Prompt Chemoenzymatic Synthesis of Diverse Complex-Type Oligosaccharides and Its Application to the Solid-Phase Synthesis of a Glycopeptide with Asn-Linked Sialyl-undeca- and Asialo-nonasaccharides

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Abstract: We describe herein the preparation of 24 pure asparagine-linked oligosaccharides (Asn-oligosaccharides) from asparagine-linked biantennary complex-type sialylundecasaccharide [(NeuAc-α-2,6-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, **2**] obtained from egg yolk. Our synthetic strategy aimed at adapting branch specific exo-glycosidases digestion (β-D-galactosidase, N-acetyl-β-D-glucosaminidase and α-D-mannosidase) of the individual asialo-branch after preparation of monosialyloligosaccharides obtained from **2** by acid hydrolysis of NeuAc. In order to perform branch specific exo-glycosidase digestion, isolation of pure monosialyloligosaccharides obtained was essential. However, isolation of two kinds of monosialyloligosaccharides are difficult by HPLC due to their highly hydrophilic nature. Therefore, we examined chemical protection with hydrophobic protecting (Fmoc and benzyl) groups. These chemical protection enabled us to separate the monosialyloligosaccharides by use of a HPLC column (ODS) on synthetic scales. Using these pure monosialiloligosaccharides enable us to obtain 24 Asn-linked oligosaccharides (100 mg scale) within a few weeks by branch specific exo-glycosidase digestions (α-D-neuraminidase, β-D-galactosidase, N-acetyl-β-D-glucosaminidase and α-D-mannosidase). In addition, solid-phase synthesis of glycopeptide having Asn-linked sialyl-undeca- and asialo-nonasaccharides thus obtained, was also performed on an acid labile HMPA-PEGA resin.

Keywords: carbohydrates • glycoproteins • oligosaccharides

Introduction

Cell-surface oligosaccharides are responsible for several biological processes such as cell–cell adhesion, differentiation, inflammation and immune responce.[1] Oligosaccharides are mainly divided into two groups, glycolipids and glycoproteins. In the case of glycoproteins, oligosaccharides are known to be O-glycans and N-glycans. The N-glycans are further divided into highmannose-, complex- and hybrid-types. These N-glycans on the protein surface play essential roles to physicochemical- and chemical-behavior such as protein folding, resistance to proteolysis, and clearance of glycoproteins.[1d] N-Glycans are attached to the asparagine residue of the Asn-X-Thr/Ser (X is any amino acid except for proline) sequence in the protein backbone as a post-translational modification[2] and are then transported to the golgi apparatus for further modification by glycosyltransferases. However, most glycoproteins have many various oligosaccharide structures (glycoforms) depending on the structural diversity of their N-glycans. N-Glycans show microheterogeneity at the nonreducing terminal and can show over one hundred of glycoforms. However, there is no evidence for why glycoproteins have glycoforms. In order to investigate the roles of the oligosaccharides, chemical and chemoenzymatic syntheses of these oligosaccharides have been performed.[3] Especially, in the past decade, synthetic technologies for the synthesis of large oligosaccharides have advanced considerably.[4] In order to study why the glycoproteins show different glycoforms, N-glycans of diverse structure must be promptly prepared and then tested in bioassays. Although N-glycans have been generally synthesized from the corresponding monosaccharides, their complex

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structures result in time consuming syntheses due to repetitive protection and deprotection of hydroxyl groups.

Recently, semisynthetic methods have been developed.[5] In this approach, oligosaccharides from natural sources such as commercially available glycoproteins are used to afford large quantities of pure oligosaccharides. This methodology has advantages compared with chemical and chemoenzymatic synthesis, because most glycosylation reactions and protection/deprotection steps can be omitted. On the other hand, the disadvantage is the inability to introduce branch specific modification of N-glycan structure. Complex type N-glycans generally form bi-, tri- and tetra-branch structures in which the branches consist of NeuAc-α-2,6/3-Gal-β-1,4-GlcNAc-β-1,2-Man-α sequences. In order to synthesize diverse oligosaccharides by the semisynthetic method, branch specific removal of the sugar residues from a single NeuAc-α-2,6/3-Gal-β-1,4-GlcNAc-β-1,2-Man-α structure is necessary. Therefore, we set out to synthesize over 20 pure asparagine-linked oligosaccharides (Asn-oligosaccharides) using branch-specific exo-glycosidase digestion from the asparagine-linked biantennary complex-type sialylundecasaccharide **2** obtained in large scale from egg yolk (Figure 1). If we could prepare pure monosialyloligosaccharides from **2** by selective acid hydrolysis of a NeuAc, we would be able to obtain diverse pure Asn-oligosaccharides by subsequent branch specific exo-glycosidase digestion (β-D-galactosidase, N-acetyl-β-D-glucosaminidase and α-D-mannosidase) of the individual asialo-branches. However, this simple strategy has troublesome drawbacks as shown in Figure 2. Acid hydrolysis of the NeuAc may not be selective and may afford various products (step 1 in Figure 2): the disialyloligosaccharide (substrate), two kinds of monosialyoligosaccharides and the asialooligosaccharide (the over-reaction product). If these Asn-oligosaccharides can be purified on a synthetic scale (>100 mg scale), subsequent branch specific exo-glycosidase digestions would afford many kinds of diverse Asn-oligosaccharides. However, oligosaccharides have a highly hydrophilic nature, therefore the purification by HPLC is known to be very difficult on a synthetic scale. In order to purify these oligosaccharides, we added hydrophobic protecting groups to increase the degree of interaction between the oligosaccharides and ODS-HPLC column (step 2 in Figure 2). Successful purification would enable us to obtain many di-

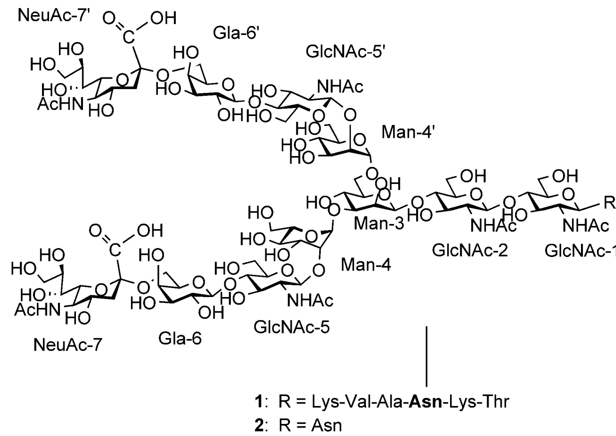

Figure 1. Structure of sialylglycopeptide.

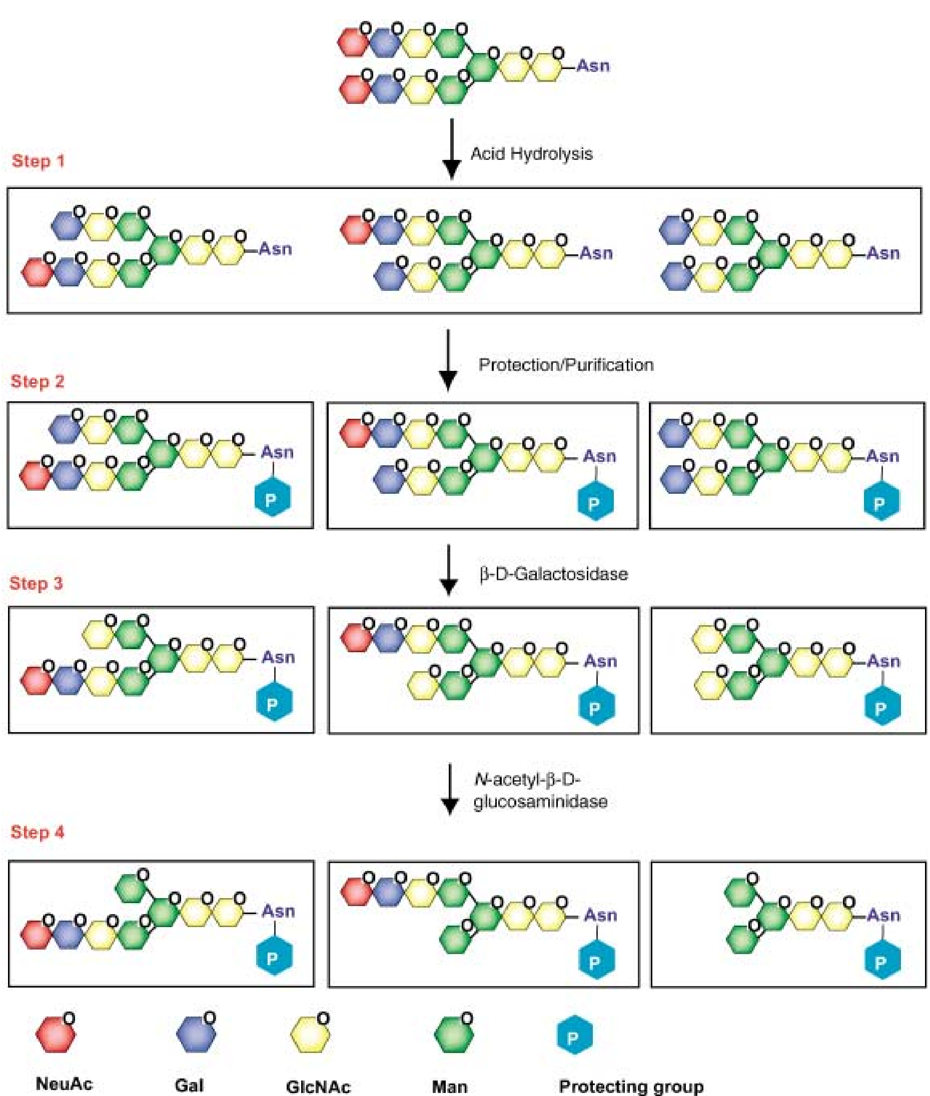

Figure 2. Synthetic strategy of diverse Asn-linked oligosaccharides.

