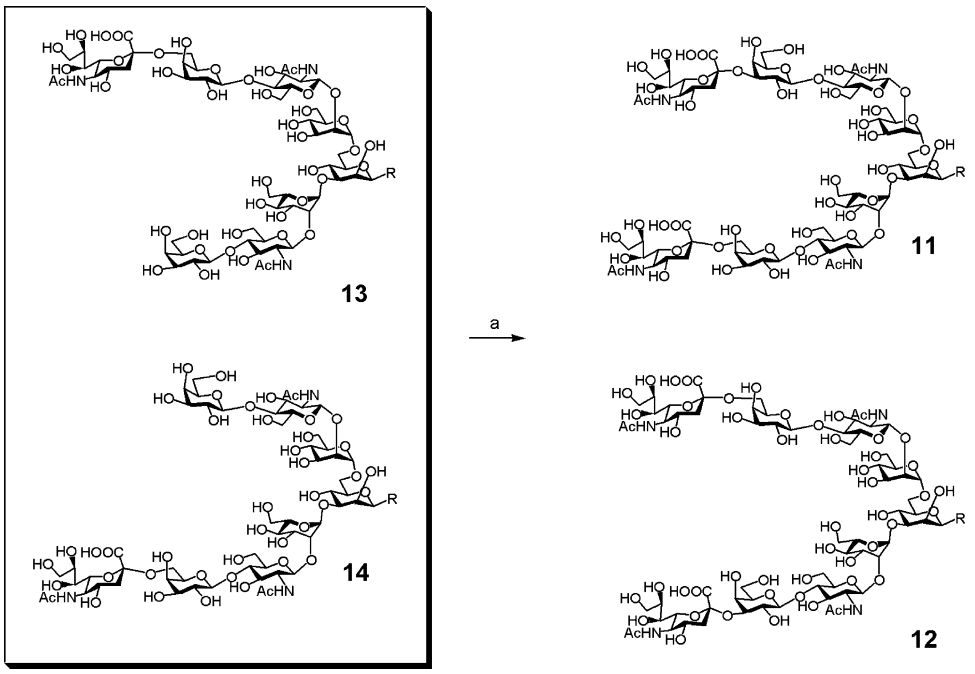
Discussion

Sialyl linkage is divided into three types, α-(2,3)-, α-(2,6)- and α-(2,8), and these are frequently found at the terminal of oligosaccharides in both glycolipid and glycoprotein. In order to investigate the role of the sialyl residue, stereospecific chemical synthesis of sialyl oligosaccharides has been performed. In addition, semisynthesis of the sialyloligosaccharides has also been developed. This semisynthesis is advantageous compared to chemical synthesis, because formation of most glycosyl linkages is not needed. We have established a chemoenzymatic semisynthesis of diverse Asn-linked dibranched complex type oligosaccharides, but its sialyl linkage was limited to be α-(2,6)-type. In order to obtain further diverse sialyloligosaccharides, we examined sialylation toward dibranched asialooligosaccharide **1** using α-(2,3)-sialyltransferase and subsequent branch-specific glycosidase digestion toward asialobrach by β-D-galactosidase, *N*-Acetyl-β-D-glucosamindase and α-D-mannosidase. Preparation of the asialooligosaccharide **1** is feasible and is now at over gram scale. Sialyltransferases used in this examination are *rat recombinant* and *Neisseria meningitidis* types, and both enzymes are suitable for multiscale synthesis of sialyloligosaccharides. On the sialyl transfer reaction toward asialooligosaccharide, *rat recombinant* sialyltransferase shows branch specificity toward a galactoside at the terminal of the Man-α-1,6-Man-β-arm, but bacterial

enzyme did not show such branch specificity. Branch specificity of sialyltransferases has been extensively studied by van den Ejinden and Vliegenthart groups. In the case of *bovine colostrum* α-(2,6)-sialyltransferase [7], the enzyme prefers transfer of a sialyl residue to the galactoside at the terminal of the Man-α-1,3-Man-β-arm. For α-(2,3)-sialyltransferase from human placenta, transfer to the Gal-β-1,4-GlcNAc-β-1,4-Man-α-1,3-Man-β-arm was superior in the tribranched complex type oligosaccharide, but the Gal-β-1,4-GlcNAc-β-1,4-Man-α-1,6-Man-β-arm might be specific in the dibranched complex type oligosaccharides [8,9]. For rat recombinant enzyme in our experiment, therefore, branch specificity was almost similar. On the other hand, a bacterial enzyme, *neisseria meningitidis* α-(2,3)-sialyltransferase, did not show branch specificity and was suitable for preparation of both monosialyloligosaccharides. However, the transfer reaction could not

