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Chemical Synthesis of a Glycoprotein Having an Intact Human Complex-Type Sialyloligosaccharide under the Boc and Fmoc Synthetic Strategies

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Abstract: The chemical synthesis of complex glycoproteins is an ongoing challenge in protein chemistry. We have examined the synthesis of a single glycoform of monocyte chemotactic protein-3 (MCP-3), a CC-chemokine that consists of 76 amino acids and one N-glycosylation site. A three-segment native chemical ligation strategy was employed using unprotected peptides and glycopeptide. Importantly, the synthesis required the development of methods for the generation of sialylglycopeptide-$^{\alpha}$thioesters. For the sialylglycopeptide-$^{\alpha}$thioester segment, we examined and successfully implemented approaches using Fmoc-SPPS and Boc-SPPS. To avoid use of hydrogen fluoride, the Boc approach utilized minimal side chain protection and direct thiolysis of the resin bound peptide. Using these strategies, we successfully synthesized a glycoprotein having an intact and homogeneous complex-type sialyloligosaccharide.

Introduction

A number of proteins are co- or post-translationally modified with oligosaccharides in the endoplasmic reticulum (ER) or the Golgi apparatus. Importantly, it has been found that oligosaccharides play important roles in many biological processes.[1−4] For example, high mannose-type oligosaccharides are essential for efficient protein folding in the ER.[5−7] In addition, complex-type oligosaccharides are important for protein function involving glycoprotein half-life in the blood and consequently affect diabetes, antigen-dependent cellular cytotoxicity, and lung disease.[1−4] These studies clearly show the importance of complex-type oligosaccharides for protein function. However, due to the microheterogeneity (glycoform) of the oligosaccharides that reside on natural proteins, it is difficult to identify the specific oligosaccharide structures that are essential for an individual biological process or protein function. To obtain single protein glycoforms, a number of strategies have been developed. For the preparation of glycoproteins having mature oligosaccharides, expression systems using CHO cells or yeast[8] have been developed. However, these methods are limited by the heterogeneity of the resultant oligosaccharide products. To complement these biological methods, an efficient chemical synthesis can yield glycoproteins in which both the oligosaccharide structure and location in the polypeptide sequence can be precisely controlled. However, such a preparation method has yet to be established.

To efficiently synthesize glycoproteins, synthetic control over both the N-linked oligosaccharide and the polypeptide is required. In previous work, we prepared 35 different complex-type asparagine-linked oligosaccharides and examined their utility in the syntheses of sialylglycopeptides.[9−12] In addition, over the past few years, it has been shown that full-length nonglycosylated proteins can be synthesized using the native chemical ligation (NCL) strategy[13] enabling analysis of the functional and conformational properties of synthetic proteins. In this approach, two peptides are separately prepared: one bearing a C-terminal $^{\alpha}$thioester and the other, an N-terminal cysteine. Using these unique functional groups, we ligated the two unprotected segments in neutral aqueous solution.[13] The critical challenge in applying this technique to the preparation of glycoproteins is the synthesis of a peptide-$^{\alpha}$thioester having complex-type oligosaccharides.[14−19] These peptides are chal-

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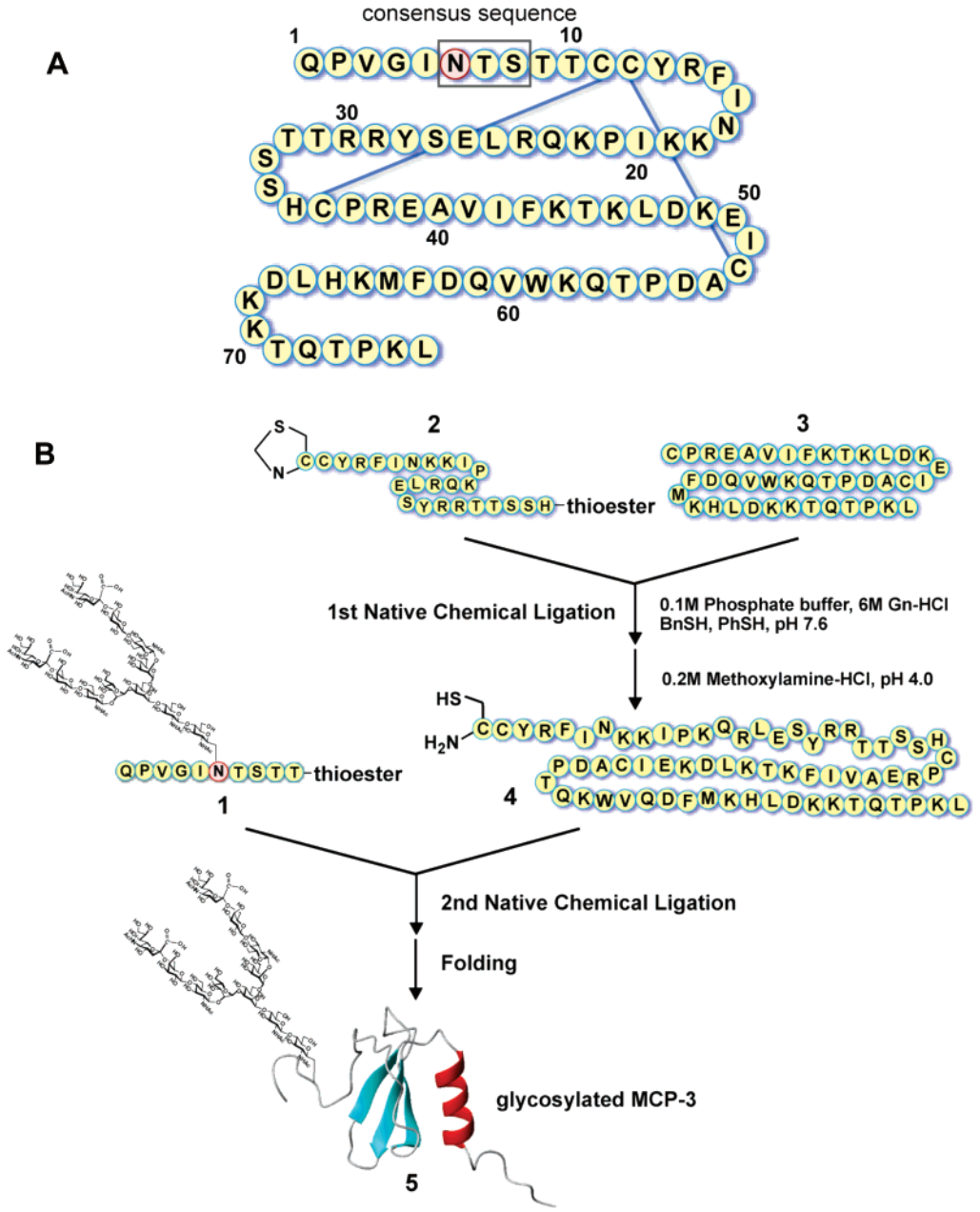

Figure 1. (A) Amino acid sequence of MCP-3. Disulfide bonds between cysteines are indicated as blue line. The N-terminus, Q, is a pyroglutamic acid. (B) Synthetic strategy of glycosylated MCP-3 **5** using native chemical ligation. Oligosaccharide is the complex-type disialyloligosaccharide.

lenging since oligosaccharides are sensitive to the strong acids used in Boc-solid-phase peptide synthesis (SPPS) and $^{\alpha}$thioesters are poorly compatible with base treatments in Fmoc-SPPS. To investigate new approaches for glycopeptide-$^{\alpha}$thioester synthesis and glycoprotein assembly, we selected the glycoprotein monocyte chemotactic protein-3 (MCP-3). MCP-3 consists of 76 amino acid residues with two disulfide bonds[20,21] and has a single consensus sequence for N-glycosylation (Asn-X-Thr).[21] Here, we report the development of methods to synthesize glycopeptide-$^{\alpha}$thioesters that are sufficiently robust to enable the chemical synthesis of glycoproteins having a mature, human, complex-type sialyloligosaccharide.

