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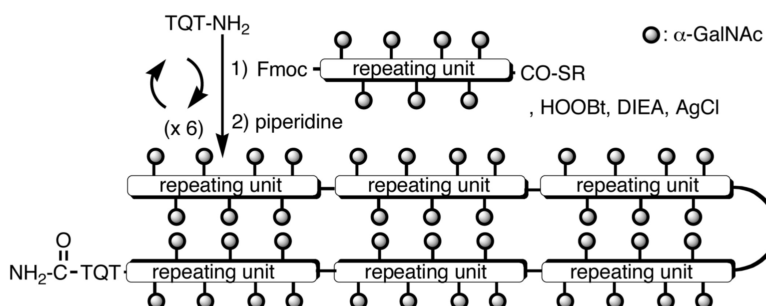

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Chemical Synthesis of 23 kDa Glycoprotein by Repetitive Segment Condensation: A Synthesis of MUC2 Basal Motif Carrying Multiple O-GalNAc Moieties

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Abstract: Peptide thioester corresponding to a MUC2 tandem repeat unit, which retains seven GalNAc moieties, was prepared by the Fmoc method followed by the low TfOH treatment to remove benzyl groups at the carbohydrate portions. The glycosylated peptide thioester was then consecutively joined by the activation of a thioester group by silver ions to obtain a MUC2 tandem repeat model composed of 141 amino acids with 42 GalNAc moieties.

Introduction

The carbohydrate on proteins, which is usually attached to the peptide backbone via N- or O-linkage, has been shown to play essential roles in many biological processes, such as protein folding, cell−cell interaction, and tumor metastasis.[1] However, the details of these functions are not yet known, which mainly derive from the microheterogeneity of the carbohydrate portion. In the case of N-linked carbohydrate, the glycosylation usually occurs at the consensus sequence of Asn-X-Ser/Thr. In addition, the structure around the reducing end region is common to all N-linked glycans. In contrast, the site of O-glycosylation, which is typically found in the tandem repeat region of mucins, is solely determined by the action of glycosyl transferases, which cannot be predicted at present. Furthermore, the mucin-type O-glycan has a more diverse structure compared to that of the N-glycan and usually exists in a dense clustered form. Due to these complex features, the functional and structural analysis of mucins remains a difficult task.

To overcome these difficulties, various homogeneous glycopeptides have been synthesized as models of natural mucins.[2−12] It is known that in the course of malignant transformation, the synthesis of O-glycan becomes incomplete and short carbohydrates, such as Tn (α-GalNAc), T (Gal-GalNAc), and sialyl-Tn antigen, are highly expressed on mucins. Synthetic models have been used to characterize the epitope structure of these tumor-associated mucins[13,14] and to develop efficient cancer vaccines.[15,16] Synthetic models have also been used as substrates to analyze the specificity of various glycosyl transferases as well as for structural characterization of mucins.[17−24] Most of these studies use glycopeptide models composed of a single repeat sequence or a sequence within a repeat unit to eliminate difficulties associated with the repeating character of mucins. It has been made clear that the binding of monoclonal antibodies

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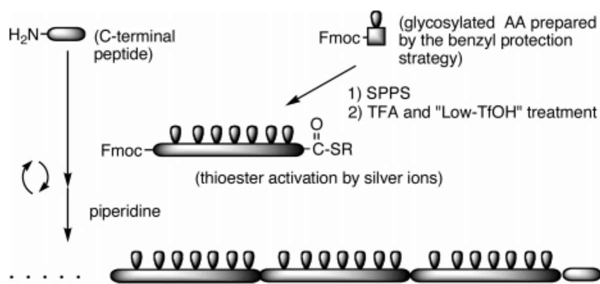

Figure 1. A general scheme for the preparation of homogeneous tandem repeat of mucins by the thioester method combined with benzyl-protection strategy at the carbohydrate portion.

created against tumor-associated mucins to the repeating sequence of MUC1 becomes stronger as the number of repeating unit increases,[25,26] which means that the conformation and the function of the tandem repeat are dependent on the length of the peptide chain. Thus, if the repeating glycopeptide with sufficient length can be prepared, such a model would clarify a more accurate function and structure of mucins. In addition, the model would be a candidate to create effective vaccines in point of its stable conformation as well as of its sufficient size for immunization without carrier proteins. We have been establishing a facile method of glycoprotein synthesis based on the thioester method,[27,28] combining the benzyl-protection strategy at the carbohydrate portion.[29,30] Here, we report the extension of this procedure for the preparation of MUC2 basal structure carrying multiple O-GalNAc moieties at specific sites.