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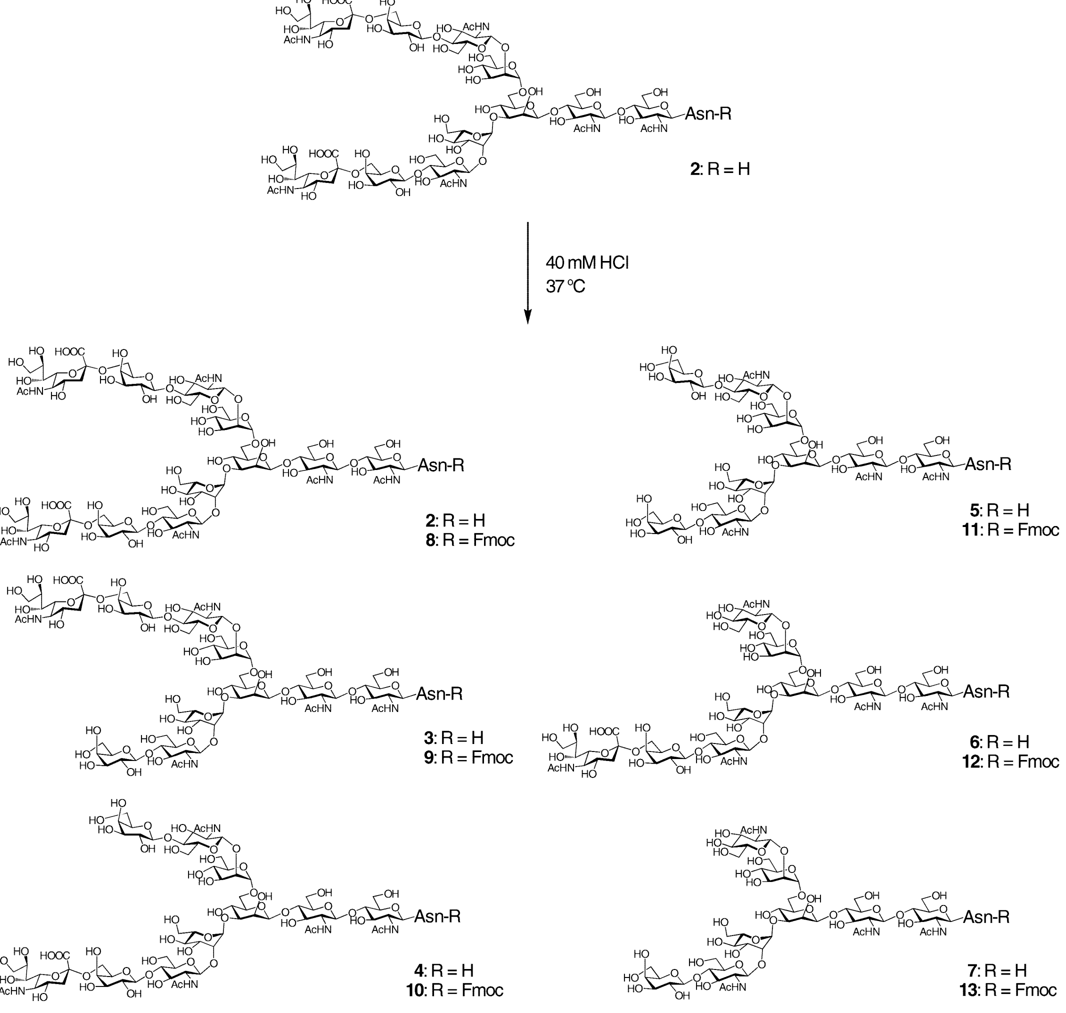
verse oligosaccharides by branch specific exo-glycosidase digestion as shown in step 3 and 4 of Figure 2.

In this paper, we report the highly efficient chemoenzymatic semisynthesis of 24 different, diverse Asn-oligosaccharides and its application to the solid-phase synthesis of sialylglycopetides by use of the asparagine linked oligosaccharides thus obtained.

Results and Discussion

It is known that sialylglycopeptide **1** can be easily prepared from egg yolk with large scale.[6] As shown in Figure 1, this oligosaccharide has general biantennary complex-type structure. If this sialylglycopeptide can be used to make diverse oligosaccharides, this sialyloligosaccharide would be a valuable source for large scale preparation. However, in order to use this sialylglycopeptide as a versatile oligosaccharide block, unnecessary amino acid sequences should be removed (Figure 1, bottom right). Therefore, we first had to remove the amino acids in order to convert the asparagine-linked sialyloligosaccharide **2**[7] by protease digestion (Actinase-E). The asparagine-linked biantennary oligosaccharide **2** thus obtained was then treated by acid hydrolysis to remove one of the two NeuAc residues of **2** (Scheme 1). For these acidic conditions, we have found a 40 mM of HCl solution to be the most suitable condition for removing the NeuAc residue.[8] However, as expected, this acid hydrolysis afforded four different Asn-oligosaccharides **2**–**5** along with **6** and **7** as side-products. In order to use these oligosaccharides, the Asn-oligosaccharides needed to be purified at this point. However, since these Asn-oligosaccharides are very hydrophilic, purification by HPLC with an ODS-column was unsuccessful on scales greater than a 1 mg. Indeed, it is known that large scale separation (mg scale) of oligosaccharide mixtures prepared from natural sources is very difficult even using several different HPLC columns. Therefore, we examined the addition of hydrophobic protecting groups to these Asn-oligosaccharides in order to increase the interaction between hydrophobic protected Asn-oligosaccharides and the ODS column. Among several protecting groups examined, the 9-fluorenylmethyl (Fmoc) group was found to be suitable for hydrophobicity as well as its application to Fmoc solid phase glycopeptide synthesis. 9-Fluorenylmethyl-*N*-succimidylcarbonate[9] was added to a mixture of Asn-oligosaccharides **2**–**7**, affording the corresponding Fmoc-Asn-oligo-



Scheme 1.

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saccharides **8–13** in almost quantitative yields. As shown in Figure 3, all the Fmoc-Asn-oligosaccharides can be purified on a HPLC column except for **9** and **10**. This purification scale is limited to a 30 mg mixture **8–13** per purification (ODS column ∅20 mm × 250 mm). In order to obtain each Fmoc-Asn-oligosaccharides in a few hundred mg scale, we performed this purification repeatedly. Since the purification of a mixture takes less 1 h, this semisynthetic method is preferable compared with the chemical synthesis which takes a few months up to a half year. If a large ODS column can be used for this purification, a large scale purification could be easily performed. In order to separate monosialyl-oligosaccharides **9** and **10**, further protection with hydrophobic groups was then examined. Since NeuAc residues have a carboxylic group, this functional group should be protected selectively by esterification. For this protection, we have found benzylesterification to occur selectively on the carboxylic acid of the two NeuAc in **8** without protection of the asparagine.[10] Therefore, this benzylesterification condition was applied to the mono-benzyl esterification of the monosialyloligosaccharides **9** and **10** to afford the corresponding products **14** and **15** in moderate yields. The increased hydrophobicity of the benzyl esters enabled us to separate Fmoc-Asn-oligosaccharides **14** and **15** (Scheme 2).

9, **10**

1) Cs_2CO_3, H_2O
2) BnBr, DMF

14: R = Fmoc

15: R = Fmoc

Scheme 2.

The limit of this purification is about 10 mg per purification (ODS column ∅20 mm × 250 mm). The structure of oligosaccharide **14** and **15** was determined by conversion into **16** and **12**, respectively, by galactosidase digestion and subsequent deprotection. The Fmoc-Asn-oligosaccharide **14** and **15** can also be used in synthesis of glycopeptides with monosialyl biantennary oligosaccharides which are abundant among cell surface glycoproteins.

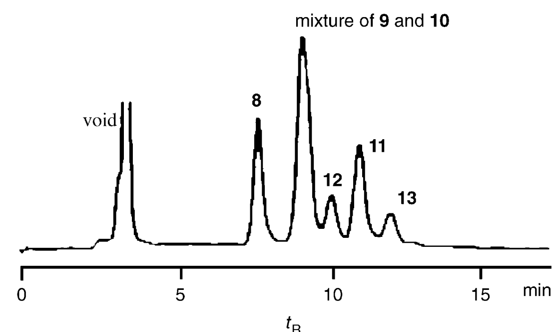
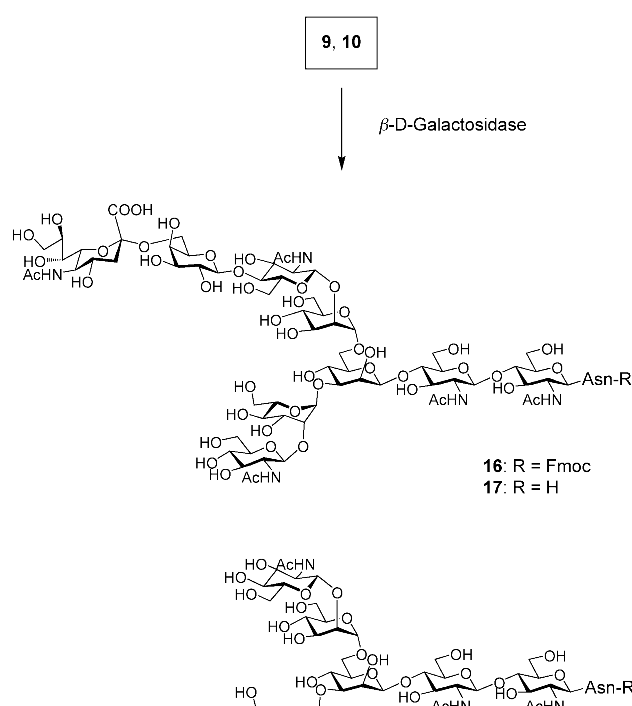


Figure 3. HPLC profile (analytical scale) of acid hydrolysis reaction.