Results

Synthetic Strategy for Glycosylated MCP-3. The peptide sequence of MCP-3 is shown in Figure 1A. The N-terminus is a pyroglutamate (Pyr), and residues 6−8 encompass an NTS

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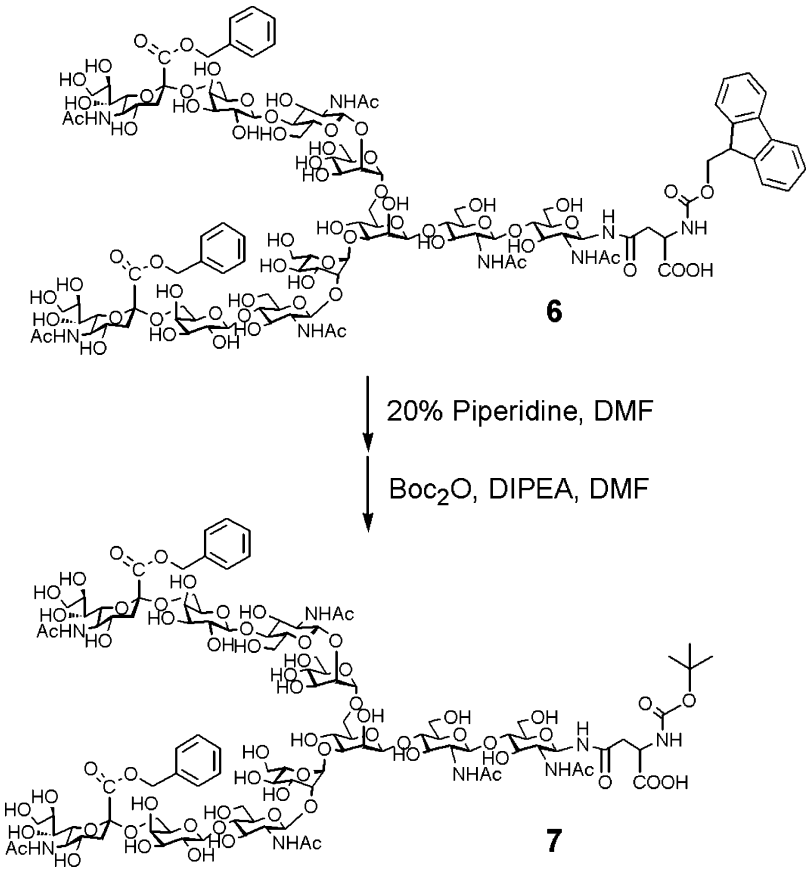
Scheme 1. Synthesis of Boc-Asn(BnCHO)-OH **7**



consensus sequence for N-glycosylation. In addition, two disulfide bonds are formed between Cys11−Cys36 and Cys12−Cys52 (Figure 1A). For the synthesis of glycosylated MCP-3 **5**, we employed a repetitive NCL strategy using three unprotected polypeptides. Using the native chemical ligation strategy at Cys residues, we could assemble MCP-3 from three peptides, **1**(1−10), **2**(11−35), and **3**(36−76) (Figure 1B). This strategy places the glycosylated Asn in peptide **1**, which must be synthesized as a C-terminal peptide-$^{\alpha}$thioester. In addition, segment **2** must be protected to prevent intramolecular cyclization; therefore, we used the 1,3-thiazolidine-4-carboxoyl (Thz) group.[22] Since segments **2** and **3** contain no post-translational modifications, they were synthesized by standard Boc- SPPS.[23] The critical step for this synthetic strategy was the preparation of a sialylglycopeptide-$^{\alpha}$thioester. Therefore, we developed synthetic approaches to synthesize segment **1** using both a minimal protection Boc-SPPS strategy and an efficient Fmoc strategy.[18]

A Minimal Protection Strategy Approach for a Sialylglycopeptide-$^{\alpha}$thioester, Segment 8 Based on Boc-SPPS. Although Boc-SPPS is the method of choice for peptide-$^{\alpha}$-thioesters,[23−26] the strong acid deprotection (HF) limits its use for glycopeptide-$^{\alpha}$thioesters. However, Boc-SPPS avoids treatment of the growing polypeptide chain with strong bases or nucleophiles, so it is advantageous for $^{\alpha}$thioester peptides. Although the Boc strategy has been considered unsuitable for the synthesis of acid labile glycopeptides, we aimed to develop a method for the synthesis of acid-sensitive glycopeptide-$^{\alpha}$-thioesters taking advantage of the *N*-Boc protection. Such an approach would expand the synthetic utility of Boc methods for glycopeptide synthesis. To carry out the Boc strategy in the synthesis of the glycopeptide-$^{\alpha}$thioester, side chain deprotection and cleavage from the resin with HF must be avoided. Resin-bound peptide-$^{\alpha}$thioesters can be treated directly with thiols to yield free-peptide-$^{\alpha}$thioesters, providing an efficaciously mild method for their cleavage from the resin.[27] In addition, side chain protection groups requiring HF must be avoided. Previously, we demonstrated the peptide-coupling conditions necessary to avoid esterification of hydroxyl groups in oligosaccharide during Fmoc solid-phase glycopeptide synthesis.[19] As a result, we expected these conditions would not acylate Ser and Thr hydroxyl groups. Since the peptide sequence of segment **1** (QPVGIN(CHO)TSTT) has no amino acid residue requiring the side chain protection during peptide elongation, a Boc-SPPS glycopeptide approach was pursued. For more complex peptides, there are alternative protection strategies compatible with thioesters (for example, Alloc) that could be envisioned for side chain protection.[28] With this minimal protection scheme in mind, we investigated a novel synthetic procedure using Boc chemistry to obtain segment **1**, a sialylglycopeptide-$^{\alpha}$thioester using side chain unprotected Boc-Ser-OH and Boc-Thr-OH on a mercap-

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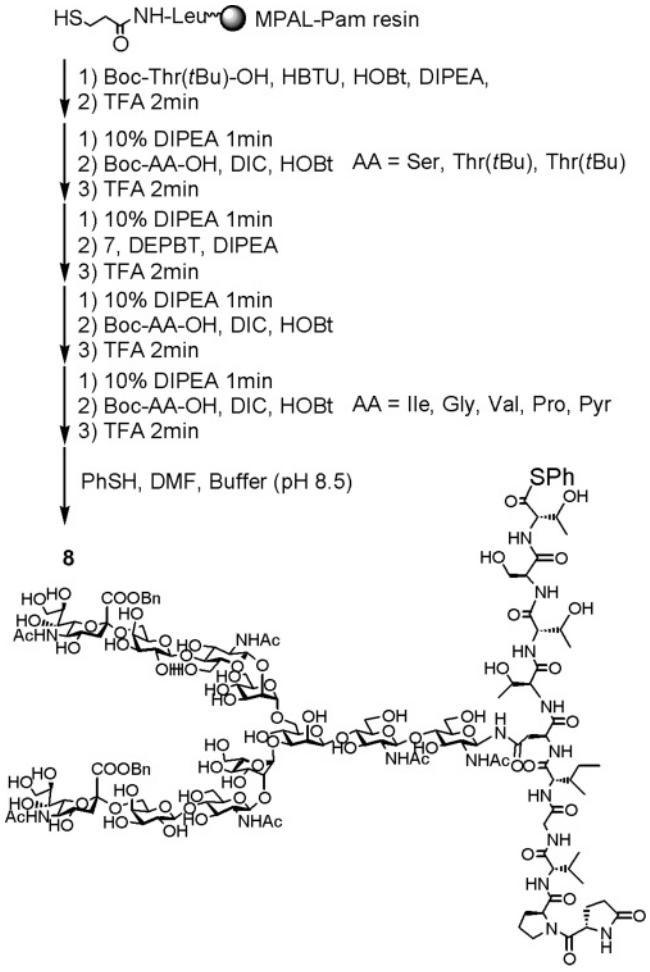
Scheme 2. Synthesis of Segment **1** as a Sialylglycopeptide-$^{\alpha}$thiophenylester **8** by Use of Boc Chemistry and Thioester Exchange Reaction for the Cleavage from the Resin



toproprionyl-Leu-Pam-resin. First, we prepared Boc-Asn(BnCHO)-OH **7**, as shown in Scheme 1. The Fmoc group of compound **6** was removed by 20% piperidine, and then Boc anhydride was applied to afford Boc-Asn(BnCHO)-OH **7** in good yield (Scheme 1).