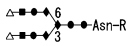
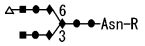
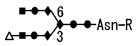
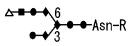
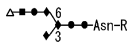
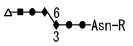
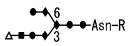
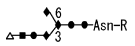
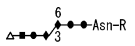
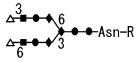
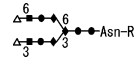


Scheme 3. (a) CMP-Neu5Ac, *rat recombination* α-2,6-sialyltransferase.



Scheme 4. (a) CMP-Neu5Ac, *rat recombination* α-2,3-sialyltransferase.

Table 1. ^{1}H NMR data of diverse Asn-oligosaccharides[a]

											
	2 R = Fmoc	**3** R = Fmoc	**4** R = Fmoc	**5** R = Fmoc	**6** R = Fmoc	**7** R = Fmoc	**8** R = Fmoc	**9** R = Fmoc	**10** R = Fmoc	**11** R = Fmoc	**12** R = Fmoc
1H-1	5.09	5.09	5.09	5.09	5.09	5.09	5.09	5.09	5.09	5.09	5.09
2H-1	4.65	4.65	4.66	4.66	4.65	4.65	4.65	4.67	4.66	4.70–4.64	4.70–4.64
3H-1	4.85	4.85	4.85	4.85	4.85	4.84	4.86	4.87	4.86	4.85	4.86
4H-1	5.21	5.21	5.21	5.21	5.19	–	5.21	5.21	5.21	5.22	5.20
4′H-1	5.01	5.01	5.02	5.02	5.02	5.01	5.01	5.01	–	5.01	5.03
5H-1	4.65	4.65	4.66	4.66	–	–	4.65	4.67	4.66	4.70–4.64	4.70–4.64
5′H-1	4.65	4.65	4.66	4.66	4.65	4.65	4.65	–	–	4.70–4.64	4.70–4.64
6H-1	4.65	4.56	4.63	–	–	–	4.65	4.63	4.63	4.53	4.63
6′H-1	4.65	4.62	4.56	4.65	4.65	4.65	–	–	–	4.63	4.54
3H-2	4.33	4.33	4.34	4.34	4.34	4.17	4.34	4.34	4.31	4.34	4.33
4H-2	4.28	4.28	4.28	4.28	4.16	–	4.28	4.28	4.28	4.28	4.28
4′H-2	4.20	4.20	4.19	4.20	4.20	4.19	4.19	–	–	4.19	4.20
6H-3	4.21	–	4.22	–	–	–	4.20	4.20	4.20	<4.00	4.20
6′H-3	4.21	4.22	–	4.21	4.22	4.20	–	–	–	4.21	<4.00
7H-3_{ax}	1.89	–	1.89	–	–	–	1.89	1.89	1.88	1.80	1.89
7H-3_{eq}	2.85	–	2.85	–	–	–	2.85	2.85	2.85	2.76	2.85
7′H-3_{ax}	1.89	1.89	–	1.89	1.89	1.89	–	–	–	1.89	1.81
7′H-3_{eq}	2.85	2.85	–	2.85	2.85	2.85	–	–	–	2.85	2.76
Asn-CH_2	2.81, 2.61	2.81, 2.62	2.81, 2.61	2.81, 2.63	2.81, 2.67	2.82, 2.61	2.81, 2.70	2.80, 2.68	2.80, 2.66	2.79, 2.68	2.80, 2.71
Ac	2.16, 2.14	2.16, 2.14	2.16, 2.14	2.16, 2.15	2.16, 2.13	2.16, 2.14	2.16, 2.14	2.16, 2.14	2.14, 2.13	2.16, 2.13	2.16, 2.15
	2.13, 2.12	2.13, 2.12	2.12, 1.98	2.14, 2.12,	2.12, 1.98	2.12, 1.98	2.12, 1.99	2.12, 1.98	2.12, 1.98	2.12, 1.98	2.14, 2.12
	1.98	1.98		1.98							1.98
Fmoc	8.01, 7.81	8.01, 7.80	8.01, 7.80	8.01, 7.81	8.01, 7.80	8.01, 7.81	8.00, 7.79	8.01, 7.80	8.00, 7.79	8.01, 7.80	8.00, 7.80
	7.58, 7.53	7.60, 7.53	7.60, 7.53	7.60, 7.53	7.60, 7.53	7.60, 7.53	7.59, 7.52	7.60, 7.53	7.59, 7.52	7.60, 7.52	7.59, 7.52