Since the limit per purification of **14** and **15** is about 10 mg, we examined another condition to afford more monosialooligosaccharides easily. Therefore we also examined purification of monosialyloligosaccharides each other after galactosidase digestion toward mixture of **9** and **10**. We performed exo-β-D-galactosidase digestion on a mixture of **9** and **10**. We used commercially available β-D-galactosidase containing less than 5 % of other glycosidases as impurities. This galactosidase digestion afforded monosialyl Asn-oligosaccharides **12** and **16** in 48 and 41 % yield, respectively (Scheme 3). Purification of these Asn-oligosaccharides can also be performed by HPLC as shown in Figure 4. This purification (ODS column ∅20 mm × 250 mm) can be performed on a 20 mg scale of mixture of **12** and **16**. Indeed, we obtained each monosialyloligosaccharides on a 200 mg scale by repetitive purification. In this purification, the retention time of **16** was faster than that of **12**. The structures of Asn-oligosaccharides **12** and **16** were determined by ^{1}H NMR signals of the reporter group,[11] after conversion into **41** and **19**, respectively, by glycosidase digestions. Because the anomeric protons of the three mannosides are empirically observed with the corresponding chemical shifts in the NMR spectrum, the structures of **19** and **41** can be identified based on disappearance of 4H-1 and 4′H-1, respectively. As shown in Figure 5 and Table 1, although differences in ^{1}H NMR data of **12** and **16** are only small, the retention time by HPLC is clearly visible (Figure 4).

In order to modify pure Asn-oligosaccharide **16**, sequential exo-glycosidase digestions were performed as shown in Scheme 4. These exo-glycosidase digestions can be performed in > 100 mg scale with yields in the range 60–90 % and reactions finished within one day depending on the enzyme quantities added. Each reactions can be easily monitored by HPLC and their products **18–22** were easily purified by the same HPLC conditions. We also examined deprotection of the N-Fmoc group by morphorine after isola-



Scheme 3.

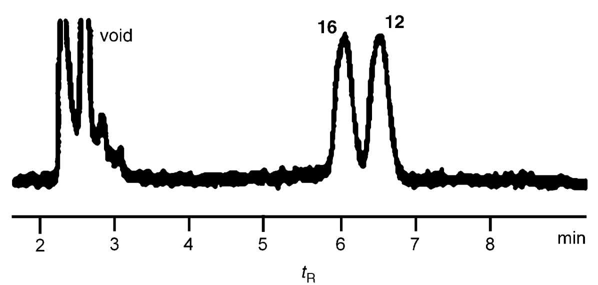


Figure 4. HPLC profile (analytical scale) of galactosidase digestion of **9** and **10**.

tion of the each Fmoc-Asn-oligosaccharides **18–22**. Complete deprotection reactions afforded Asn-oligosaccharides **23–27** in good yields.

In order to further modify the Asn-oligosaccharides, we examined a variety of sequence for glycosidases digestion as shown in Scheme 5. These digestions can be performed on a 100 mg scale and no over-reaction products were observed in HPLC and NMR analysis by good commercially available glycosidase (less than 5 % of other glycosidases as contaminant). For the asialooligosaccharides **11** and **34** (Scheme 5), slight branch specificity was observed. However since excess glycosidase were used, the digestions proceeded smoothly. The diverse Asn-oligosaccharides shown in Scheme 5 were also deprotected by morphorine to afford Asn-oligosaccharides **29**, **32**, **33**, **5**, **36**, and **37** in good yield.

In Figure 6, several Asn-oligosaccharides are shown which were prepared from pure Asn-monosialyloligosaccharide **12**. Since those structures are positional isomers to those in Schemes 4 and 5, that is, **40** and **18**, **41** and **19**, the structurally diverse Asn-oligosaccharides shown in Figure 6 were prepared by the same procedure shown in Schemes 4 and 5. For Jack beans α-D-mannosidase digestion, large excess of α-D-mannosidase is essential. Hydrolysis of 1→6 branched α-D-mannoside in Asn-oligosaccharide **40** (Figure 6) to afford **41** required a 27 fold excess of enzyme over substrate **18** (Scheme 4). This branch specificity of Jack beans α-D-mannosidase digestion is reversed compared with Asn-penta-oligosaccharide **37**.[12] Brossmer reported that the 1→6 branched in mannoside in **37** was easier digested than the 1→3-branched mannoside. Attachment of the branched NeuAc-α-2,6-Gal-β-1,4-GlcNAc-β-1,2 sequence to the mannoside-4 may alter the substrate specificity of mannosidase digestion. In the NMR analysis, a remarkable phenomenon was observed. When GlcNAc-5′ residue of **12**, **13** and **34**, respectively, was removed by N-acetyl-β-D-glucosaminidase, the H-2 of Man-4′ was remarkably shifted upfield. This tendency have been also reported by Vliegenthart.[11] As seen in Schemes 4 and 5, Asn-oligosaccharides were obtained by glycosidase digestion from the pure Asn-monosialyloligosaccharide **16**. Glycosidase digestion to obtain Asn-oligosaccharides can also be performed by use of a mixture of **9** and **10** as a starting substrate, and then purified by HPLC as shown in Scheme 6. In this one-pot procedure, β-D-galactosidase and N-acetyl-β-D-glucosaminidase were added to a mixture of **9** and **10** and the reaction was monitored by HPLC. After removal of N-acetyl-β-D-glucosaminide, the reaction mixture was heated under reflux for 5 min in order to deactivate glycosidases and then α-D-neuraminidase was added to the reaction mixture. If β-D-galactosidase and N-acetyl-β-D-glucosaminidase activities remained after α-D-neuraminidase digestion, the reaction mixture would afford

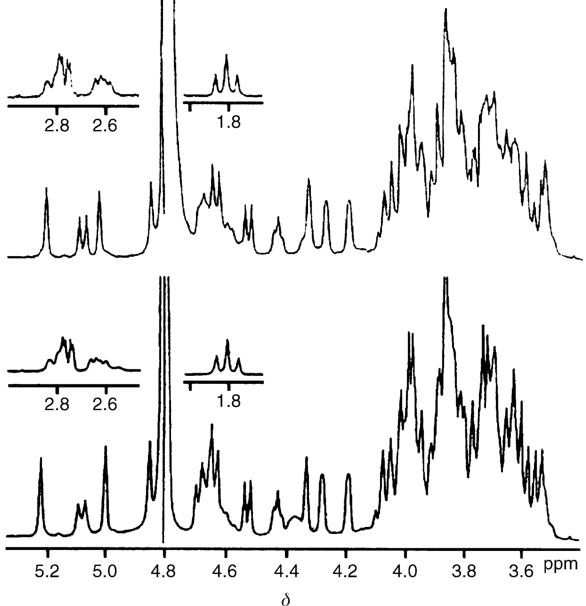


Figure 5. 1H NMR spectra of monosialo-nonasaccharides **12** (top) and **16** (bottom).

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Scheme 4. a) N-Acetyl-β-D-glycosaminidase; b) α-D-mannosidase; c) α-D-neuraminidase; d) β-D-galactosidase.

multiple compounds including over-reaction products, such as **35**. The reflux time is dependent on the quantity of glycosidases added. A series of those one-pot glycosidase digestions afforded the structural isomers **30** and **38**. These isomers can be easily purified by HPLC to afford pure **30** and **38**. Although the separation of **9** and **10** is a little difficult, the other positional isomers can easily be separated from one another.

As shown in Schemes 2–6 and Figure 6, this procedure can easily afford more than 100 mg of these Asn-oligosaccharides by repetitive enzymatic reactions. Since these Asn-oligosaccharides contain α-sialyl- and β-mannosyl linkages which are the most difficult during chemical glycosylation, our semisynthetic method is the first efficient procedure to prepare such a variety of Asn-oligosaccharides. Although some Asn-oligosaccharides were prepared by a chemical method consisting of many steps, our method can easily yield the desired biantennary Asn-oligosaccharides in a few weeks. In order to increase the variety of Asn-oligosaccharides, use of glycosyltransferases and sugar nucleotide analogues should be employed.

Solid-phase synthesis of glycopeptide with several Asn-oligosaccharides: Glycoproteins with N-glycan or O-glycan linkages, have several different oligosaccharides on their peptide backbones. Therefore, a synthetic technology for glycopeptides consisting of several oligosaccharides is essential in order to elucidate the roles of glycopeptides and glycoproteins. However, although synthetic methods for glycopeptides have been developed,[13] preparation of oligosaccharides is also time consuming, which hinders further biological investigations. Especially, the synthesis of Asn-linked oligosaccharide such as **8** require sophisticated synthetic techniques and is very time consuming. In addition, solid-phase synthesis of sialylglycopeptides is very difficult, because the sialyl linkage in the glycopeptide is very labile during trifluoroacetic acid (or HF in Boc method) treatment to release the sialylglycopeptide from the solid phase. In this respect, α-2,6 sialyl linkages are more labile than α-2,3-sialyl linkages. In order for α-2–6 sialyloligosaccharide to resist acid hydrolysis, an electron-withdrawing group such as an acetyl group should be used as a protecting group.[13n]

Our semisynthetic method solved the above time consuming problems during preparation of oligosaccharide and provides adequate quantities. In addition, asparagine is already protected with the Fmoc group which is convenient for Fmoc solid phase peptide synthesis. However, in order to use Fmoc-Asn sialyloligosaccharide **8** for the synthesis of sialylglycopeptides, selective protection of the carboxylic acid groups in NeuAc is essential. For this problem, we have already found a selective benzyl esterification method for the carboxylic acid of NeuAc to afford benzyl esterified Fmoc-Asn-sialyloligosaccharide **52** (Scheme 7)[10] In addition, we also found that benzyl esterified Fmoc-Asn-sialyloligosaccharide **52** is stable towards treatment with 95% TFA for 3 h; this is a conventional condition in solid-phase synthesis to cleave the peptide from the solid phase. These new finding enabled us to synthesize sialyloligopeptide (ALLVNSS: N is Asn-oligosaccharide **8**) on a solid phase.[10]

Since native proteins have several different oligosaccharides on the peptide backbone, we next examined the synthesis of a glycopeptide with two kinds of large Asn-oligosaccharides, **8** and **11**, prepared using our semisynthetic

16

α-D-Neuraminidase

28: R = Fmoc
29: R = H

18

α-D-Neuraminidase

30: R = Fmoc
32: R = H

β-D-Galactosidase

31: R = Fmoc
33: R = H

11: R = Fmoc
5: R = H

β-D-Galactosidase

34: R = Fmoc
36: R = H

N-Acetyl-β-D-glucosaminidase

35: R = Fmoc
37: R = H

Scheme 5.