Next, we examined the synthesis of segment **8**, an $^{\alpha}$thiophenylester type of segment **1**, as shown in Scheme 2. The first amino acid, Thr, was attached to the thiol group of the mercaptopropionic acid-leucine-linked Pam resin (2.0 μmol scale) using HBTU/HOBt/diisopropylethylamine (DIPEA). Subsequent peptide elongation was performed using DIC/HOBt to avoid esterification of the sugar hydroxyl groups by activated amino acids.[19] Introduction of the critical Boc-(BnCHO)Asn-OH **7** to the peptide resin was carried out using DEPBT/DIPEA (2 equiv),[19] and the coupling reaction was repeated three times, yielding an 80% coupling yield. As shown in our Fmoc approach,[19] we believe this poor coupling is due to the nature of the polystyrene solid support used here, and hydrophilic resins such as PEGA are more amenable to the coupling of large carbohydrates to peptides. After construction of the desired peptide using the DIC/HOBt coupling method, we examined the cleavage procedure utilizing thioester exchange reaction with thiophenol (PhSH). When the resin was treated with the cocktail (DMF/0.1 M phosphate buffer containing 6 M Gn-HCl (pH 8.5)/PhSH = 16:4:1), we succeeded in obtaining segment **8** as a sialylglycopeptides-$^{\alpha}$thiophenylester in 13% recovered yield

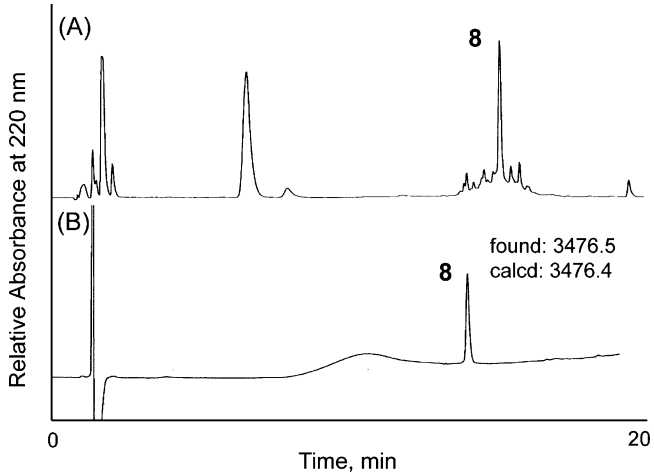


Figure 2. RP-HPLC profiles and ESI-mass of obtained products. (A) Crude sample of **8** after cleavage from the resin. (B) Purified desired product, QPVGIN(BnCHO)TSTT-SPh **8**. HPLC elution condition: column, Cadenza CD-18 (3 μm, 4.6 × 75 mm) at a flow rate of 1.0 mL min^{-1}. (A) Isocratic elution for 5 min and then linear gradient of 4.5−67.5% CH_3CN containing 0.09% TFA in 0.1% TFA aqueous over 15 min. (B) Isocratic elution for 2 min and then linear gradient same as that in (A).

(Figure 2). The obtained compound was characterized by ESI mass spectrometry and HPLC. To confirm its purity, we measured the ^{1}H NMR spectrum of this glycopeptide-$^{\alpha}$-thiophenylester **8**. However, $^{\alpha}$thioester peptide **8** exhibited a slight decomposition during analysis. Therefore, we used the corresponding glycopeptide-$^{\alpha}$thioethylester for the NMR, due to its greater stability toward nucleophiles.[29] To obtain the glycopeptide-$^{\alpha}$thioethylester, a cleavage solution containing ethanthiol (DMF/0.1 M phosphate buffer containing 6 M Gn-HCl (pH 8.5)/PhSH/EtSH = 16:4:1:5) was used. This condition afforded the glycopeptide-$^{\alpha}$thioethylester in good yield. Using this stable $^{\alpha}$thioethylester peptide, we measured the ^{1}H NMR spectrum of glycopeptide and confirmed its purity (Supporting Information).

Synthesis of Segment 1 as a Sialylglycopeptide-$^{\alpha}$thiobenzylester 11 Based on Fmoc-SPPS. We have reported an efficient synthetic procedure for glycopeptide-$^{\alpha}$thioesters using a Fmoc strategy.[18] First, a glycopeptide with protecting groups on the side chain of the peptide is synthesized using standard Fmoc-SPPS on a mild acid labile HMPB resin,[30] enabling the fully side chain protected glycopeptide to be efficiently removed from the resin. After cleavage from the resin, the free C-terminal carboxylic acid is converted to the corresponding thioester using optimized coupling conditions, thus minimizing epimerization at the C-terminal amino acid residue. The use of this approach for glycopeptides is especially attractive since the carbohydrate moiety greatly improves the solubility of the fully protected peptides. As shown in Scheme 3, the first amino acid residue, Thr, was introduced to the HMPB-PEGA resin using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT). The peptide elongation was carried out using a DIC/HOBt coupling method except for the oligosaccharyl asparagine. Fmoc-Asn(BnCHO)-OH **6** was coupled (>95%) with peptide resin using DEPBT/DIPEA. After elongation of the peptide, the resin was treated with acetic acid and trifluoroethanol solution to cleave the side

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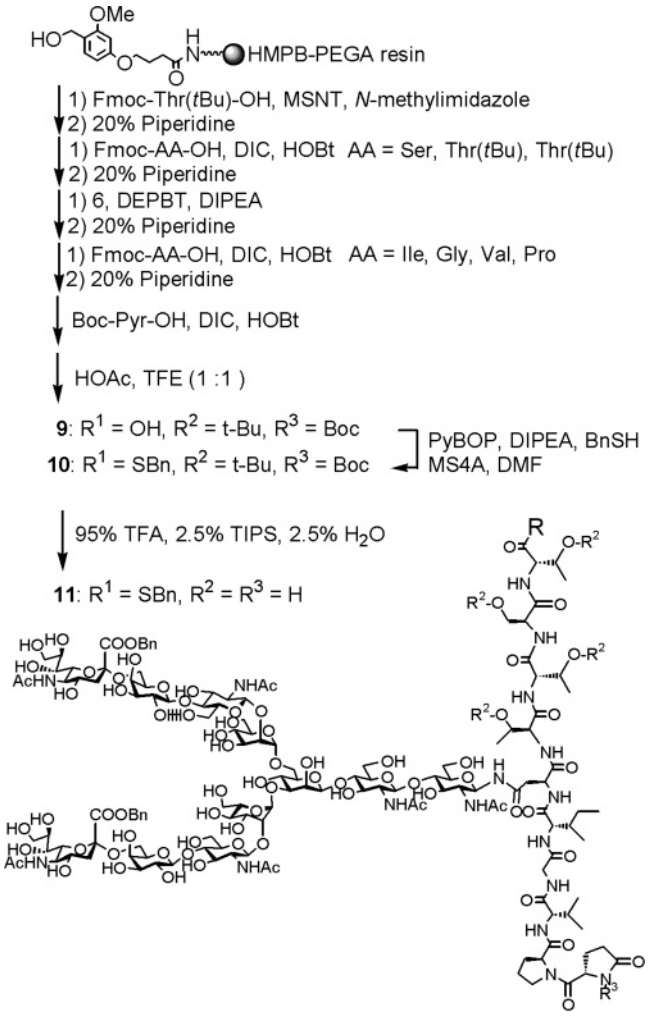
Scheme 3. Synthesis of Segment **1** as a Sialylglycopeptide-$^{\alpha}$thiobenzylester **11** Using Fmoc Approach



chain protected glycopeptide **9** from the resin. This cleavage reaction was repeated three times, and the combined mixture was evaporated in vacuo. Compound **9** was characterized by HPLC and ESI mass analysis (Figure 3A). Without further purification, $^{\alpha}$thioesterification with benzylmercaptan (BnSH) was performed at the C-terminus of protected glycopeptide **9** using an optimized condition, PyBOP/DIPEA at −20° for 2 h, avoiding epimerization at the C-terminus[18] (Figure 3B). After the $^{\alpha}$thioesterification, the crude product **10** was treated with 95% TFA, 2.5% triisopropylsilane (TIPS), and 2.5% water to remove the protecting groups of the side chain (Figure 3C). Finally, HPLC purification afforded segment **1** as a sialylglycopeptide-$^{\alpha}$thiobenzylester **11**, in 43% yield. Compound **11** was characterized by NMR and ESI mass analysis. The resulting glycopeptide was homogeneous as determined by HPLC and ^{1}H NMR spectrum analysis (Figure 4).