[a]Each proton signals in NeuAc-α-2,3-Gal-β-1,4-GlcNAc-β-1,2-Man-α-1,6 are expressed as a prime.

be regulated to avoid formation of disialyloligosaccharide [10] and the substrate, asialooligosaccharide **1**, remained unconsumed. Purification of those oligosaccharides was surprisingly easier compared to that of mono-α-(2,6)-sialyloligosaccharides and all four components could be isolated as a pure form in sufficient scale. In the case of separation of mono-α-(2,6)-sialyloligosaccharides **13** and **14** from each other, an additional protecting group such as benzyl ester, was essential in order to increase molecular hydrophobicity, which was expected to show more interaction with ODS HPLC column chromatography. Therefore, structural difference between α-(2,6)- and α-(2,3)-sialyl linkages affected the characteristic nature in the polarity of oligosaccharide. Structural identification of two mono-α-(2,3)-sialooligosaccharides was difficult by ^{1}H NMR; therefore, we performed identification after α-D-mannosidase digestion to obtain **7** and **10**. Since proton signals of each mannose H1 are easily identified by the reporter group, structural identification of **7** and **10** was feasible.

Glycosidase digestion toward these monosialyloligosaccharides was smoothly performed within 12 h and all reactions can be monitored by HPLC. There were no overreaction products by use of commercially available glycosidases and the examination afforded a sufficient quantity of desired products **5**–**12**.

Since dibranched sialyloligosaccharide having α-(2,6)- and α-(2,3)-sialyl linkage are abundant in many mammalian tissues, we examined further modification by α-(2,6)-sialylation toward the asialo-branch in the mono-α-(2,3)-sialyloligosaccharide **3** and **4**. The products were easily obtained, and we noted that the two kinds of disialyloligosaccharides in which sialyl linkages are a mixture of α-(2,3) and α-(2,6) types, were well separated on HPLC analysis, resulting in easy purification. These results enabled us to examine another convenient synthetic method of **11** and **12**. We examined the first preparation of a mixture of mono-α-(2,6)-sialyloligosaccharides by partial acid hydrolysis of α-(2,6)-disialooligosaccharide and subsequent transfer reaction of sialic acid by α-(2,3)-sialyltransferase. This sequential treatment also afforded the desired disialooligosaccharides **11** and **12** and waste of an original attached α-(2,6)-sialyl linkage in the oligosaccharide material can be avoided. On monitoring this transfer reaction by HPLC, there was no substrate specificity of α-(2,3)-sialyltransferase toward the original α-(2,6) sialyl residue attached.

Experimental section

General methods

NMR spectra were measured with Bruker Avance 400 (30°, internal standard HOD = 4.81) instrument. MS spectra were measured with Bruker Daltonics AutoFLEX instrument.