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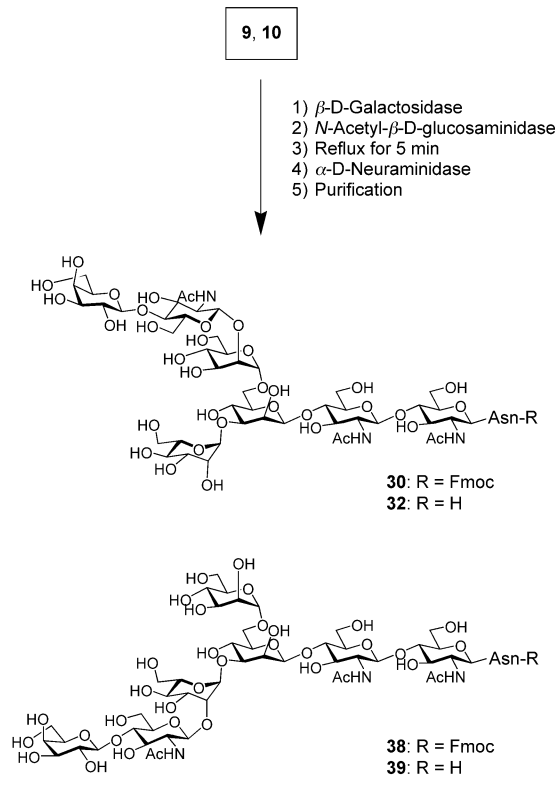

Scheme 6.

method. The peptide backbone we selected contains twelve amino acids, ALLV N(asialooligosaccharide)ALLVN(sialyloligosaccharide)SS. The sequence ALLVNSS is a fragment of erythropoietin 85–95.[14] Although the natural type erythropoietin has Asn-linked triantennary oligosaccharide at the asparagine in ALLVNSS, we added a biantennary oligosaccharide for use as a model compound.

In addition, we added ALLVN(asialo-biantennary oligosaccharide) to the ALLVN(sialyloligosaccharide)SS in order to perform a model solid-phase synthesis of a glycopeptide with two different large oligosaccharides. In this amino acid sequence, valine is attached to the asparagine. Because steric hindrance between the isopropyl residue of valine and the Asn-oligosaccharide may occur, this synthesis also gives the limitations of this method. The synthetic technique is based on the Meldal procedure.[5b] After two serines were attached to the HMPA-PEGA resin, protected sialyloligosaccharide **52** was attached by 2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HATU) and diisopropylethylamine in DMSO/DMF for 24 h (yield 38%).[10] The peptide was then elongated by addition of valine, leucine, alanine using Fmoc-amino acid activated by pentafluorophenol (OPfp) esters in the presence of 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) as the catalyst.[5b] Coupling of Fmoc-Val-OPfp was stopped at 85% yield by monitoring the disappearance of the yellow Dhbt color. If coupling of Val is continued, esterification by amino acid toward hydroxyl groups on oligosaccharide occurred. This undesired esterification was observed by HPLC analysis after release of the glycopeptide from the solid phase. When the sample was treated with $NaOH_{aq}$ (pH 11), many peaks appeared simplify in the HPLC analysis. This indicates that undesired esterification occurred. Therefore, we terminated the coupling reaction of Val at 85% yield. The problem may result from steric hindrance between Val and the oligosaccharide. Coupling of other amino acids (Leu, Leu, Ala) by the OPfp method occurred in quantitative yield by monitoring the Dhbt color, and no esterification of the hydroxyl group of the oligosaccharide was observed. Subsequently the Fmoc-Asn-asialooligosaccharide **11** was attached in 48% yield under the same conditions as the disialyloligosaccharide **52**. Coupling of Val, Leu, Leu, Ala was also performed by the same pentafluorophenol method. In this step, the coupling yields of Val, Leu, Leu, Ala showed quantitative yield. In this case, coupling of a second valine to asialooligosaccharyl-Asn might be good yield, since the terminal asialooligosaccharide appear to be flexible compared with the sialyloligosaccharide due to the distance from the solid phase. After construction of the glycopeptide, the glycopeptide was deprotected and cleaved from the resin by treatment with TFA/water (95:5) for 3 h. In this case, the sialyl linkage was stable toward acid treatment. Removal of the benzyl ester was carefully performed using aqueous sodium hydroxide at pH 11. The crude products were then subjected to RP-HPLC purification. Pure compound **53** was obtained and characterized by ^{1}H NMR spectroscopy (2D-TOCSY). WatergateTOCSY shows corresponding amide hydrogens in the 2D NMR spectra (Supporting Information). Since the coupling yield of Asn-oligosaccharide and valine was not high on the solid phase, several glycopeptides fragments were afforded in the final reaction mixture released from solid phase. Nevertheless, the desired glycopeptide with two different large Asn-oligosaccharides can be synthesized by the solid-phase method.

Conclusion

In the past decade, many kinds of oligosaccharides have been synthesized by chemical and chemoenzymatic methods. However, since asparagine linked oligosaccharides have complicated structures, synthesis of these oligosaccharides is very difficult. In addition, in order to examine solid phase glycopeptide synthesis, at least 100 mg of the oligosaccharide should be necessary to optimize several coupling reactions as well as for obtaining an adequate quantity of target glycopeptide. Furthermore, in order to record the ^{1}H,^{15}N HSQC and its HSQC-TOCSY and HSQC-NOESY spectra, which is a general method in structural biology of peptides and proteins, a minimum 10 mM concentration of glycopeptide should be prepared if natural abundance of ^{15}N is used for the measurement.[15]

Our semisynthetic method has solved these problems for the synthesis of biantennary complex type oligosaccharides and affords 24 different, diverse Asn-oligosaccharides. Although this semisynthetic method is based on a laboratory HPLC purification procedure for the preparation of Asn-oligosaccharides as a starting materials, we believe that this method is the most convenient chemoenzymatic method for

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Figure 6. Structure of oligosaccharide derivatives.

diverse Asn-oligosaccharides, which can then be used for further biological research and synthesis of other glycoconjugates. For this application, we demonstrated the solid-phase synthesis of sialyloligopeptides with two large Asn-oligosaccharides. Research to increase the quantity of several target glycopeptides is in progress. We have applied our method to the synthesis of glycoproteins by use of native chemical ligation or the intein method[16] in order to investigate the influence of oligosaccharides on glycoprotein conformation and bioactivity.

Experimental Section

General methods: Jack beans β-D-galactosidase was purchased from Seikagakukogyo Co. Ltd. (Japan). Jack beans *N*-acetyl-β-D-glucosaminidase, Jack beans α-D-mannosidase and vibrio cholera α-D-neuraminidase were purchased from SIGMA (USA). NMR spectra were measured with Bruker Avance 400 (30 °C, internal standard HOD = 4.718 ppm; external (or internal) standard acetone = 2.225 ppm)[11] instrument. Pure sialylglycopeptide **1** was prepared by the reported method.[6]

Asparagine linked sialylundecasaccharide 2

Method A: Actinase-E (9 mg) was added to a solution of sialylglycopeptide **1**[6] (60 mg) and NaN_3 in a Tris-HCl buffer (50 mM, $CaCl_2$ 10 mM, pH 7.5, 3 mL) and this mixture was incubated for 2 d at 37 °C. During incubation, the pH was kept at 7.5. This reaction was monitored by TLC (1 M NH_4OAc/isopropanol 1:1). After the reaction was finished, the mixture was lyophilized and purification of the residue by gel permeation (Sephadex-G-25, ∅2.0 cm × 20 cm, H_2O) afforded pure asparagine-linked sialyloligosaccharide **2**[7] (42 mg, 86 %).