Synthesis of Glycosylated MCP-3 Using Repetitive Native Chemical Ligation. Segments **2** and **3** were synthesized by manual Boc-SPPS. The first native chemical ligation between segments **2** and **3** to afford segment **4** was performed in 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine·HCl, 1% (vol/vol) BnSH, and 1% (vol/vol) PhSH (final pH ≈ 6.5). This ligation proceeded cleanly and afforded the desired ligation product. Without further purification, conversion of Thz to Cys at the N-terminus of the ligation product was performed using

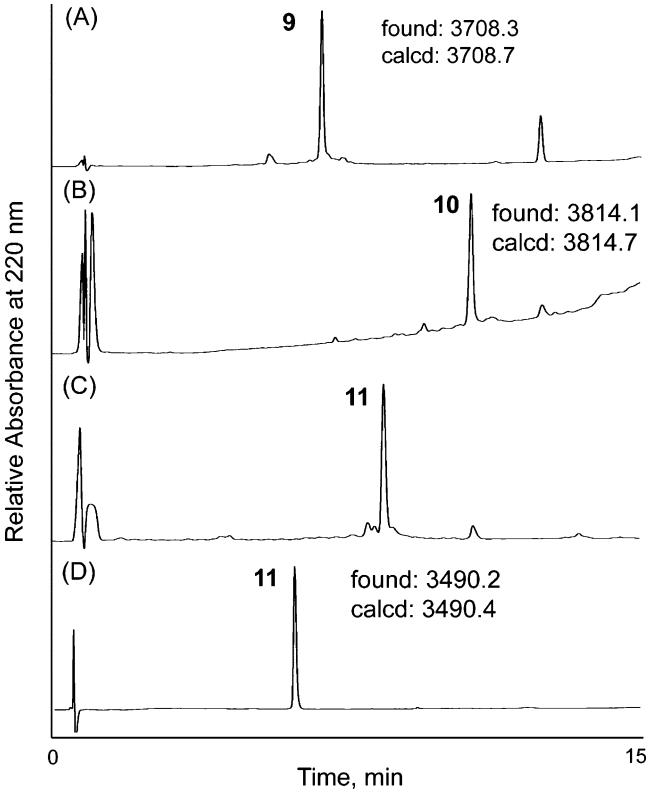


Figure 3. RP-HPLC profiles and ESI-mass of obtained products. (A) Crude sample of **9** after cleavage from the resin, (B) crude sample after $^{\alpha}$thiobenzyl esterification, (C) crude sample after 95% TFA treatment, (D) purified segment **11**, QPVGIN(BnCHO)TSTT-SBn. HPLC elution condition: column, Cadenza CD-18 (3 μm, 4.6 × 75 mm) at a flow rate of 1.0 mL min^{-1}, linear gradient of 36−90% CH_3CN containing 0.09% TFA in 0.1% TFA aqueous over 15 min for (A) and (B), 4.5−67.5% for (C), 18−54% for (D).

methoxylamine hydrochloride, as described by Bang and Kent,[22] to afford segment **4**. After purification of segment **4** by HPLC, a second native chemical ligation was carried out using segments **11** and **4** (Figure 5). Segments **11** and **4** were dissolved in 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine·HCl, 1% (vol/vol) BnSH, and 1% (vol/vol) PhSH (final pH ≈ 6.5). The concentrations of **11** and **4** were 1 and 2 mM, respectively. The reaction was performed at 37 °C. After 17 h, segment **11** was added to the reaction mixture again (final concentration of segment **11**, 2 mM) and incubated for an additional 17 h at 37 °C. The second native chemical ligation reaction afforded the desired product **12**, which was characterized by mass spectrometry and HPLC (Figure 5B). When glycopeptide-$^{\alpha}$-thiophenylester **8** was used for this second native chemical ligation, the ligation between **8** and **4** was finished within 20 h (Supporting Information). Oxidative folding was undertaken without purification by diluting the ligation mixture with five volumes of 0.1 M Tris/HCl buffer (pH 8.0) and bubbling air for 1 min. The reaction mixture was then incubated for 24 h at room temperature.

HPLC and ESI mass spectrometry analysis of the reaction mixture revealed that the main peak of the desired product **13** eluted at an earlier time than that of original sample **12** (Figure 5C). It is known that the folded form elutes earlier than that of the unfolded form in the case of a chemokine (nonglycosylated protein).[31] This clean conversion, monitored by HPLC, suggested that the folding reaction occurred smoothly and afforded the folded form even in the presence of a large and highly

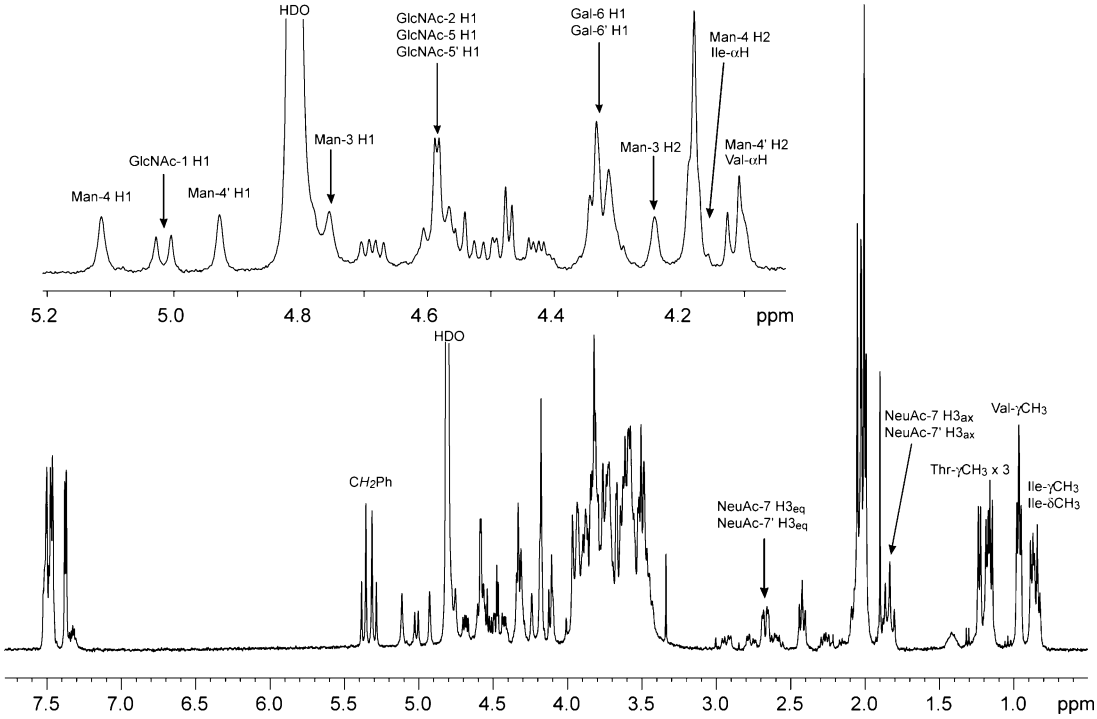


Figure 4. ^{1}H NMR spectrum of sialylglycopeptide-$^\alpha$thiobenzylester **11**.

hydrophilic oligosaccharide moiety such as a complex-type *N*-glycans on the peptide backbone. The multiply charged ion peaks of **13** also indicated the loss of 4 Da compared with that of the linear form **12**, and these mass data indicated that glycoprotein **13** adopted two disulfide bonds. After HPLC purification of the folded form **13** (Figure 5D), it was treated with 50 mM NaOH solution for 10 min to remove benzyl ester groups on the NeuAc residues of glycoprotein **13**, which was characterized by ESI mass spectrometry and HPLC (Figure 5E,F).