Synthesis of oligosaccharide derivatives **2, 3** and **4**

Rat recombinant α-(2,3)-sialyltransferase

To a solution of asialooligosaccharide derivative **1** (24.4 mg, 12.34 μmol) in a cacodylate buffer (50 mM, pH = 5.0, 1.22 ml) was added BSA (2 mg), CMP-sialic acid disodium salt (32.5 mg, 49.4 μmol) alkaline phosphatase (50 Units, Roche) and α-(2,3)-sialyltransferase (45 mU, *Rat Recombinant*, Calbiochem). This mixture was incubated for 3 h at 37°C. The reaction was monitored by HPLC (YMC AM-312, 1.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 20/80). After the solution was filtered by membrane filter (0.45 μm, Advantec), purification was performed by HPLC system (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 20/80) to afford α-(2,3)-disialyloligosaccharide **2** (retention time (R.T.) = 16 min), α-(2,3)-monosialyl oligosaccharide **3** (R.T. = 20 min), α-(2,3)-monosialyl oligosaccharide **4** (R.T. = 24 min), and recovered starting material **1** (R.T.= 30 min). Each fraction were pooled and then concentrated. After desalting with ODS column (cosmosil 75C_{18}-OPN, nacalai tesque, eluted with H_2O then 25% CH_3CN), the solution containing oligosaccharide was lyophilized. These procedure afforded pure oligosaccharides **2** (13.6 mg, 43.1%), **3** (8.6 mg, 30.7%), **4** (1.0 mg, 3.6%), and recovered **1** (1.0 mg, 4.1%).

Bacterial (Neisseria meningitidis) α-(2,3)-sialyltransferase

To a solution of asialooligosaccharide derivative **1** (29.9 mg, 15.1 μmol) in a cacodylate buffer (50 mM, pH = 5.0, 7.5 ml) was added BSA (20 mg), CMP-sialic acid disodium salt (30.0 mg, 46.9 μmol) alkaline phosphatase (250 Units, Roche) and α-(2,3)-sialyltransferase (150 μl, *Neisseria meningitidis*). This mixture was incubated for 36 h at 37°C. The reaction was monitored by HPLC (YMC AM-312, 1.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 20/80). Purification were performed with the same manner in the case of *rat recombinant* sialyltransferase reaction. These procedure afforded pure oligosaccharides **2** (2.8 mg, 7.2%), **3** (5.8 mg, 16.9%), **4** (10.3 mg, 30.0%), and recovered **1** (7.1 mg, 23.7%).

α-(2,3)-Disialyl oligosaccharide **2**

MALDI-MS (positive mode) calcd for $C_{103}H_{154}N_8NaO_{66}$ $[M + Na^+]$ 2581.883, found 2581.817.

α-(2,3)-Monosialyl oligosaccharide **3**

MALDI-MS (positive mode) calcd for $C_{92}H_{137}N_7NaO_{58}$ $[M + Na^+]$ 2290.788, found 2290.731.

α-(2,3)-Monosialyl oligosaccharide **4**

MALDI-MS (positive mode) calcd for $C_{92}H_{137}N_7NaO_{58}$ $[M + Na^+]$ 2290.788, found 2290.723.

β-D-Galactosidase digestion of monosialyl decasaccharide **3**

To a solution of monosialyldecasaccharide **3** (5.8 mg, 2.6 μmol) in HEPES buffer (50 mM, pH = 6.0, 580 μl) containing BSA (1.0 mg) was added β-D-galactosidase (150 mU, Seikagakukogyo, *Jack. Beans.*). This mixture was incubated at 37°C for 5.5 h (monitored by HPLC). After almost starting material was

consumed, the reaction mixture was filtered (membrane, 0.45 μm). Then the filtrate was purified by HPLC system (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 18/82) to afford monosialyl nonasaccharide **5** (R.T. = 30 min). The fraction containing an oligosaccharide **5** was concentrated and then desalting with ODS column (cosmosil 75C_{18}-OPN, nacalai, eluted with H_2O then 25% CH_3CN) was performed. The solution containing oligosaccharide was pooled and then lyophilized to afford a pure monosialyl nonasaccharide **5** (5.4 mg, 99%). MALDI-MS (positive mode) calcd for $C_{86}H_{127}N_7NaO_{53}$ [M+Na^+] 2128.735, found 2128.742.