Method B: Crude sialylglycopeptide **1** containing egg yolk peptides (ca. 1:1 weight %) was also used for the preparation of **2**. The crude material was easily prepared by repetitive purification (2–3 times) with Sephadex G-25 (H_2O) after extraction from ca. 80 egg yolks.[6] Actinase-E (263 mg, Tris-HCl buffer 8 mL) was added to a solution of this crude sialylglycopeptide **1** (809 mg) and NaN_3 in a Tris-HCl buffer (50 mM, $CaCl_2$ 10 mM,

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

	8: R = Fmoc	**2**: R = H	**14**: R = Fmoc	**15**: R = Fmoc	**12**: R = Fmoc	**6**: R = H	**16**: R = Fmoc	**17**: R = H	**40**: R = Fmoc	**46**: R = H
1 H-1	5.00	5.07	4.99	4.99	5.00	5.06	5.00	5.07	5.00	5.06
2 H-1	4.60	4.61	4.58	4.58	4.61	4.61	4.60	4.62	4.62–4.49	4.61
3 H-1	4.77	4.77	4.76	4.76	4.77	4.77	4.77	4.78	4.78	4.77
4 H-1	5.14	5.13	5.12	5.12	5.14	5.13	5.12	5.13	5.14	5.12
4′H-1	4.95	4.95	4.93	4.93	4.92	4.91	4.94	4.95	4.92	4.91
5 H-1	4.60	4.60	4.58	4.58	4.56	4.60	4.55	4.56	4.62–4.49	4.59
5′H-1	4.60	4.60	4.58	4.58	4.56	4.55	4.55	4.62	–	–
6 H-1	4.45	4.44	4.46	4.33	4.45	4.44	–	–	4.44	4.43
6′H-1	4.45	4.44	4.33	4.47	–	–	4.45	4.52	–	–
3 H-2	4.25	4.25	4.24	4.24	4.25	4.24	4.25	4.25	4.25	4.24
4 H-2	4.20	4.20	4.19	4.19	4.20	4.19	4.19	4.19	4.20	4.18
4′H-2	4.11	4.12	4.11	4.11	4.11	4.10	4.11	4.12	<4.0	<4.0
7 H-3$_{ax}$	1.72	1.71	–	1.84	1.72	1.71	–	–	1.72	1.70
7 H-3$_{eq}$	2.67	2.67	–	2.68	2.67	2.66	–	–	2.67	2.66
7′H-3$_{ax}$	1.72	1.71	1.84	–	–	–	1.79	1.72	–	–
7′H-3$_{eq}$	2.67	2.67	2.68	–	–	–	2.67	2.68	–	–
Asn-CH$_2$	2.73, 2.52	2.94, 2.85	2.72, 2.52	2.72, 2.52	2.72, 2.54	2.90, 2.80	2.72, 2.52	2.94, 2.85	2.73, 2.52	2.91, 2.81
Ac	2.07, 2.07	2.07, 2.06	2.06, 2.05	2.06, 2.05	2.07, 2.07	2.07, 2.06	2.06, 2.06	2.08, 2.07	2.07, 2.07	2.06, 2.06
	2.07, 2.03	2.06, 2.02	2.04, 2.02	2.04, 2.02	2.05, 2.03	2.05, 2.02	2.05, 2.03	2.06, 2.04	2.03, 1.89	2.02, 2.00
	1.89	2.02	1.89	1.89	1.89	2.01	1.89	2.02		
Fmoc	7.92, 7.72		7.91, 7.71	7.91, 7.71	7.92, 7.71		7.92, 7.71		7.92, 7.72	
	7.51, 7.44		7.53–7.41 (Fmoc and Ph)	7.53–7.41 (Fmoc and Ph)	7.51, 7.43		7.51, 7.44		7.50, 7.44	
other			5.38, 5.31 (CH$_2$)	5.38, 5.31 (CH$_2$)						

	18: R = Fmoc	**23**: R = H	**41**: R = Fmoc	**47**: R = H	**19**: R = Fmoc	**24**: R = H	**11**: R = Fmoc	**5**: R = H	**34**: R = Fmoc	**36**: R = H	**35**: R = Fmoc	**37**: R = H
1 H-1	5.00	5.08	5.00	5.07	5.00	5.07	5.00	5.07	4.99	5.07	4.99	5.07
2 H-1	4.62–4.56	4.62	4.63–4.50	4.61	4.64–4.53	4.61	4.58	4.62	4.55	4.61	4.58–4.52	4.61
3 H-1	4.77	4.78	4.78	4.78	4.77	4.76	4.76	4.76	4.76	4.77	4.77	4.78
4 H-1	5.11	5.11	5.14	5.14	–	–	5.12	5.12	5.11	5.11	5.10	5.10
4′H-1	4.95	4.95	–	–	4.94	4.94	4.93	4.92	4.91	4.91	4.91	4.91
5 H-1	–	–	4.63–4.50	4.60	–	–	4.58	4.58	4.55	4.55	–	–
5′H-1	4.62–4.56	4.62	–	–	4.64–4.53	4.59	4.58	4.58	4.55	4.55	–	–
6 H-1	–	–	4.45	4.44	–	–	4.47	4.47	–	–	–	–
6′H-1	4.45	4.45	–	–	4.45	4.44	4.47	4.47	–	–	–	–
3 H-2	4.25	4.26	4.23	4.23	4.28	4.07	4.24	4.24	4.24	4.24	4.24	4.25
4 H-2	4.07	4.08	4.20	4.19	–	–	4.19	4.19	4.18	4.18	4.06	4.06
4′H-2	4.12	4.12	–	–	4.11	4.10	4.11	4.12	4.10	4.10	3.97	3.97
7 H-3$_{ax}$	–	–	1.72	1.71	–	–	–	–	–	–	–	–
7 H-3$_{eq}$	–	–	2.67	2.67	–	–	–	–	–	–	–	–
7′H-3$_{ax}$	1.72	1.72	–	–	1.72	1.71	–	–	–	–	–	–
7′H-3$_{eq}$	2.68	2.68	–	–	2.68	2.67	–	–	–	–	–	–
Asn-CH$_2$	2.73, 2.53	2.94, 2.85	2.73, 2.52	2.92, 2.83	2.73, 2.55	2.93, 2.85	2.72, 2.52	2.93, 2.84	2.72, 2.51	2.80, 2.63	2.72, 2.53	2.79, 2.61
Ac	2.07, 2.07	2.09, 2.07	2.07, 2.05	2.06, 2.06	2.07, 2.07	2.08, 2.06	2.07, 2.05	2.08, 2.05	2.06, 2.05	2.07, 2.05	2.05, 1.88	2.07, 2.01
	2.03, 1.89	2.04, 2.02	2.03, 1.89	2.03, 2.01	2.03, 1.89	2.02, 2.01	2.05, 1.88	2.05, 2.01	2.05, 1.89	2.05, 2.01		
Fmoc	7.92, 7.72		7.92, 7.72		7.93, 7.72		7.91, 7.70		7.91, 7.71		7.91, 7.70	
	7.51, 7.44		7.51, 7.44		7.51, 7.44		7.50, 7.43		7.50, 7.43		7.50, 7.43	

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Table 1. (Continued)

	13: R = Fmoc	**7**: R = H	**28**: R = Fmoc	**29**: R = H	**38**: R = Fmoc	**39**: R = H	**30**: R = Fmoc	**32**: R = H	**45**: R = Fmoc	**51**: R = H	**31**: R = Fmoc	**33**: R = H
1 H-1	5.00	5.06	4.99	5.06	4.99	5.07	4.99	5.07	4.99	5.06	4.99	5.06
2 H-1	4.57	4.60	4.58	4.61	4.57	4.61	4.58	4.61	4.55	4.60	4.55	4.61
3 H-1	4.76	4.76	4.76	4.75	4.77	4.77	4.76	4.76	4.76	4.77	4.76	4.76
4 H-1	5.12	5.11	5.12	5.11	5.12	5.11	5.10	5.10	5.11	5.11	5.10	5.09
4′H-1	4.92	4.91	4.92	4.92	4.91	4.91	4.92	4.92	4.91	4.91	4.91	4.91
5 H-1	4.55	4.57	4.55	4.57	4.57	4.57	–	–	4.55	4.54	–	–
5′H-1	4.55	4.55	4.55	4.55	–	–	4.58	4.57	–	–	4.55	4.54
6 H-1	4.46	4.46	–	–	4.46	4.46	–	–	–	–	–	–
6′H-1	–	–	4.47	4.46	–	–	4.47	4.47	–	–	–	–
3 H-2	4.24	4.28	4.24	4.23	4.24	4.24	4.24	4.24	4.24	4.24	4.24	4.24
4 H-2	4.19	4.18	4.18	4.18	4.19	4.18	4.07	4.06	4.18	4.18	4.06	4.06
4′H-2	4.11	4.10	4.11	4.10	<4.0	<4.0	4.11	4.10	3.97	3.96	4.10	4.10
7 H-3_{ax}	–	–	–	–	–	–	–	–	–	–	–	–
7 H-3_{eq}	–	–	–	–	–	–	–	–	–	–	–	–
7′H-3_{ax}	–	–	–	–	–	–	–	–	–	–	–	–
7′H-3_{eq}	–	–	–	–	–	–	–	–	–	–	–	–
Asn-CH_2	2.72, 2.51	2.88, 2.77	2.72, 2.52	2.87, 2.76	2.72, 2.52	2.90, 2.80	2.72, 2.52	2.90, 2.81	2.72, 2.52	2.88, 2.77	2.72, 2.52	2.87, 2.76
Ac	2.06, 2.05	2.07, 2.04	2.06, 2.05	2.07, 2.04	2.06, 2.05	2.07, 2.04	2.06, 2.04	2.07, 2.04	2.06, 2.05	2.06, 2.04	2.06, 2.05	2.07, 2.04
	2.05, 1.89	2.04, 2.00	2.04, 1.89	2.04, 2.00	1.89	2.01	1.89	2.01	1.89	2.00	1.89	2.00
Fmoc	7.92, 7.72 7.51, 7.44		7.91, 7.71 7.51, 7.43		7.92, 7.71 7.51, 7.43		7.92, 7.71 7.51, 7.44		7.90, 7.70 7.49, 7.42		7.91, 7.70 7.50, 7.43	