To confirm the conformational properties of the synthetic glycosylated MCP-3 **5**, we examined the disulfide bond connectivity and measured the CD spectrum. To give insight into the disulfide bond pattern, we employed a standard strategy using chymotrypsin digestion and subsequent HPLC and mass analysis of the resultant peptide fragments (Supporting Information). Chymotrypsin treatment of glycosylated MCP-3 afforded major fragment **14** along with a minor product **15** (Figure 6). The major product purified by HPLC was found to have a molecular weight corresponding to the desired glycopeptide fragment **14**. This analysis clearly indicated that the synthetic glycosylated MCP-3 has two disulfide bonds between Cys11/12 and Cys 36/52. However, the minor product **15** (ca. 7% purity based on HPLC analysis) was found to have an undesired disulfide bond between Cys36 and Cys52. The major glycopeptide fragment **14** was further reduced by tris(2-carboxy ethyl)phosphine to remove one of the two disulfide bonds. The reduction afforded suitable glycopeptide fragments **16** and **17** along with two peptide fragments **18** and **19**. The molecular weights analyzed by ESI-mass were found to correspond with the molecular weight of the desired fragments **16**−**19** shown in Figure 6. In addition, CD spectrum of glycosylated MCP-3 **5** was measured. CC chemokines have been shown to display a weak CD spectrum with minima at approximately 207 and 220 nm.[32] As shown in SI Figure 6, synthetic glycosylated MCP-3 exhibited the expected CD spectrum that is consistent with a single α helix and a more poorly formed β sheet that is characteristic of these proteins. Furthermore, we examined an ELISA assay using anti MCP-3 monoclonal antibodies under sandwich ELISA procedure (SI Figure 7). This assay clearly indicated that synthetic glycosylated MCP-3 has the two correct epitope structures. Taken together, these analyses support the correct folding of the protein with greater than 90% purity. These studies demonstrate the successful synthesis of glycosylated MCP-3 **5** having an intact complex-type N-linked sialyloligosaccharide, representing glycoprotein synthesis incorporating a pure and intact human complex-type sialyloligosaccharide.

Discussion

In the preparation of synthetic glycoprotein, the synthesis of glycopeptide intermediates having intact oligosaccharides has been a long-standing problem. Our development of methods for the preparation of complex N-linked oligosaccharide asparagine derivatives at the gram scale coupled with convenient synthetic methods to incorporate these residues into peptides is a promising strategy for glycoprotein synthesis.[9−12] To incorporate these oligosaccharide derivatives into the larger polypeptide chains that compose proteins, efficient and practical methods for synthesizing glycopeptide-$^\alpha$thioester need to be developed. Here we have investigated two approaches, one based on Boc minimal side chain protection and the other based on Fmoc methods. Together, these approaches promise to greatly extend synthetic access to complex glycoproteins.

In the case of Boc-SPPS, Pam resin was used as a solid support. Although Pam resin seemed to not be ideal for glycopeptide synthesis using the highly hydrophilic Boc-Asn-(CHO)-OH **7**, the desired glycopeptide was attained using

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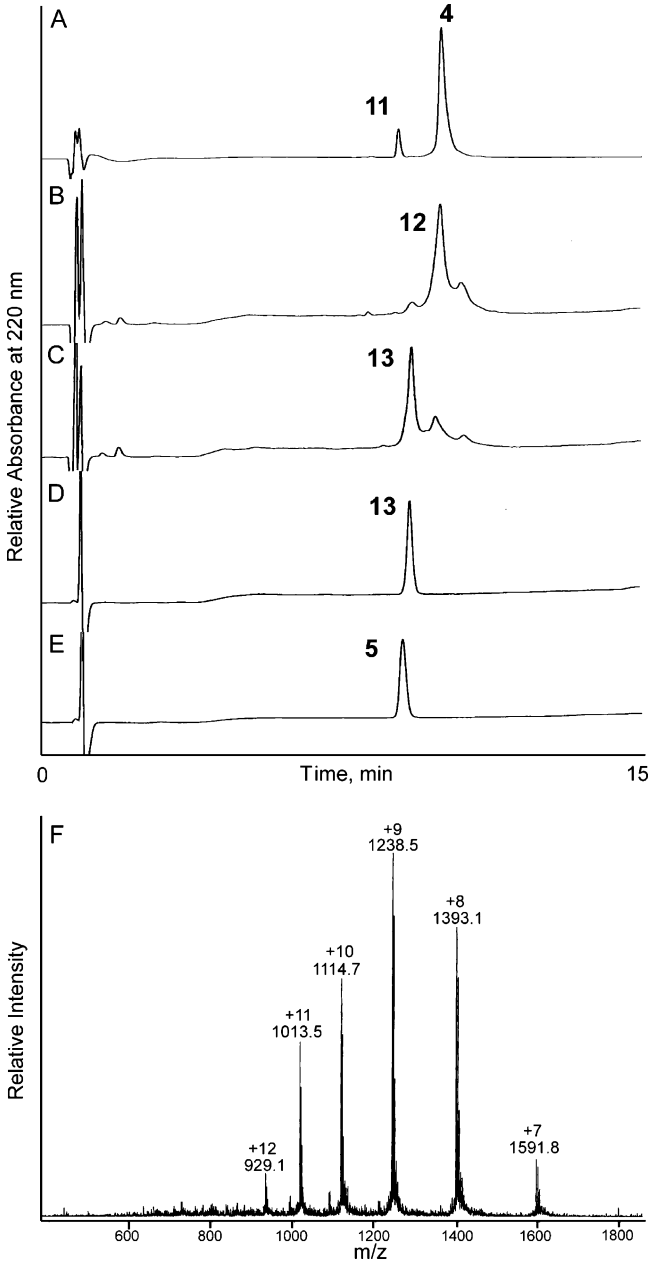

Figure 5. RP-HPLC profiles and observed mass of final product. (A) Ligation reaction mixture after 0 min, (B) ligation reaction mixture after 48 h, (C) crude sample after folding, (D) purified product after folding, (E) purified glycosylated MCP-3 after saponification of benzyl ester of NeuAc. HPLC elution condition: column, Cadenza CD-18 (3 μm, 4.6 × 75 mm) at a flow rate of 1.0 mL min^{-1}, linear gradient of 18−54% CH_3CN containing 0.09% TFA in 0.1% TFA aqueous over 15 min. (F) ESI mass spectra of glycosylated MCP-3 **5**. The Na^+ and K^+ adducted ion peaks were also observed.

repetitive coupling of Boc-Asn(CHO)-OH **7**. After attaching oligosaccharyl-Asn to the peptide resin, we carefully performed elongation of the peptide, avoiding esterification of the sugar hydroxyl groups by the activated *t*Boc-amino acids. Previously, we found a peptide elongation condition, which could avoid esterification of the free hydroxyl groups on oligosaccharides using activated Fmoc-amino acids at 40 mM.[19] Synthesis of segment **8** under these conditions, but using *t*Boc-amino acids, avoided the undesired esterification of both the unprotected sugar and Ser hydroxyl groups. As shown in Scheme 2 and Figure 2, construction of glycopeptide **8** was performed smoothly, and the desired glycopeptide was obtained as the major product. The critical element of this strategy was releasing the glycopeptide-$^{\alpha}$thioester using a nucleophilic substitution reaction with an additional free thiol. Using several thiols and reaction times, we developed an optimal condition of 8 h with thiophenol (25 equiv). In addition, thiols, such as benzylmercaptan and ethanethiol, released the desired glycopeptide-$^{\alpha}$thioester in good yield.

Although the peptide used in this work was compatible with Boc-SPPS without the use of any protecting groups, many peptides will need some side chain protection. To examine this strategy for any peptide sequence, alternative side chain protecting groups are required. In addition, Alloc- and allyl-type groups could be used for the side chain protection of Arg, Lys, Asp, and Glu. Some commonly used group such as His(DNP) and Cys(S-tBu) can be removed by thiol treatment. Future studies will focus on using alternative side chain protection and hydrophilic peptide resins.[27] The advantage of the Boc-SPPS method is that the C-terminal $^{\alpha}$thioester is obtained without overactivation of the C-terminus of the polypeptide, which often leads to epimerization.