*N-acetyl-β-*D*-glucosamindase digestion of monosialyl nonasaccharide* **5**

To a solution of monosialyl nonaoligosaccharide **5** (4.0 mg, 1.9 μmol) in HEPES buffer (50 mM, pH = 6.0, 400 μl) containing BSA (1.0 mg) was added *N*-Acetyl-β-D-glucosamindase (250 mU, SIGMA, *Jack. Beans.*). This mixture was incubated at 37°C for 15 h (monitored by HPLC). After almost starting material was consumed, the reaction mixture was filtered (membrane, 0.45 μm). Then the filtrate was purified by HPLC system (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 20/80). The fraction containg an oligosaccharide **6** (R.T.= 23 min) was concentrated and then desalted with ODS column (cosmosil 75C_{18}-OPN, nacalai, eluted with H_2O then 25% CH_3CN) to afford a pure monosialyl octasaccharide **6** (3.1 mg, 86%). MALDI-MS (positive mode) calcd for $C_{78}H_{114}N_6NaO_{48}$ [M+Na^+] 1925.656, found 1925.721.

α-D-*mannosidase digestion of monosialyl octasaccharide* **6**

To a solution of monosialyl octaoligosaccharide **6** (1.2 mg, 0.63 μmol) in HEPES buffer (50 mM, pH = 6.0, 120 μl) containing BSA (1.0 mg) was added α-D-mannosidase (342 mU, SIGMA, *Jack. Beans.*). This mixture was incubated at 37°C for 21 h (monitored by HPLC). After almost starting material was consumed, the reaction mixture was filtered. Then the filtrate was purified by HPLC system (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 22/78) to monosialyl heptasaccharide **7** (R.T.= 21 min). The fraction containing an oligosaccharide **7** was concentrated and then desalted with ODS column (cosmosil 75C_{18}-OPN, nacalai, eluted with H_2O then 25% CH_3CN) to afford a pure monosialyl heptasaccharide **7** (0.8 mg, 73%). MALDI-MS (positive mode) calcd for $C_{72}H_{104}N_6NaO_{43}$ [M+Na^+] 1763.603, found 1763.690.

β-D-*galactosidase digestion of monosialyl decasaccharide* **4**

β-D-Galactosidase digestion (300 mU) of monosialyl decaoligosaccharide **4** (10.3 mg, 4.5 μmol) in HEPES buffer (50 mM, pH = 6.0, 1030 μl) and purification (**8**: R.T.= 33 min) were performed with the same manner in the preparation of **5**. A pure monosialyl nonasaccharide **8** (8.6 mg) was obtained in 90% yield. MALDI-MS (positive mode) calcd for $C_{86}H_{127}N_7NaO_{53}$ [M+Na^+] 2128.735, found 2128.719.

*N-acetyl-β-*D*-glucosamindase digestion of monosialyl nonasaccharide* **8**

N-Acetyl-β-D-glucosamindase (250 mU) digestion of monosialyl nonaoligosaccharide **8** (4.2 mg, 2.0 μmol) in HEPES buffer (50 mM, pH = 6.0, 420 μl) and purification (R.T.= 25 min) were performed with the same manner in the preparation of **6**. A pure monosialyl octasaccharide **9** (3.8 mg) was obtained in 99% yield. MALDI-MS (positive mode) calcd for $C_{78}H_{114}N_6NaO_{48}$ [M + Na^+] 1925.656, found 1925.680.

α-D-*mannosidase digestion of monosialyl octasaccharide* **9**

α-D-Mannosidase (20 U) digestion of monosialyl octaoligosaccharide **9** (1.5 mg, 0.79 μmol) in HEPES buffer (50 mM, pH = 6.0, 150 μl) and purification (R.T. = 26 min) were performed with the same manner in the preparation of **7**. A pure monosialyl octasaccharide **10** (1.3 mg) was obtained in 95 % yield. MALDI-MS (positive mode) calcd for $C_{72}H_{104}N_6NaO_{43}$ [M+Na^+] 1763.603, found 1763.704