Results and Discussion

Synthetic Strategy. Our synthetic strategy is shown in Figure 1. Fmoc-serine or threonine carrying various carbohydrates are prepared based on our benzyl-protection strategy and are introduced during the solid-phase synthesis of the peptide thioester by the Fmoc method. After the chain assembly, the resin is treated with TFA to deprotect the peptide portion and is followed by "low trifluoromethanesulfonic acid (TfOH)"[31] to remove the benzyl groups. The latter treatment has been shown to deprotect various N- and O-linked carbohydrates, with remaining glycosidic linkages intact.[32−35] Then, the glycopeptide thioester is consecutively joined by the thioester method to obtain homogeneous tandem repeat structure.

To accomplish this strategy, there are two points which need consideration. First is the preparation of a peptide thioester carrying multiple carbohydrates. In mucins, Ser or Thr residues often exist consecutively. The bulky glycosylated amino acids generally retain reduced reactivity during the coupling reaction, and thus their repeated introduction might yield much defective peptides during the solid-phase synthesis. The sequence of the repeating unit of MUC2 is an extreme case, as it retains five consecutive Thr at around 10 residues from the C-terminal, where the reactivity of the resin-bound peptide itself is decreased because of the β-sheet formation.[36] In addition, all benzyl groups have to be completely removed from the dense clustered carbohydrates. The second point is whether the repetition of segment coupling proceeds efficiently. Although three consecutive segment condensations by the thioester method have already been achieved in the synthesis of many proteins,[28] the reaction here is more challenging because the repeating unit retains many free hydroxyl groups at the carbohydrate portions, which potentially receive O-acylation depending on reaction conditions. If these requirements are fulfilled, this strategy will be generally applicable for the synthesis of tandem repeated glycoproteins and also for globular glycoproteins. Here, we demonstrate the potential of our method by synthesizing the hexa-repeats of the MUC2 unit carrying multiple Tn antigens at specific sites. This appears to be the largest glycoprotein ever synthesized by a fully chemical procedure.

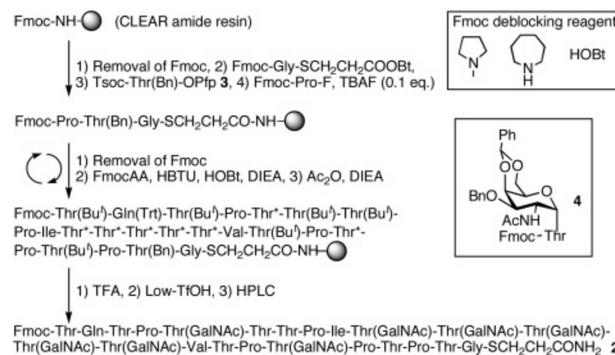

Figure 2. Synthetic route for the repeating unit of MUC2 carrying seven GalNAcs **2**. The asterisk denotes the protected GalNAc moiety.

Solid-Phase Synthesis of a Repeating Unit. The synthetic route for the MUC2 unit is shown in Figure 2. The sequence of this unit is reported to be PTTTPITTTTTVTPTPTPTGTQT.[37] To avoid the epimerization of the C-terminal amino acid residue during the segment coupling reaction, the original sequence was designed to shift three residues so that the carboxyl terminal of this sequence is made to be glycine. Among 14 potential O-glycosylation sites within this sequence, we selected seven sites and introduced Tn-antigen for this initial study. The consecutive five Thr residues were included for the glycosylation site for the mimicry of the dense cluster of carbohydrates of mucins as well as for the synthetic challenge described above. The glycosylated peptide thioester of the repeating unit was prepared by the modified Fmoc strategy reported previously for peptide thioester synthesis.[34] First, C-terminal glycine was introduced using Fmoc-Gly-SCH_2CH_2COOH by the 1,3-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method. The Fmoc group was then cleaved by the mixture of 1-methylpyrrolidine, hexamethyleneimine, and HOBt, which effectively cleaves the Fmoc groups with the thioester linkage remaining intact.[38] The amino group was then reacted with the second amino acid Tsoc-Thr(Bn)-OPfp **3**, which is protected