The second approach for glycopeptide-$^{\alpha}$thioester synthesis is to assemble the glycopeptide by Fmoc-SPPS on a mild-acid labile HMPB-PEGA resin. We have found that PEGA resin is essential for the preparation of glycopeptides in good yield.[19] The high swelling and hydrophilic nature of the solid support may contribute to this result. The side chain protected glycopeptide can then be converted into a thioester with optimized conditions using PyBOP and diisopropylethylamine. The key to this approach is the solubilizing properties of the unprotected carbohydrate moiety for the side chain protected peptide. This enhanced solubility enables high concentrations of peptide to be attained, increasing the reaction rate and reducing epimerization. As the polypeptide segment size increases, the solubility may decrease, making conversion to the glycopeptide-$^{\alpha}$thioester more challenging. In such cases, the Boc-SPPS approach may find greater utility. As a result, the further development of the two complementary methods for glycopeptide-$^{\alpha}$thioester synthesis will greatly facilitate synthetic access to glycoproteins.

The MCP-3 synthesis was completed by first ligating segments **2** and **3**, synthesized by a conventional Boc strategy, affording the desired MCP-3 11−76 polypeptide. A second native chemical ligation between glycopeptide-$^{\alpha}$thioester **8** or **11** and segment **4** afforded the desired full-length glycopeptide sequence of glycosylated MCP-3 in good yield, as shown in Figure 5B. These data clearly show that a peptide having a large oligosaccharide chain at the polypeptide termini can be used for ligation reactions. Indeed, this ligation was performed at a sterically hindered C-terminal Thr residue, known to have slow ligation kinetics.[23] In the case of ligation using a glycopeptide-$^{\alpha}$thiophenylester **8**, the reaction velocity was 2 times faster than that of the glycopeptide-$^{\alpha}$thiobenzylester **11**, as we expected. This may indicate that reactivity of $^{\alpha}$thioester affects ligation rather than steric hindrance of the oligosaccharide chain. The resulting MCP-3 glycopeptide was folded using air oxidation, as previously described for several chemokines.[31] As shown in Figure 5C, the folded product **13** eluted earlier than the unfolded product **12** by HPLC, as has been observed for other chemokines. In addition, the high efficiency of the folding process is similar to nonglycosylated chemokines. After saponification of

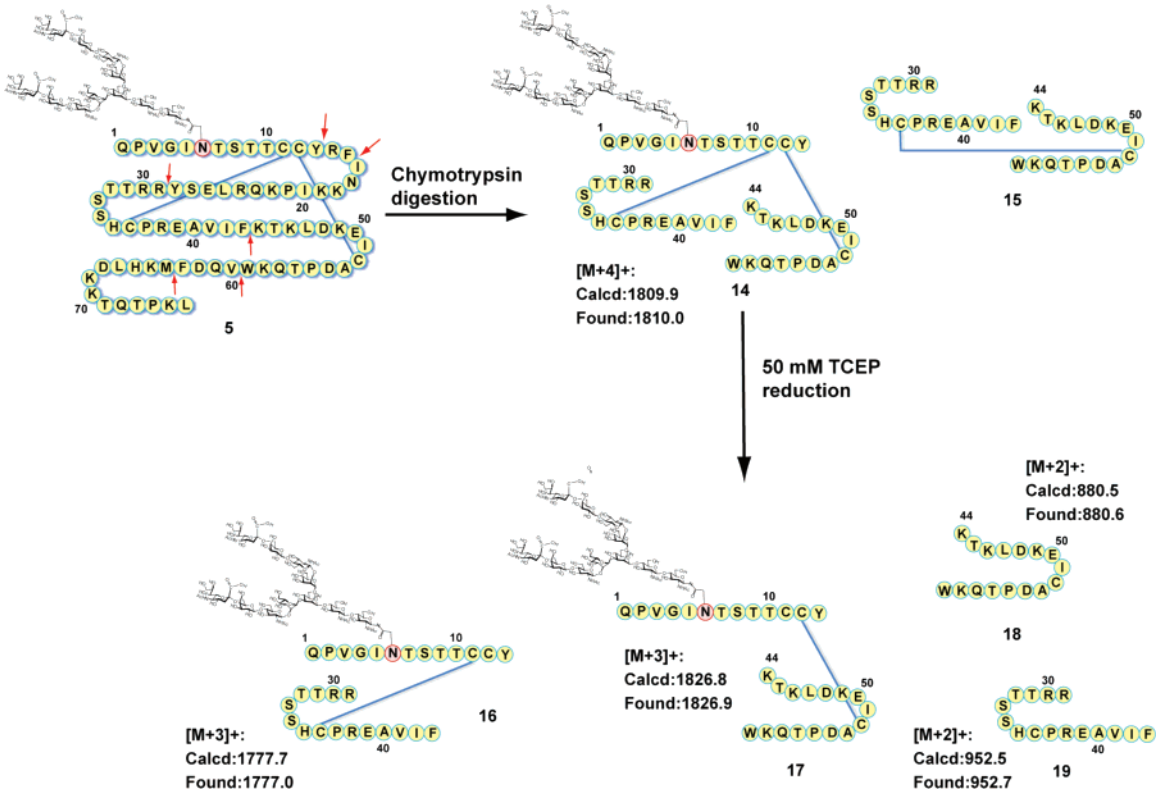

Figure 6. Evaluation of disulfide bonds by chymotrypsin digestion.

the benzyl group of the sialyloligosaccharide, we obtained the synthetic complex glycoprotein, glycosylated MCP-3.

The oxidative folding procedure yielded a largely homogeneous folded product. A minor product **15** (ca. 7% purity based on HPLC analysis) was observed through chymotrypsin digestion, and this product was found to have an incorrect disulfide bond (Figure 6). Because disulfide bond formation during protein folding is an equilibrium reaction between correct folding and misfolding, contamination of undesired disulfide bond formation might not be avoidable in the synthesis. In addition, proteolysis can induce scrambling of the disulfide bond during enzyme digestion.[33] Therefore, we could not confirm when this undesired disulfide bond formation was formed. In terms of a major folded glycoprotein, chymotrypsin digestion afforded a single glycopeptide fragment **14** first and then this fragment **14** was gradually digested to another fragment, concurrently decreasing the first glycopeptide fragment **14**. If the undesired fragment is contaminated, the HPLC profile might display an additional HPLC peak. However, a major glycopeptide fragment **14** was a single product under several HPLC analytical conditions. Although we could not perfectly distinguish between the desired and misfolded protein, our investigation for disulfide bond positions using mass analysis, CD spectrum, and simple ELISA assay was consistent with desired glycosylated MCP-3. Our synthetic methodology may be useful for research into such glycoprotein folding experiments and conformational analysis through chemical approaches.

Conclusion

Here we have achieved the synthesis of glycopeptide-$^{\alpha}$-thioesters using both Boc and Fmoc strategies. Significantly, this Boc method will afford a new approach to the preparation of oligosaccharyl peptide-$^{\alpha}$thioesters. Optimizing protection methods for all amino acid side chains during Boc-SPPS needs to be established and will be solved through extensive optimization, as has been done for previous strategies that seek to combine peptide and oligonucleotide synthesis.[34] In this work, we have achieved the chemical synthesis of a glycoprotein with a full complex-type sialyloligosaccharide, a structure that has not previously been incorporated in a synthetic glycoprotein. In addition, we conducted a simple bioassay, ELISA, with this synthetic glycoprotein. This means that the technology should be useful for research into functional glycomics through a chemical approach. Currently, research is underway to generate several different glycoforms and to analyze their functional and conformational properties.