	42: R = Fmoc	**48**: R = H	**20**: R = Fmoc	**25**: R = H	**43**: R = Fmoc	**49**: R = H	**21**: R = Fmoc	**26**: R = H	**44**: R = Fmoc	**50**: R = H	**22**: R = Fmoc	**27**: R = H
1 H-1	4.99	5.06	5.00	5.07	4.99	5.07	4.99	5.07	4.99	5.06	5.00	5.07
2 H-1	4.57	4.61	4.58	4.62	4.55	4.61	4.55	4.62	4.57	4.61	4.58	4.62
3 H-1	4.77	4.77	4.75	4.75	4.76	4.77	nd	4.76	4.77	4.77	4.76	4.76
4 H-1	5.12	5.11	–	–	5.12	5.12	–	–	5.11	5.10	–	–
4′H-1	–	–	4.92	4.92	–	–	4.91	4.91	–	–	4.91	4.91
5 H-1	4.57	4.57	–	–	4.55	4.54	–	–	–	–	–	–
5′H-1	–	–	4.58	4.58	–	–	4.55	4.55	–	–	–	–
6 H-1	4.46	4.46	–	–	–	–	–	–	–	–	–	–
6′H-1	–	–	4.47	4.47	–	–	–	–	–	–	–	–
3 H-2	4.22	4.22	4.07	4.08	4.22	4.22	4.07	4.07	4.22	4.22	4.07	4.08
4 H-2	4.19	4.18	–	–	4.18	4.18	–	–	4.07	4.07	–	–
4′H-2	–	–	4.10	4.09	–	–	4.09	4.10	–	–	3.97	3.97
7 H-3_{ax}	–	–	–	–	–	–	–	–	–	–	–	–
7 H-3_{eq}	–	–	–	–	–	–	–	–	–	–	–	–
7′H-3_{ax}	–	–	–	–	–	–	–	–	–	–	–	–
7′H-3_{eq}	–	–	–	–	–	–	–	–	–	–	–	–
Asn-CH_2	2.72, 2.52	2.91, 2.82	2.72, 2.52	2.91, 2.81	2.72, 2.54	2.89, 2.78	2.72, 2.56	2.89, 2.79	2.72, 2.52	2.90, 2.80	2.72, 2.52	2.92, 2.83
Ac	2.05, 2.05	2.05, 2.04	2.07, 2.05	2.08, 2.04	2.05, 2.05	2.06, 2.05	2.07, 2.05	2.07, 2.05	2.05, 1.89	2.05, 2.01	2.07, 1.89	2.07, 2.01
	1.89	2.01	1.89	2.01	1.88	2.01	1.89	2.01				
Fmoc	7.92, 7.71 7.50, 7.43		7.92, 7.71 7.51, 7.44		7.87, 7.67 7.48, 7.41		7.89, 7.68 7.49, 7.42		7.91, 7.70 7.50, 7.43		7.91, 7.71 7.50, 7.43	

[a] ● NeuAc, ● Gal, ● GlcNAc, ● Man.

pH 7.5, 32 mL) and this mixture was incubated for 60 h at 37 °C. During incubation, the pH was kept at 7.5. After 60 h, to this mixture was added Actinase-E (25 mg) and this mixture was further incubated for 55 h. This reaction was monitored by TLC (1 M NH_4OAc/isopropanol 1:1). After the reaction was finished, the mixture was lyophilized and purification of the residue by gel permeation (Sephadex-G-25, ∅2.5 cm × 100 cm, H_2O) afforded pure asparagine-linked sialyloligosaccharide **2** (301 mg).

Fmoc-oligosaccharides 8–11: An HCl solution (80 mM, 11.4 mL) was added to a solution of pure Asn-oligosaccharide **2** (1.07 g, 456 μmol) in water (11.4 mL) and this mixture was stirred at 37 °C. After 6 h, the mixture was cooled to 4 °C and neutralized with aq $NaHCO_3$, and lyophilized. Purification of the residue by gel permeation column (Sephadex G-25: ∅2.5 cm × 100 cm, water) afforded a mixture (778 mg) of disialo substrate **2**, monosialyl oligosaccharides **3**, **4** and asialooligosaccharide **5**[5a] along with **6** and **7**. This mixture was then used for next protecting reaction after lyophilization. To a solution of this mixture (778 mg) in H_2O/acetone (3.8–5.7 mL) was added $NaHCO_3$ (162 mg, 1.93 mmol) and 9-fluorenylmethyl-*N*-succimidylcarbonate (432 mg, 1.28 mmol), and the mixture was stirred at room temperature. After 2 h, the mixture was evaporated to remove acetone, and desalted with ODS-column (∅20 mm ×

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HMPA

1) Fmoc-Ser(*t*Bu)-OH, MSNT, *N*-methylimidazole
2) 20% Ac_2O
3) 20% piperidine
4) Fmoc-Ser(*t*Bu)-OH, HOBt, DIPCDI
5) 20% Ac_2O
6) 20% piperidine

1) **52**, HATU, DIPEA
2) 20% Ac_2O in *i*PrOH:MeOH (1:1)
3) 20% piperidine

1) Fmoc-Val-OPfp, Dhbt
2) 20% Ac_2O
3) 20% piperidine

1) Fmoc-AA-OPfp, Dhbt
2) 20% piperidine 3 x (AA = Leu, Leu, Ala)

1) **11**, HATU, DIPEA
2) 20% Ac_2O in *i*PrOH:MeOH (1:1)
3) 20% piperidine

1) Fmoc-Val-OPfp, Dhbt
2) 20% Ac_2O
3) 20% piperidine

1) Fmoc-AA-OPfp, Dhbt
2) 20% piperidine 3 x (AA = Leu, Leu, Ala)

95% TFA aq

NaOH aq, pH 11

53

52

Scheme 7.

250 mm; eluted with H_2O, 100 mL and then 25% MeCN, 200 mL) afforded a mixture of Fmoc-oligosaccharides **8–13** (681 mg). This mixture was further purified by HPLC: ODS column (YMC packed column D-ODS-5-A 120A, ∅20 mm × 250 mm, 50 mM NH_4OAc/MeCN 82:18, 7.5 mL min^{-1}, monitoring at 215 nm) to obtain disialyloligosaccharide **8** (t_R = 15.7 min), mixture of monosialyloligosaccharide **9**, **10** (t_R = 19.5 min) and asialooligosaccharide **11**[5a] (t_R = 25.5 min). In addition, monosialylnonasaccharide **12** (t_R = 23.3 min) and asialooctasaccharide **13** (28.2 min) were also obtained. This purification was performed repeatedly with ca. 30 mg of mixture each time (total ca. 680 mg). The individual oligosaccharides thus obtained were combined, lyophilized and then desalted with HPLC (ODS-column: ∅5 mm × 150 mm; eluted with H_2O, 100 mL then 25% MeCN, 200 mL) afforded pure oligosaccharides **8** (148 mg, 13%), mixture of **9** and **10** (249 mg, 24%), **11** (101 mg, 11%), **12** (68 mg, 7%) and **13** (35 mg, 4%).

Disialooligosaccharide 8: HRMS: calcd for $C_{103}H_{154}N_8NaO_{66}$: 2581.8838; found: 2581.8821 $[M+Na]^+$.

Asialooligosaccharide 11: HRMS: calcd for $C_{81}H_{120}N_6NaO_{50}$: 1999.6930; found: 1999.6939 $[M+Na]^+$.

Monosialyloligosaccharide 12: HRMS: calcd for $C_{86}H_{125}N_7Na_3O_{53}$: 2172.6995; found: 2172.7084 $[M+Na]^+$.

Asialooctasaccharide 13: HRMS: calcd for $C_{75}H_{110}N_6NaO_{45}$: 1837.6402; found: 1837.6418 $[M+Na]^+$.

Benzyl esterification of monosialyloligosaccharides 14 and 15: A solution of a mixture of Fmoc-monosialyldecasaccharide **9** and **10** (5.0 mg) in cold H_2O (1 mL, 4 °C) was passed through to a column (∅0.5 cm × 5 cm containing Dowex-50W × 8(H^+) resin), and the column was washed with cold water (10 mL). The eluant and the washing were pooled and lyophilized. This residue was dissolved in H_2O (0.22 mL) and neutralized (pH 7) by stepwise addition of a solution of Cs_2CO_3 (2.5 mg mL^{-1}) monitoring with pH meter, and lyophilized. The residue was dissolved in dry DMF (0.43 mL) and was mixed with a solution of BnBr (6.6 μL) in DMF (20 μL) and stirred at room temperature under argon atmosphere. After 48 h, diethyl ether (5 mL) was added and the precipitate formed was collected. Purification of the precipitated material by HPLC (ODS column, ∅20 mm × 250 mm, 50 mM NH_4OAc/MeCN 78:22) afforded monobenzyl-sialyloligosaccharides **14** (91 min) and **15** (88 min). Desalting of the each product by HPLC (ODS-column: ∅5 mm × 150 mm; H_2O 50 mL and then 25% MeCN, 100 mL) afforded pure monobenzyl-sialyloligosaccharides **14** (1.6 mg) and **15** (1.8 mg).

Saccharide 14: FAB-MS: calcd for $C_{99}H_{143}N_{17}NaO_{58}$: 2380.8; found: 2380.1 $[M+H]^+$.

Saccharide 15: FAB-MS: calcd for $C_{99}H_{143}N_{17}NaO_{58}$: 2380.8; found: 2380.5 $[M+H]^+$.

Galactosidase digestion of monosialyldecasaccharide 9 and 10: β-D-Galactosidase (390 mU in 50 mM HEPES buffer, 100 μL, pH 6.0) was added to a mixture of monosialyldecasaccharides **9** and **10** (135 mg, 59.4 μmol) in HEPES buffer (50 mM, pH 6.0, 5.6 mL) containing bovine serum albumin (1.0 mg) and this mixture was incubated at 37 °C for 19 h, and lyophilized. Purification of the residue by HPLC (YMC packed column D-ODS-5-A120A, ∅20 mm × 250 mm, 50 mM NH_4OAc/MeCN 82:18, 7.5 mL min^{-1}) afforded monosialyloligosaccharide **16** (36 min) and **12** (39.2 min). This purification was performed repeatedly with ca. 10–20 mg portions of the reaction mixture (total ca. 120 mg). Desalting of each product by HPLC (ODS-column: ∅5 mm × 150 mm; H_2O 100 mL and then 25% MeCN solution 200 mL) yielded pure oligosaccharides **16** (51 mg, 41%) and **12** (60 mg, 48%).

Monosialylnonasaccharide 16: HRMS: calcd for $C_{86}H_{127}N_7NaO_{53}$: 2128.7356; found: 2128.7363 $[M+Na]^+$.