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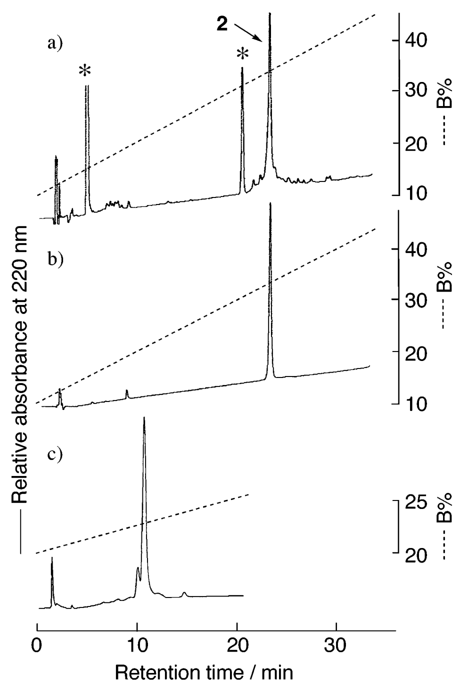

Figure 3. HPLC profile of glycopeptide thioester **2**: (a) crude glycopeptide **2**; (b) purified glycopeptide **2**; (c) re-chromatogram of glycopeptide **2**. The asterisked peaks are derived from scavengers. Elution conditions of (a) and (b): column, Mightysil RP-18 GP (4.6 × 150 mm) at a flow rate of 1 mL min^{-1}; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA. Elution conditions of (c): column, Mightysil RP-18 GP (4.6 × 150 mm) at a flow rate of 1 mL min^{-1}; eluent, A, 20 mM $AcONH_4$ (pH 5.7), B, acetonitrile containing 10% of solution A.

by a silyl carbamate group (Tsoc, *N*-triisopropylsilyloxycarbonyl).[39] Using acid fluoride,[40] the third amino acid was then condensed in the presence of a catalytic amount of the fluoride ion. N-Deprotection by the fluoride ion generates a free amino group, which is immediately acylated by the third amino acid fluoride. Thus, the formation of diketopiperazine, which is a well-known side reaction observed at the dipeptide stage, is effectively prevented.[39] If this precaution was not taken, 40% of the defective peptide thioester, lacking the C-terminal two amino acids, was formed in our previous study.[34] Further elongation of the peptide chain was carried out by the Fmoc procedure using *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. The carbohydrate portion was introduced using Fmoc-Thr carrying benzyl-protected GalNAc **4** by the same activation method. After the completion of the peptide chain assembly, the resin was first treated with TFA (Reagent K[41]) to deprotect the peptide portion. Then, the crude peptide was further treated with "low TfOH"[32−35] to remove the benzyl groups. Despite the introduction of five consecutive GalNAc moieties, and of the presence of multiple benzyl groups, the desired peptide thioester carrying seven GalNAcs was obtained in good purity, as demonstrated by the HPLC data in Figure 3a. The crude product was purified by HPLC using aqueous acetonitrile containing 0.1% TFA as an eluent, as shown in Figure 3b. MALDI mass measurement shows that the separated glycopeptide has a desired mass number (m/z = 4069.2, calcd for $(M + Na)^+$: 4068.8), which supports the success of this synthesis. However, a weak signal was also observed at m/z 3871.2, which was derived from the des-Pro-Thr peptide (calcd. for $(M + Na)^+$: 3870.7). This contaminant was overlapped with the desired peptide by this eluent system and was only unveiled by a shallow gradient of acetonitrile in 20 mM $AcONH_4$ (pH 5.7), as shown in Figure 3c. This fact shows that in the case of a tandem repeat of mucins, where the sequence is composed of limited amino acids with high frequency, complete purification is difficult using a single RPHPLC purification. If the stepwise synthesis was continued to obtain two or three tandem repeat peptides, the separation of the impurity could no longer be possible. Thus, in the synthesis of the tandem repeat peptide, a segment coupling method is essential. The impurity was removed by a second HPLC using the buffer system and was desalted using aqueous acetonitrile containing 0.1% TFA. The yield of peptide **2** was 5.9% based on the amino groups on the initial resin.