Experimental Section

Materials and Methods. NMR spectra were measured with a Bruker Avance 400 (internal standard HOD = 4.81 ppm in D_2O solution) instrument. Boc-Leu-OCH_2Pam-resin, HMPA-PEGA resin, Fmoc-AA-OH derivatives, Boc-AA-OH derivatives, and *S*-tritylmercaptopropionic acid were purchased from Merck, Applied Biosystems, and Peptide Institute Inc. Other chemical reagents were also commercially available. RP-HPLC analyses were carried out on a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Cadenza column (Imtakt Corp., 3 μm, 75 $\times$ 4.6 mm) at a flow rate of 1.0 mL min^{-1}. ESI mass measurement was carried out on a Bruker Daltonics/Esquire3000 plus. The CD spectrum was acquired in the range 190−275 nm with 1-mm path length on a JASCO J-720 spectrometer.

Synthesis of Dibenzyl-Esterified Sialyloligosaccharyl Boc-Asparagine 7. Dibenzyl-esterified sialyloligosaccharyl Fmoc-asparagine **6** (63

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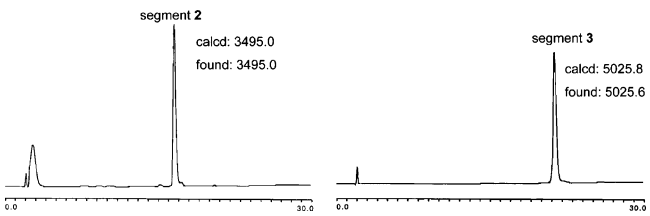
mg, 23 μmol) was dissolved in 20% piperidine/DMF (4.6 mL, 5 mM) and then stirred for 1 h at room temperature. The reaction mixture was poured into ethyl ether (60 mL), and the precipitate was filtrated. The precipitate was dissolved into 0.1% formic acid solution, and then the solution was lyophilized or evaporated in vacuo. Purification of the residue by gel permeation (Sephadex-G25, 20 mm × 900 mm) afforded dibenzyl-esterified disialyloligosaccharyl asparagine (43 mg, 74%). ^{1}H NMR (400 MHz, 295 K in D_2O, HOD = δ 4.81) δ 7.50−7.40 (m, 10H, Ph), 5.34 (d, 2H, J = 11.8 Hz, PhCH_2), 5.27 (d, 2H, J = 11.8 Hz, PhCH_2), 5.08 (s, 1H, Man4-H-1), 5.03 (d, 1H, J = 9.7 Hz, GlcNAc1-H-1), 4.90 (s, 1H, Man4′-H-1), 4.73 (s, 1H, Man3-H-1), 4.55 (m, 3H, GlcNAc2, 5, 5′-H-1), 4.29 (bd, 2H, Gal6, 6′-H-1), 4.22 (bd, 1H, Man3-H-2), 4.15 (bd, 1H, Man4′-H-2), 4.07 (bd, 1H, Man4- H-2), 2.90 (dd, 1H, J = 17.0 Hz, 4.5 Hz Asn-βCH$_2$), 2.83 (dd, 1H, J = 17.0 Hz, 6.8 Hz, Asn-βCH$_2$), 2.64 (bdd, 2H, NeuAc7, 7′-H$_{3eq}$), 2.04 (s, 3H, Ac), 2.00 (s, 6H, Ac × 2), 1.98 (s, 6H, Ac × 3), 1.81 (dd, 2H, J = 12.4, 13.0 Hz, NeuAc7, 7′-H-3$_{ax}$); ESI-MS m/z calcd for $[M + H]^+$ 2518.4, found 2518.0 (deconvoluted).

To the solution of dibenzyl-esterified disialyloligosaccharyl asparagine (43 mg, 17 μmol) in DMF (4.2 mL, 4 mM), diisopropylethylamine (22 mg, 170 μmol) and Boc_2O (37 mg, 170 μmol) were added, and then the mixture was stirred for 1.5 h at room temperature. Then the reaction mixture was poured into ethyl ether (120 mL). The precipitate was filtrated and then was dissolved into water. The solution was lyophilized or evaporated in vacuo. Purification of the residue by gel permeation (Sephadex-G25, 20 mm × 900 mm) afforded dibenzyl-esterified sialyloligosaccharyl Boc-asparagine **7** (41 mg, 93%). ^{1}H NMR (400 MHz, 295 K in D_2O, HOD = δ 4.81) δ 7.54−7.42 (m, 10H, Ph,), 5.38 (d, 2H, J = 11.8 Hz, PhCH_2), 5.31 (d, 2H, J = 11.8 Hz, PhCH_2), 5.12 (s, 1H, Man4-H-1), 5.07 (d, 1H, J = 9.6 Hz, GlcNAc1-H-1), 4.94 (s, 1H, Man4′-H-1), 4.77 (s, 1H, Man3-H-1), 4.60 (m, 3H, GlcNAc2, 5, 5′-H-1), 4.33 (bd, 2H, Gal6, 6′- H-1), 4.30 (m, 1H, Asn-αH), 4.25 (bs, 1H, Man3-H-2), 4.19 (bd, 1H, Man4′-H-2), 4.11 (bd, 1H, Man4- H-2), 2.78 (dd, 1H, J = 15.1, 3.7 Hz Asn-βCH$_2$), 2.68 (bdd, 2H, NeuAc7, 7′-H$_{3eq}$), 2.60 (dd, 1H, J = 15.1, 8.4 Hz, Asn-βCH$_2$), 2.07 (s, 3H, Ac), 2.04 (s, 6H, Ac × 2), 2.02 (s, 6H, Ac × 3), 1.84 (dd, 2H, J = 12.4, 12.4 Hz, NeuAc7, 7′-H-3$_{ax}$); ESI-MS m/z calcd for $[M + H]^+$ 2618.5, found 2618.0 (deconvoluted).

Synthesis of Sialylglycopeptide-$^\alpha$thiophenylester 8, QPVGIN(Bn-CHO)TSTT-SPh. Trityl-associated mercaptopropionic acid-leucine (TAMPAL) Pam resin was prepared according to the reported procedure.[23] After removal of the trityl protecting group with two 1-min treatments with 2.5% triisopropylsilane and 2.5% H_2O in TFA, mercaptopropionic acid-leucine (2.0 μmol, MPAL) resin was incubated for 1 h with Boc-Thr(*t*Bu)-OH (11 μmol), HBTU (10 μmol), HOBt (10 μmol), and DIPEA (30 μmol) in DMF (100 μL). Further peptide elongation (Thr-Ser-Thr) was carefully performed using 5.0 equiv of Boc-amino acid derivatives to the resin with DIC/HOBt under the low concentration condition (concentration of amino acid was estimated to adjust to 40 mM in the reaction mixture) to avoid unexpected esterification toward hydroxyl groups on the oligosaccharide. Each synthetic cycle was repeated as follows: (1) removal of Boc group by a 2-min treatment with neat TFA, (2) washing with DCM and then DMF, (3) neutralizing with 10% DIPEA in DMF for 1 min, (4) second washing by DMF, (5) 1 h coupling time with 10 μmol of preactivated Boc-amino acid derivative, and (6) third washing with DMF and then DCM. For introduction of oligosaccharyl asparagines to the peptide resin, Boc-Asn(CHO)-OH **7** (4 μmol) was coupled using DEPBT (8 μmol) and DIPEA (4 μmol) in DMF (100 μL) for 16 h. This coupling reaction was repeated three times. Further peptide elongation was carefully performed using the above-mentioned procedure. After the complete assembly of the sequence (QPVTGIN(Bn-CHO)TSTT), the resin was treated with DMF (400 μL), 0.1 M phosphate buffer containing 6 M Gn/HCl (pH 8.5, 100 μL), and PhSH (25 μL) for 20 h. This cleavage procedure by use of thioester exchange reaction was repeated three times. The combined mixture was diluted with 30% acetonitrile in water containing 1% TFA and then lyophilized. Purification of the crude product by RP-HPLC afforded pure sialylglycopeptide-$^\alpha$thiophenylester **8** (1 mg, 13%). ESI-MS m/z calcd for $[M + H]^+$ 3476.4, found 3476.5 (deconvoluted).