General purification method for exo-glycosidase digestion: After exo-glycosidase digestion, the mixture was lyophilized,

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and fractionated by HPLC (YMC packed column D-ODS-5 S-5 120A, ∅20 mm × 250 mm, eluted with 50 mM NH_4OAc/MeCN 8:2, 4.0 mL min^{-1}, monitoring at 215 nm) afforded the oligosaccharide. The fraction containing the desired product was pooled and lyophilized. Removal of NH_4OAc was accomplished with HPLC (ODS-column: ∅5 mm × 150 mm; H_2O, 100 mL then 25% MeCN, 200 mL, monitoring at 215 nm) afforded the pure oligosaccharide.

General procedure for N-acetyl-β-D-glucosaminidase digestion: N-Acetyl-β-D-glucosaminidase (ca. 1.6 U in 50 mM HEPES buffer, 100 μL, pH 6.0) was added to a solution of substrate (48 μmol) in HEPES buffer (50 mM, pH 6.0, 1.8 mL) containing bovine serum albumin (1 mg) and incubated at 37 °C. After completion of the digestion (ca. one day) as monitored by HPLC (ODS: ∅5 mm × 150 mm, 50 mM NH_4OAc/MeCN 8:2, monitoring at 215 nm), the mixture was lyophilized.

General procedure for α-D-mannosidase digestion: α-D-Mannosidase (ca. 0.5 U in 50 mM HEPES buffer, 50 μL, pH 6.0) was added to a solution of substrate (24 μmol) in HEPES buffer (50 mM, pH 6.0, 0.9 mL) containing bovine serum albumin (1 mg) and the solution was incubated at 37 °C. In the case of substrate **40**, 16 U of enzyme was used. After completion of the digestion (ca. one day) as monitored by HPLC (ODS: ∅5 mm × 150 mm, 50 mM NH_4OAc/MeCN 8:2, monitoring at 215 nm), the mixture was lyophilized.

General procedure for α-D-neuraminidase digestion: α-D-Neuraminidase (ca. 130 mU in 50 mM HEPES buffer, 50 μL, pH 6.0) was added to a solution of substrate (18.4 μmol) in HEPES buffer (50 mM, pH 6.0, 0.72 mL) containing bovine serum albumin (1 mg) and this solution was incubated at 37 °C. After completion of the digestion (ca. one day) as monitored by HPLC (ODS: ∅5 mm × 150 mm, 50 mM NH_4OAc/MeCN 8:2, monitoring 215 nm), the mixture was lyophilized.

General procedure for β-D-galactosidase digestion: β-D-Galactosidase (ca. 180 mU in 50 mM HEPES buffer, 50 μL, pH 6.0) was added to a solution of substrate (6.2 μmol) in HEPES buffer (50 mM, pH 6.0, 0.6 mL) containing bovine serum albumin (1 mg) and this solution was incubated at 37 °C. After completion of the digestion (ca. 30 h) as monitored by HPLC (ODS ∅5 mm × 150 mm, 50 mM NH_4OAc/MeCN 8:2, monitoring at 215 nm), the mixture was lyophilized.

Compound 13: Substrate **12** (20 mg, 9.4 μmol); α-D-neuraminidase: 141 mU; reaction time: 18 h; **13** (13 mg, 76%).

Compound 18: Substrate **16** (102 mg, 48 μmol); *N*-acetyl-β-D-glucosaminidase: 1.6 U; reaction time: 28 h; **18** (63 mg, 69%); HRMS: calcd for $C_{78}H_{114}N_6NaO_{48}$: 1925.6562; found: 1925.6539 [*M*+Na]$^+$.

Compound 19: Substrate **18** (45 mg, 24 μmol); α-D-mannosidase: 585 mU; reaction time: 20 h; **19** (28 mg, 69%); HRMS: calcd for $C_{72}H_{104}N_6NaO_{43}$: 1763.6034; found: 1763.6074 [*M*+Na]$^+$.

Compound 20: Substrate **19** (32 mg, 18.4 μmol); α-D-neuraminidase: 134 mU; reaction time: 17 h; **20** (13 mg, 52%); FAB-MS: calcd for $C_{61}H_{88}N_5O_{35}$: 1450.5; found: 1450.3 [*M*+H$^+$].

Compound 21: Substrate **20** (9 mg, 6.2 μmol); β-D-galactosidase: 186 mU; reaction time: 32 h; **21** (5.4 mg, 68%); FAB-MS: calcd for $C_{55}H_{77}N_5NaO_{30}$: 1310.5; found: 1310.2 [*M*+Na]$^+$.

Compound 22: Substrate **21** (3.4 mg, 2.6 μmol); *N*-acetyl-β-D-glucosaminidase: 144 mU; reaction time: 24 h; **22** (2.1 mg, 75%); FAB-MS: calcd for $C_{47}H_{65}N_4O_{25}$: 1085.4; found: 1085.3 [*M*+Na]$^+$.

Compound 28: Substrate **16** (28 mg, 21 μmol); α-D-neuraminidase: 198 mU; reaction time: 20 h; **28** (17 mg, 70%); HRMS: calcd for $C_{75}H_{110}N_6NaO_{45}$: 1837.6402; found: 1837.6471 [*M*+Na$^+$].

Compound 30: Substrate **18** (45 mg, 24 μmol); α-D-neuraminidase: 134 mU; reaction time: 14 h; **30** (28 mg, 74%); HRMS: calcd for $C_{67}H_{97}N_5NaO_{40}$: 1634.5608; found: 1634.5564 [*M*+Na]$^+$.

Compound 31: Substrate **30** (11 mg, 6.8 μmol); β-D-galactosidase: 275 mU; reaction time: 14 h; **31** (6.3 mg, 64%); FAB-MS: calcd for $C_{61}H_{88}N_5O_{35}$: 1450.5; found: 1450.4 [*M*+H]$^+$.

Compound 34:[13q] Substrate **11** (123 mg, 62 μmol); β-D-galactosidase: 612 mU; reaction time: 61 h; **34** (71 mg, 70%); HRMS: calcd for $C_{69}H_{100}N_6NaO_{40}$: 1675.5873; found: 1675.5841 [*M*+Na$^+$].

Compound 35: Substrate **34** (50 mg, 30 μmol); *N*-acetyl-β-D-glucosaminidase: 2.1 U; reaction time: 48 h; **35** (25 mg, 66%); MALDI-TOF: calcd for $C_{53}H_{75}N_4O_{30}$: 1247.4, found 1248.0 [*M*+H]$^+$.

Compound 38: Substrate **40** (47 mg, 25 μmol); α-D-neuraminidase: 369 mU; reaction time: 37 h; **38** (26 mg, 65%); HRMS: calcd for $C_{67}H_{97}N_5NaO_{40}$: 1634.5608; found: 1634.5644 [*M*+Na]$^+$.

Compound 40: Substrate **12** (100 mg, 47 μmol); N-acetyl-β-D-glucosaminidase: 1.65 U; reaction time: 20 h; **40** (63 mg, 70%); HRMS: calcd for $C_{78}H_{114}N_6NaO_{48}$: 1925.6562; found: 1925.6533 [*M*+Na]$^+$.

Compound 41: Substrate **40** (51 mg, 26 μmol); α-D-mannosidase: 16 U; reaction time: 25 h; **41** (22 mg, 48%); HRMS: calcd for $C_{72}H_{104}N_6NaO_{43}$: 1763.6034; found: 1763.6041 [*M*+Na]$^+$.

Compound 42: Substrate **41** (28 mg, 16 μmol); α-D-neuraminidase: 117 mU; reaction time: 17 h; **42** (15 mg, 68%); FAB-MS: calcd for $C_{61}H_{88}N_5O_{35}$: 1450.5; found: 1450.3 [*M*+H]$^+$.

Compound 43: Substrate **42** (10 mg, 6.9 μmol); β-D-galactosidase: 205 mU; reaction time: 20 h; **43** (5.6 mg, 64%); FAB-MS: calcd for $C_{55}H_{78}N_5O_{30}$: 1288.5; found: 1288.3 [*M*+H]$^+$.

Compound 44: Substrate **43** (3.6 mg, 2.8 μmol); N-acetyl-β-D-glucosaminidase: 195 mU; reaction time: 24 h; **44** (2.3 mg, 77%); FAB-MS: calcd for $C_{47}H_{65}N_4O_{25}$: 1085.4; found: 1085.3 [*M*+H]$^+$.

Compound 45: Substrate **38** (12 mg, 7.4 μmol); β-D-galactosidase: 297 mU; reaction time: 46 h; **45** (6.6 mg, 61%); FAB-MS: calcd for $C_{61}H_{88}N_5O_{35}$: 1450.5; found: 1450.3 [*M*+H]$^+$.

General procedure for Fmoc deprotection: Morpholine (160 μL) was added to a solution of the Fmoc-substrate (1 μmol) in DMF (240 μL) and this mixture was stirred at room temperature. After finish the reaction (ca. 7–10 h), to this mixture was added Et_2O (4.0 mL) and then precipitate was collected. Purification of the residue by HPLC (ODS-column: ∅5 mm × 150 mm; H_2O, 100 mL then 25% MeCN, 200 mL, monitoring at 215 nm) afforded Asn-oligosaccharide.

Compound 6: FAB-MS: calcd for $C_{71}H_{117}N_7NaO_{51}$: 1906.7; found: 1906.1 [*M*+Na]$^+$.

Compound 7: FAB-MS: calcd for $C_{60}H_{101}N_6O_{43}$: 1593.6; found: 1593.8 [*M*+H]$^+$.

Compound 17: FAB-MS: calcd for $C_{71}H_{118}N_7O_{51}$: 1884.7; found: 1884.5 [*M*+H]$^+$.

Compound 23: FAB-MS: calcd for $C_{63}H_{104}N_6NaO_{46}$: 1703.6; found: 1703.1 [*M*+Na]$^+$.

Compound 24: FAB-MS: calcd for $C_{57}H_{94}N_6NaO_{41}$: 1541.5; found: 1541.3 [*M*+Na]$^+$.