Segment Condensation. Unit **2** was then condensed with $TQT-NH_2$ by the thioester method,[27,28] as shown in Figure 4. This condensation forms the original repeat sequence from the C-terminus. Glycopeptide thioester **2** and $TQT-NH_2$ were dissolved in DMSO, and the thioester group was activated by silver chloride. As shown in Figure 5, the reaction was almost completed within 6 h without serious side reactions. The following Fmoc removal was achieved by adding 5% piperidine to the reaction mixture within 15 min. The crude peptide was purified by HPLC, and the desired product **5** was obtained in 62% yield. Then, the coupling, piperidine treatment, and the purification by gel filtration chromatography (GFC) were repeated five more times by the thioester method. All coupling reactions proceeded without serious side reactions within 6 h to obtain [TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_n$TQT-NH_2 (n = 2, **6**; n = 3, **7**; n = 4, **8**; n = 5, **9**; n = 6, **1**), as shown in Figure 5. Even in case of the last coupling reaction, which yielded a glycoprotein of over 20 kDa carrying 42 GalNAc moieties of **1**, the high efficiency of the coupling was maintained. The yields of these coupling reactions after purification by GFC were about 70% on average. The final purification was achieved by RPHPLC, and the desired product **1** was successfully obtained in 55% yield. The product was well characterized by the amino acid analysis and ESI mass analysis, as shown in Figure 6. These results demonstrate this method to be highly efficient in the synthesis of a tandem repeat structure.

Conclusion

The peptide thioester carrying seven GalNAc moieties was successfully prepared by the Fmoc procedure in high purity. The obtained thioester was repeatedly condensed to the amino component peptide with good yield and efficiency. These successes demonstrate the general applicability of this procedure for the synthesis of tandem repeated as well as globular glycoproteins of 20 kDa. This method also enables us, in principle, to synthesize glycopeptide thioesters carrying different carbohydrates *within* a peptide sequence. Therefore, by condensing various heterogeneously glycosylated peptide thioesters consecutively, the tandem repeat model having heterogeneous carbohydrate *between* the repeating units can be synthesized. Such glycopeptides will simulate the real mucin surfaces. Together with homogeneous models these heterogeneous models will contribute to the structural and functional analysis of natural

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Figure 4. The synthesis of homogeneous tandem repeat model **1** by the consecutive segment coupling by the thioester method.

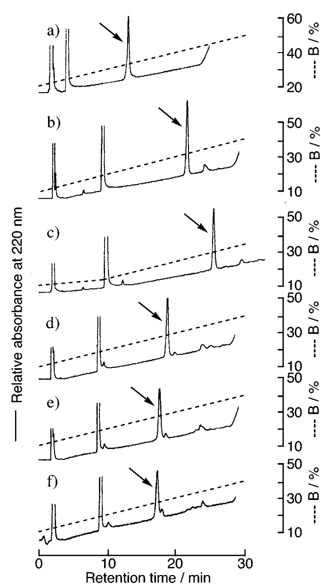

Figure 5. RPHPLC profile of the crude peptide obtained by the segment couplings. Elution conditions: column, Mightysil RP-18 GP (4.6 × 150 mm) at a flow rate of 1 mL min^{-1}; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA. The arrows in (a)−(f) show peptides **5**, **6**, **7**, **8**, **9**, and **1**, respectively, before Fmoc removal.

mucins. Along this line, further studies are currently being undertaken, and the results will be presented in due course.