Synthesis of Sialylglycopeptide-$^\alpha$thiobenzylester 11, QPVGIN-(Bn-CHO)TSTT-SBn. Synthesis of sialylglycopeptide in which peptide side chains were protected was performed by a manual Fmoc procedure (2 μmol scale).[18] The first amino acid, Fmoc-Thr-(*t*Bu)-OH (3 equiv), was attached to the PEGA resin using MSNT (3.0 equiv) and *N*-methylimidazole (2.75 equiv) in CH_2Cl_2 (250 mM). The peptide elongation was performed by treatment with corresponding Fmoc-amino acid derivatives (5.0 equiv), DIC (5.0 equiv), and HOBt (5.0 equiv) in DMF (0.4 M) for 1.0 h. Fmoc-Asn(Bn-CHO)-OH **6** (2.0 equiv) was coupled with peptide resin employing DEPBT (3.0 equiv) and DIPEA (2.0 equiv) in DMF (30 mM). After introduction of **6** to the peptide resin, the peptide was elongated by the DIC/HOBt method. For this elongation, the concentration of Fmoc-amino acid was arranged to be 40 mM in DMF to avoid unexpected esterification toward hydroxyl groups on the oligosaccharide. Deprotection of the Fmoc group was performed with 20% piperidine in DMF for 20 min. After construction of glycopeptide, a glycopeptide **9** in which peptide side chains were protected was released from the resin by use of acetic acid/trifluoroethanol (1:1, 2.0 mL), and this treatment was repeated twice. The solution thus obtained was concentrated in vacuo. The residue containing a crude glycopeptide **9** was dissolved into DMF and then concentrated as a coevaporation three times. To a solution of this crude glycopeptide **9** in DMF (0.2 mL, 5 mM) were added molecular sieves 4 Å (10 mg) and benzylthiol (30 equiv), and then this mixture was stirred at −20 °C. After 1 h, PyBOP (5 equiv) and DIPEA (5 equiv) were added to this mixture, and the mixture was stirred at −20 °C. After 4 h, the solution was filtrated, and ethyl ether (6.0 mL) was added to this filtrate to give a precipitate of glycopeptide-$^\alpha$thioester **10**. The precipitate was collected by centrifugation. Toward this precipitate was added a solution containing 95% TFA, 2.5% TIPS, and 2.5% H_2O to remove protecting groups for 2 h. Then the solution was concentrated in vacuo. Purification of the residue by RP-HPLC afforded the desired sialylglycopeptide-$^\alpha$thiobenzylester **11** (3.0 mg, 43%). ^{1}H NMR (400 MHz, 295 K in D_2O, HOD = δ 4.81) δ 7.54−7.35 (m, 15H, Ph,), 5.38 (d, 2H, J = 11.8 Hz, PhCH_2), 5.28 (d, 2H, J = 11.8 Hz, PhCH_2), 5.10 (s, 1H, Man4-H-1), 5.01 (d, 1H, J = 9.6 Hz, GlcNAc1-H-1), 4.92 (s, 1H, Man4′-H-1), 4.77 (s, 1H, Man3-H-1), 4.70 (dd, 1H, Pyr-αH), 4.25 (bs, 1H, Man3-H-2), 2.91 (bdd, 1H, Asn-βCH$_2$), 2.75 (bdd, 1H, Asn-βCH$_2$), 2.65 (bdd, 2H, NeuAc7, 7′-H$_{3eq}$), 1.84 (m, 3H, Ile-β CH$_2$, NeuAc7, 7′-H-3$_{ax}$), 1.23, 1.15, 1.13 (each d, each 3H, Thr-γCH$_3$), 1.97, 1.93 (each d, each 3H, Val-γCH$_3$), 1.86 (d, 3H, Ile-γCH$_3$), 1.82 (dd, 3H, Ile-δCH$_3$); ESI-MS m/z calcd for $[M + H]^+$ 3490.4, found 3490.2 (deconvoluted).

Synthesis of Glycosylated MCP-3 Using Repetitive Native Chemical Ligation. A peptide segment **3** and a peptide-$^\alpha$thioester segment **2** were manually prepared by solid-phase peptide synthetic methods based on "in situ neutralization" Boc chemistry[35] toward -OCH$_2$-Pam resin and MPAL-Pam resin,[23] respectively.



The first native chemical ligation reaction was performed in a phosphate buffer (pH7.5, 100 mM) solution containing 6 M Gn/HCl, 1% thiophenol (v/v) and 1% benzylmercaptan (v/v). In this ligation, concentration of each peptide and segments **2** and **3** was adjusted to 2 mM. After the reaction was completed, Thz was converted into Cys

peptide by 0.2 M methoxyamine/HCl (pH 4.0). The HPLC purification of the reaction mixture afforded the desired polypeptide segment **4** (ESI; *m*/*z* calcd for $[M + H]^+$ 7957.3, found 7957.0 (deconvoluted)). The second ligation between segment **4** and sialylglycopeptide-$^{\alpha}$thiobenzylester segment **1** was then performed. Segment **4** (0.2 mg, 24 nmol) and sialylglycopeptide-$^{\alpha}$thiobenzylester **11** (0.05 mg, 14nmol) were dissolved into 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine (40 μL). To the solution, 1% (vol/vol) BnSH and 1% (vol/vol) PhSH were added. The reaction mixture was left at 37 °C for 17 h. Then additional sialylglycopeptide-$^{\alpha}$thiobenzylester **11** (0.05 mg, 14 nmol) was added to this reaction mixture, and the mixture was further incubated for 17 h. The reaction afforded desired product **12** (ESI; *m*/*z* calcd for $[M + H]^+$ 11318.3, found 11319.5 (deconvoluted)). Without purification of the mixture, to examine a fording reaction, the mixture was diluted by adding five volumes of 0.1 M Tris/HCl buffer (pH 8.0), and then air bubbling was performed for 1 min. The reaction mixture was incubated for 24 h at room temperature. Purification of the mixture by RP-HPLC afforded the pure ligation product **13** (ESI; *m*/*z* calcd for $[M + 7H]^{7+}$ 1617.4, $[M + 8H]^{8+}$ 1415.3, $[M + 9H]^{9+}$ 1258.2, $[M + 10H]^{10+}$ 1132.4, $[M + 11H]^{11+}$ 1029.6, $[M + 12H]^{12+}$ 943.9, found 1617.5, 1415.5, 1258.4, 1132.7, 1029.9, 944.2), and subsequently, the product was treated with 50 mM NaOH for 10 min to remove the benzylester of NeuAc residues. As a result, the desired product, glycosylated MCP-3 **5** (0.05 mg, 17%), was obtained in excellent purity, which was characterized by mass spectrometry (ESI; *m*/*z* calcd for $[M + 7H]^{7+}$ 1591.6, $[M + 8H]^{8+}$ 1392.8, $[M + 9H]^{9+}$ 1238.1, $[M + 10H]^{10+}$ 1114.4, $[M + 11H]^{11+}$ 1013.2, $[M + 12H]^{12+}$ 928.9, found 1591.8, 1393.1, 1238.5, 1114.7, 1013.5, 929.1).

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In the case of the second NCL using glycopeptide-$^{\alpha}$thiophenylester, segment **4** (2 mg, 25 nmol) and sialylglycopeptide-$^{\alpha}$thiobenzylester **8** (0.9 mg, 25 nmol) were dissolved into 0.1 M phosphate buffer (pH 7.6, 495 μL) containing 6 M guanidine and 1% (vol/vol) PhSH. The reaction mixture was incubated at 37 °C for 20 h.

Chymotrypsin Digestion. Glycosylated MCP-3 (ca. 0.1 mg) was dissolved in the phosphate buffer (pH 8.6, 100 mM), and then an appropriate amount of chymotrypsin was added. After 4 h, products were isolated by HPLC (column, Cadenza CD-18 (3 μm, 4.6 × 75 mm)) at a flow rate of 1.0 mL min^{-1}, linear gradient of 18−54% CH_3-CN containing 0.09% TFA in 0.1% TFA aqueous over 15 min) and then mass analysis was examined.

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Supporting Information Available: ^{1}H NMR of glycopeptide-$^{\alpha}$thioethylester, native chemical ligation using glycopeptide-$^{\alpha}$thiophenyester **8**, chymotrypsin digestion, CD spectrum, and ELISA assay for **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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