Compound 25: FAB-MS: calcd for $C_{46}H_{77}N_5NaO_{33}$ 1250.4; found: 1250.3 [*M*+Na]$^+$.

Compound 26: FAB-MS: calcd for $C_{40}H_{67}N_5NaO_{28}$: 1088.4; found: 1088.2 [*M*+Na]$^+$.

Compound 27:[22] FAB-MS: calcd for $C_{32}H_{55}N_4O_{27}$: 863.3; found: 863.2 [*M*+H]$^+$.

Compound 29: FAB-MS: calcd for $C_{60}H_{100}N_6NaO_{43}$: 1615.6; found: 1615.0 [*M*+Na]$^+$.

Compound 32: FAB-MS: calcd for $C_{52}H_{88}N_5O_{38}$: 1390.5; found: 1390.1 [*M*+H]$^+$.

Compound 33:[19] FAB-MS: calcd for $C_{46}H_{78}N_5O_{33}$: 1228.5; found: 1228.3 [*M*+H]$^+$.

Compound 36:[13q, 22c] FAB-MS: calcd for $C_{54}H_{90}N_6NaO_{38}$: 1453.5; found: 1453.2 [*M*+Na]$^+$.

Compound 37:[23] FAB-MS: calcd for $C_{38}H_{65}N_4O_{28}$: 1025.4; found: 1025.2 [*M*+H]$^+$.

Compound 39[20]: FAB-MS: calcd for $C_{52}H_{88}N_5O_{38}$: 1390.5; found: 1390.2 [*M*+H]$^+$.

Compound 46:[18] FAB-MS: calcd for $C_{63}H_{104}N_6NaO_{46}$: 1703.6; found: 1703.0 [*M*+Na]$^+$.

Compound 47: FAB-MS: calcd for $C_{57}H_{94}N_6NaO_{41}$: 1541.5; found: 1541.2 [*M*+Na]$^+$.

Compound 48: FAB-MS: calcd for $C_{46}H_{78}N_5O_{33}$: 1228.5; found: 1228.3 [*M*+H]$^+$.

Compound 49:[22c] FAB-MS: calcd for $C_{40}H_{67}N_5NaO_{28}$: 1088.4; found: 1088.3 [*M*+Na]$^+$.

Compound 50: FAB-MS: calcd for $C_{32}H_{55}N_4O_{23}$: 863.3; found: 863.3 [*M*+H]$^+$.

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Compound 51:[21] FAB-MS: calcd for $C_{46}H_{78}N_5O_{33}$: 1228.5; found: 1228.3 $[M+H]^+$.

Synthesis of 52: A solution of Fmoc-disialyloligosaccharide **8** (20 mg) in cold H_2O (2 mL, 4°C) was passed through to a pasteur pipette column (∅0.5 cm × 5 cm) containing resin of Dowex-50W × 8(H^+). The eluant was pooled and lyophilized. To a solution of this residue in H_2O was neutralized by addition of a solution of aq Cs_2CO_3 (2.5 mg in 1 mL H_2O) and this solution was adjusted to pH 6. This solution was then lyophilized. To a solution of this residue in dry DMF (1.3 mL) was added BnBr (5.1 μL) and then the mixture was stirred at room temperature under argon atmosphere. After 45 h, to a solution of this mixture was added diethyl ether (10 mL) and then precipitate was collected. Purification of the residue by ODS-column (∅1.6 cm × 14 cm, H_2O to 40 % MeOH) afforded dibenzyl-sialyloligosaccharide **52** (18.2 mg, 85 %). ^{1}H NMR (400 MHz, 30 °C in D_2O, HOD at 4.81): δ = 8.00 (d, 2H, Fmoc), 7.80 (d, 2H, Fmoc), 7.65–7.50 (m, 12H, Ph, Fmoc), 5.46 (d, 2H, J = 11.6 Hz, $PhCH_2$), 5.40 (d, 2H, J = 11.6 Hz, $PhCH_2$), 5.21 (s, 1H, Man4-H-1), 5.08 (d, 1H, J = 9.3 Hz, GlcNAc1-H-1), 5.02 (s, 1H, Man4′-H-1), 4.86 (s, 1H, Man3-H-1), 4.67 (m, 3H, GlcNAc2,5,5′-H-1), 4.41 (brd, 3H, Gal6, 6′-H-1, Fmoc), 4.33 (brd, 1H, Man3-H-2), 4.27 (brd, 1H, Man4′-H-2), 4.20 (d, 1H, Man4-H-2), 2.79 (brd, 3H, Asn-β-CH_2, NeuAc7, 7′-H3eq), 2.61 (brdd, 1H, Asn-β-CH_2), 2.15 (s, 3H, Ac), 2.12 (s, 6H, Ac × 2), 2.10 (s, 6H, Ac × 2), 1.98 (s, 3H, Ac), 1.93 (dd, 2H, J = 12.2, 12.2 Hz, NeuAc7, 7′-H-3ax); HRMS: m/z: calcd for $C_{117}H_{165}N_8Na_2O_{66}$: 2783.9597, found 2783.9501 $[M+Na]^+$.

Glycopeptide 53: Peptide synthesis on solid phase was performed by Meldal's group procedure[5b] with the HMPA-PEGA resin. The solid phase syntheses were performed by manual procedure using a polypropylene column (Tokyo Rika, No. 183470). The HMPA-PEGA resin (35 mg) was washed with CH_2Cl_2/DMF and then dried in desiccator. The first serine was attached by addition of a solution of Fmoc-Ser(O*t*Bu)-OH (3.8 mg, 10 μmol), MSNT (1-mesitylenesulfonyl-3-nitro-1,2,4-triazole, 2.3 mg, 10 μmol), and *N*-methyimidazole (0.8 mg, 7 μmol) in CH_2Cl_2 (1 mL) and the solution was stirred. After 3 h, this resin was washed by CH_2Cl_2, isopropanol and DMF and then the resin was treated by capping reaction with 20 % acetic anhydride in DMF for 20 min. The mixture was washed with DMF and then treated with 20 % piperidine/DMF (1.5 mL) for 20 min. After washing of the resin with DMF, the second serine was attached to the first serine by the addition of a solution of Fmoc-Ser(O*t*-Bu)-OH (4.6 mg, 12 μmol), HOBt·H_2O (1-hydroxybenzotriazole monohydrate, 1.6 mg, 12 μmol) and DIPCDI (diisopopylcarbodiimide, 1.6 mg, 12 μmol) in DMF (1 mL). An identical washing procedure was performed as for the first serine. Attachment of Asn-linked disialyloligosaccharide **52** (18 mg, 6.3 μmol) was performed with HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate, 2.4 mg, 9.4 μmol) and DIPEA (diisopropylethylamine, 0.8 mg, 6.3 μmol) in DMF/DMSO (0.5–0.5 mL) at room temperature. After 24 h, washing, capping and de-N-Fmoc group were performed by the procedure above described. Attachment of valine, leucine × 2, alanine was performed with corresponding activated pentafluorophenol amino acid (Fmoc-AA-Opfp; valine (4 mg, 8.1 μmol), leucine (3.4 mg, 6.5 μmol), alanine (3.1 mg, 6.5 μmol) in the presence of Dhbt (3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl, 0.1 equiv).[5b] The reaction was monitored by the yellow color of Dhbt. Washing, capping and de-N-Fmoc group were performed by the above conventional procedure. However, capping procedure was only examined after attachment of Val. After completion of condensation with alanine, attachment of asialooligosaccharide **11** was performed using the same procedure as with **52**. Elongation of valine, leucine × 2 and alanine were also examined using the same procedure. After elongation, this glycopeptide was treated with 95 % TFA (1 mL). After 3 h, the glycopeptide was eluted from the reaction column by washing with 95 % TFA and this solution was then concentrated in vacuo at room temperature. After lyophilization of the residue, saponification (pH 11, NaOH solution) of benzyl ester afforded disialylglycopeptide. The saponification (20 min) was performed in NMR tube and monitored by ^{1}H NMR. After neutralization of this mixture by acetic acid, the mixture was lyophilized. Purification of this residue by HPLC (YMC-Pack ODS-A ∅3.0 mm × 250 mm, linear gradient: from 0.1 % TFA solution to 0.1 % TFA containing MeCN 90 % in 90 min) afforded the desired glycopeptide **53** (3.4 mg, total 3.2 % based on first serine attached). ^{1}H NMR (400 MHz, 30 °C in D_2O, HOD = δ 4.81): δ = 5.22, 5.21 (2s, 2 × 1H, Man4′-H1, Man4‴-H1), 5.11 (d, 2H, GlcNAc1-H1, GlcNAc1″-H1), 5.03, 5.01 (2s, 2 × 1H, Man4-H1, Man4″-H-1), 4.86 (dd, 2H, Asn-α), 4.69–4.66 (GlcNAc2, 2″, 5, 5′, 5″, 5‴-H1), 4.61–4.48 (Leu-α × 4, Ser-α × 2, Gal6, 6′, 6″, 6‴-H1), 4.33 (brs, 2H, Man3, 3″-H2), 4.28 (brs, 2H, Man4′, 4‴-H2), 4.20 (brs, 2H, Man4, 4″-H2), 4.20–4.17 (Val-α × 2, Ala-α × 2), 3.00 (dd, 2H, Asn-β × 2), 2.83 (dd, 2H, Asn-β × 2), 2.76 (dd, 2H, NeuAc7, 7′-H3eq), 2.16–2.10 (Ac × 10, Val-β × 2), 1.82 (dd, 2H, NeuAc7, 7′-H3ax), 1.70–1.60 (m, Leu-β, -γ), 1.60, 1.49 (2d, 2 × 3H, Ala-β), 1.02–0.96 (m, 36H, Val-γCH_3 × 4, Leu-CH_3 × 8); MALDI-TOF: m/z: calcd for $C_{200}H_{334}N_{24}O_{123}$: 5040.05, found: 5041.64 $[M+H^+]$.

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