Materials and Methods

^{1}H NMR spectra were recorded with a JEOL AL400 spectrometer (400 MHz). Chemical shifts are expressed in parts per million downfield from the signal for internal Me_4Si for a solution in $CDCl_3$. The Fmoc deblocking reagent [1-methylpyrrolidine (25%), hexamethylene imine (2%), HOBt (2%) in 1-methyl-2-pyrrolidinone (NMP)−DMSO (1:1)] was prepared according to the previously reported procedure.[38] RPHPLC purification of glycopeptide thioester **2** and glycopeptide **5** was performed using Mightysil RP-18 (10 × 250 mm, Kanto Chemical) at a flow rate of 2.5 mL/min. Gel filtration chromatography of glycopeptides **6**, **7**, **8**, **9**, and **1** was performed by G3000PW$_{XL}$ (7.8 mm × 300 mm, TOSOH) using 50% aqueous acetonitrile containing 0.1% TFA as an eluent at a flow rate of 0.5 mL/min. Amino acid composition was determined with a Lachrom amino acid analyzer (Hitachi, Tokyo) after hydrolysis with 6 M HCl at 150 °C for 2 h in an evacuated sealed tube. The amount of the peptide was calculated based on the amino acid analysis data. ESI mass measurement was carried out on an Applied Biosystems/MDS Sciex QSTAR pulsar i mass spectrometer. The glycopeptide was sprayed at a concentration of 1−2 pmol/μL in water/methanol (v/v) containing 0.1% formic acid or in water/methanol containing 0.1% TFA. MALDI mass measurement was performed by an Applied Biosystems/Voyager-DE Pro using dihydroxybenzoic acid as a matrix.

TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG-SCH$_2$CH$_2$CONH$_2$, 2. CLEAR−amide resin (290 mg, 0.35 mmol/g) was treated with the Fmoc deblocking reagent for 5 and 15 min. After washing with NMP, Fmoc-

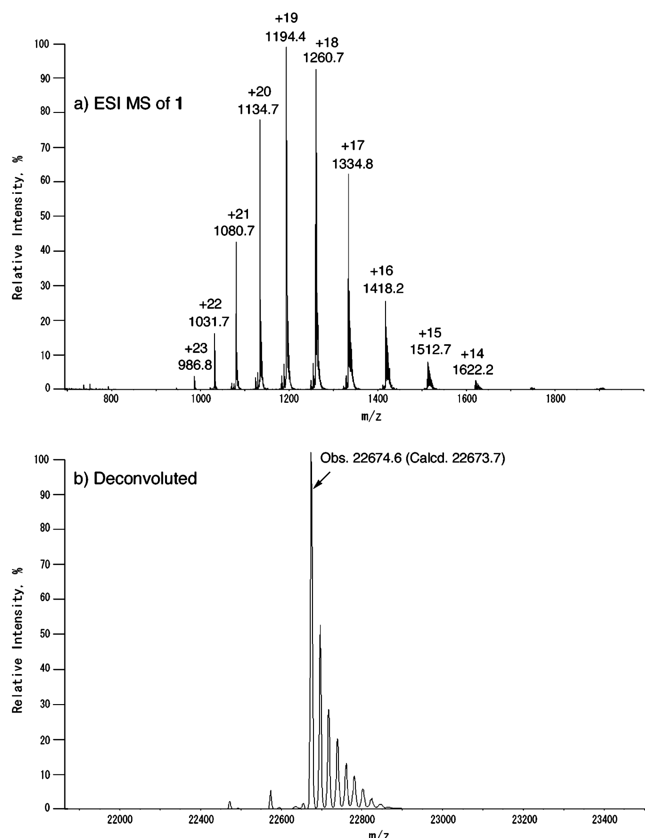

Figure 6. ESI mass spectrum of **1**. The desired peak in (b) accompanies multiple Na^+ added peaks, which might be derived from the fact that GalNAc moieties tend to coordinate Na^+.