Synthesis of oligosaccharide derivative **11**

Method A. To a solution of monosialyl oligosaccharide derivative **3** (1.2 mg, 0.53 μmol) in a cacodylate buffer (50 mM, pH = 5.0, 60 μl) was added BSA (1 mg), CMP-sialic acid disodium salt (3.4 mg, 5.3 μmol), alkaline phosphatase (25 Units, Roche) and α-(2,6)-sialyltransferase (24 mU, Rat Recombinant, Calbiochem). The mixture was incubated at 37°C. The reaction was monitored by HPLC (YMC AM-312, 1.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 18/82). Almost starting material was consumed for about 1.0 h. The solution was filtered by membrane filter (0.45 μm, Advantec), and then purified by HPLC system (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 18/82) to afford disialyloligosaccharide **11** having α-(2,3) and (2,6)-sialyl linkages (R.T.) = 24 min). The fraction was concentrated and then desalted with open-ODS column (cosmosil 75C_{18}-OPN, nacalai, eluted with H_2O then 25% CH_3CN) to afford a pure oligosaccharides **11** (1.1 mg, 81%).

Synthesis of oligosaccharide derivatives **12**

To a solution of monosialyl oligosaccharide derivative **4** (1.7 mg, 0.75 μmol) in a cacodylate buffer (50 mM, pH = 5.0, 85 μl) was added BSA (1 mg), CMP-sialic acid disodium salt (4.8 mg, 7.5 μmol), alkaline phosphatase (25 Units, Roche) and$g\alpha$-(2,6)-sialyltransferase (34 mU, Rat Recombinant, Calbiochem). The solution was incubated at 37°C. Monitoring and purification were performed with a same manner in the preparation of **11**. A pure oligosaccharides **12** (1.3 mg, R.T. = 27 min) was obtained in 68% yield.

Method B. To a mixture of mono-α-(2,6)-sialyloligosaccharide derivatives **13** and **14** (15.1 mg, 6.7 μmol) in a cacodylate buffer (50 mM, pH = 5.0, 760 μl) was added BSA (2.0 mg), CMP-sialic acid disodium salt (17.5 mg, 26.6 μmol) and alkaline phosphatase (50 Units, Roche) and α-(2,3)-sialyltransferase (28 mU, Rat Recombinant, Calbiochem). The solution was incubated at 37°C and the reaction was monitored by HPLC (YMC AM-312, 1.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 18/82). Almost starting material was consumed for about 6.5 h. The solution was filtered by membrane filter (0.45 μm, Advantec), and then purified by HPLC system repeatedly (YMC ODS-AM SH-343-5AM, 7.0 ml/min, 274 nm, CH_3CN/25 mM $AcONH_4$ aq. = 18/82). Disialyl oligosaccharide **11** and **12** having α-(2,3) and (2,6)-sialyl linkages (retention time (R.T.) = 24 min) and (R.T. = 27 min), respectively, were obtained. Each individual fractions were combined and concentrated, and then desalted with a ODS column (cosmosil 75C_{18}-OPN, nacalai, eluted with H_2O then 25% CH_3CN) to afford a pure oligosaccharides **11** (7.6 mg, 45%) and **12** (8.2 mg, 48%).

α-(2,3), (2,6)-Disialyl oligosaccharide **11**

MALDI-MS (positive mode) calcd for $C_{103}H_{154}N_8NaO_{66}$ $[M + Na^+]$ 2581.883, found 2581.787.

α (2,6), (2,3)-monosialyl oligosaccharide **12**

MALDI-MS (positive mode) calcd for $C_{103}H_{154}N_8NaO_{66}$ $[M + Na^+]$ 2581.883, found 2581.817.

Conclusion

We examined the preparation of diverse asparagine-linked α-(2,3)-sialyloligosaccharides by branch specific- and sequential-glycosidase digestion toward the asialo branch in asparagine-linked oligosaccharides. Since these oligosaccharides have already an Fmoc protecting group at the asparagine, these oligosaccharides thus obtained can be used for both solid and liquid phase glycopeptide synthesis. This examination would serve to reveal the role of sialyloligosaccharides on the protein and peptide.

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