Gly-SCH_2CH_2COOBt (0.2 mmol), which was prepared by mixing Fmoc-Gly-SCH_2CH_2COOH (77 mg, 0.2 mmol), 1 M DCC/NMP (0.2 mL), and 1 M HOBt/NMP (0.2 mL) for 30 min at room temperature, was added, and the reaction mixture was vortexed for 1 h. The resin was washed with NMP and was treated with the Fmoc deblocking reagent (2 and 5 min). After NMP washing, the resin was reacted with Tsoc-Thr(Bn)-OPfp **3** (115 mg, 0.2 mmol) in tetrahydrofuran (THF) for 15 min. The reaction was repeated with the same amount of fresh Tsoc-Thr(Bn)-OPfp. The resin was washed with CH_2Cl_2. Fmoc-Pro-F (2.2 mmol), prepared by mixing Fmoc-Pro (81 mg, 0.24 mmol), fluoro-*N*,*N*,*N*′,*N*′-tetramethylformadinium hexafluorophosphate (58 mg, 0.22 mmol), and *N*,*N*-diisopropylethylamine (DIEA, 76 μL, 0.44 mmol) in CH_2Cl_2, was then added. The reaction was initiated by adding 1 M *n*-Bu_4NF in THF (10 μL) to the reaction mixture. After vortexing for 1 h, the resin was washed with CH_2Cl_2 and NMP. The remaining amino acids were introduced manually using Fmoc-amino acid (0.5 mmol), 0.45 M HBTU−HOBt (1.0 mL, 0.45 mmol), and DIEA (0.16 mL, 0.9 mmol), except Fmoc-Thr(GalNAc) **4**, which was introduced in 0.2 mmol scale. The brief protocol is as follows. (1) NMP wash (1 min × 6), (2) Fmoc removal (2 and 18 min), (3) NMP wash (1 min × 6), (4) coupling (60 min), (5) NMP wash (1 min × 3), (6) capping by 10% Ac_2O, 5% DIEA in NMP (5 min). After completion of the chain assembly, 480 mg of protected peptide resin was obtained. A part of the resin (50 mg) was treated with Reagent K (0.75 mL) for 1 h at room temperature. TFA was removed by the nitrogen stream, and the peptide was precipitated with ether and washed with the same solvent twice. The precipitate was further treated with low-TfOH (0.5 mL) for 2 h at −10 °C. The peptide was precipitated with ether, washed with the same solvent twice, and dried in vacuo. The crude peptide was purified by RPHPLC using aqueous acetonitrile containing 0.1% TFA to obtain partially purified glycopeptide **2**. The peptide was further purified by RPHPLC using aqueous acetonitrile containing 20 mM $AcONH_4$ (pH 5.7) as an eluent and was desalted by RPHPLC using the former solvent system to give purified glycopeptide **2** (2.4 mg, 0.59 μmol, 5.9% yield based on the amino groups on the initial resin). MALDI-TOF mass: found *m/z* 4069.2, calcd for $(M + Na)^+$ *m/z* 4068.8. Amino acid analysis: $Thr_{13.09}Glu_{1.08}Pro_{4.66}Gly_{1.25}Val_{1}Ile_{0.99}$.

TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTGTQT-NH_2, 5. Glycopeptide thioester **2** (4.0 mg, 1.0 μmol), TQT−NH_2 (3.5 mg, 10 μmol), and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOOBt, 4.8 mg, 29 μmol) were dissolved in DMSO (100 μL) containing DIEA (3.4 μL, 20 μmol). AgCl (0.4 mg, 3 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (5 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by RPHPLC using aqueous acetonitrile containing 0.1% TFA as an eluent to give glycopeptide **5** (1.6 mg, 400 nmol, 40%). MALDI-TOF mass: found *m/z* 4089.0, calcd for $(M + Na)^+$ *m/z* 4088.9. Amino acid analysis: $Thr_{15.08}Glu_{2.05}Pro_{5.01}Gly_{1}Val_{0.99}Ile_{0.96}$.

[TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_2$TQT-NH_2, 6. Glycopeptide thioester **2** (2.0 mg, 500 nmol), glycopeptide **5** (1.6 mg, 400 nmol), and HOOBt (2.4 mg, 15 μmol) were dissolved in DMSO (30 μL) containing DIEA (1.7 μL, 9.8 μmol). AgCl (0.2 mg, 1.5 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (3 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by gel filtration chromatography to give glycopeptide **6** (2.3 mg, 290 nmol, 73%). MALDI-TOF mass: found *m/z* 7812.2, calcd for $(M + Na)^+$ *m/z* 7812.2. Amino acid analysis: $Thr_{28.13}Glu_{2.94}Pro_{9.58}Gly_{2}Val_{1.99}Ile_{1.93}$.

[TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_3$TQT-NH_2, 7. Glycopeptide thioester **2** (1.6 mg, 400 nmol), glycopeptide **6** (2.2 mg, 280 nmol), and HOOBt (2.0 mg, 12 μmol) were dissolved in DMSO (30 μL) containing DIEA (1.4 μL, 8.1 μmol). AgCl (0.2 mg, 1.5 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (3 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by gel filtration chromatography to give glycopeptide **7** (2.9 mg, 250 nmol, 89%). MALDI-TOF mass: found *m/z* 11536.5, calcd for $(M + Na)^+$ *m/z* 11533.0. Amino acid analysis: $Thr_{39.87}Glu_{4.05}Pro_{14.73}Gly_{3}Val_{3.00}Ile_{3.04}$.

[TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_4$TQT-NH_2, 8. Glycopeptide thioester **2** (1.3 mg, 320 nmol), glycopeptide **7** (2.9 mg, 250 nmol), and HOOBt (1.6 mg, 9.8 μmol) were dissolved in DMSO (30 μL) containing DIEA (1.1 μL, 6.3 μmol). AgCl (0.2 mg, 1.5 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (3 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by gel filtration chromatography to give glycopeptide **8** (2.9 mg, 190 nmol, 77%). MALDI-TOF mass: found *m/z* 15255.3, calcd for $(M + Na)^+$ *m/z* 15253.9. Amino acid analysis: $Thr_{51.85}Glu_{5.03}Pro_{18.93}Gly_{4}Val_{3.99}Ile_{3.98}$.

[TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_5$TQT-NH_2, 9. Glycopeptide thioester **2** (0.93 mg, 230 nmol), glycopeptide **8** (2.9 mg, 190 nmol), and HOOBt (1.1 mg, 6.7 μmol) were dissolved in DMSO (20 μL) containing DIEA (0.8 μL, 4.6 μmol). AgCl (0.2 mg, 1.5 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (2 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by gel filtration chromatography to give glycopeptide **9** (2.7 mg, 144 nmol, 77%). MALDI-TOF mass: found *m/z* 18976.4, calcd for $(M + Na)^+$ *m/z* 18974.3. Amino acid analysis: $Thr_{64.25}Glu_{5.70}Pro_{24.06}Gly_{5}Val_{4.93}Ile_{5.00}$.

[TQTPT(GalNAc)TTPIT(GalNAc)T(GalNAc)T(GalNAc)T-(GalNAc)T(GalNAc)VTPT(GalNAc)PTPTG]$_6$TQT-NH_2, 1. Glycopeptide thioester **2** (0.73, 180 nmol), glycopeptide **9** (2.7 mg, 141 nmol), and HOOBt (0.9 mg, 5.5 μmol) were dissolved in DMSO (20 μL) containing DIEA (0.6 μL, 3.5 μmol). AgCl (0.1 mg, 0.7 μmol) was then added, and the resulting mixture was stirred in the dark overnight. Piperidine (2 μL) was added, and the solution was kept in the dark for 1 h. The product was precipitated by ether and purified by gel filtration chromatography followed by RPHPLC using Protein-RP (4.6 × 150 mm, Yamamura Co., Ltd.) to give glycopeptide **1** (1.8 mg, 78 nmol, 55%). ESI mass: found m/z 22674.6, calcd for $(M + H)^+$ m/z 22673.7. Amino acid analysis: $Thr_{80.70}Glu_{7.07}Pro_{29.09}Gly_6Val_{5.75}Ile_{5.98}$.

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Supporting Information Available: Preparation of Tsoc-Thr-(Bn)-OPfp **3** and Fmoc-Thr(GalNAc) **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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