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Efficient Substitution Reaction from Cysteine to the Serine Residue of Glycosylated Polypeptide: Repetitive Peptide Segment Ligation Strategy and the Synthesis of Glycosylated Tetracontapeptide Having Acid Labile Sialyl-T_N Antigens

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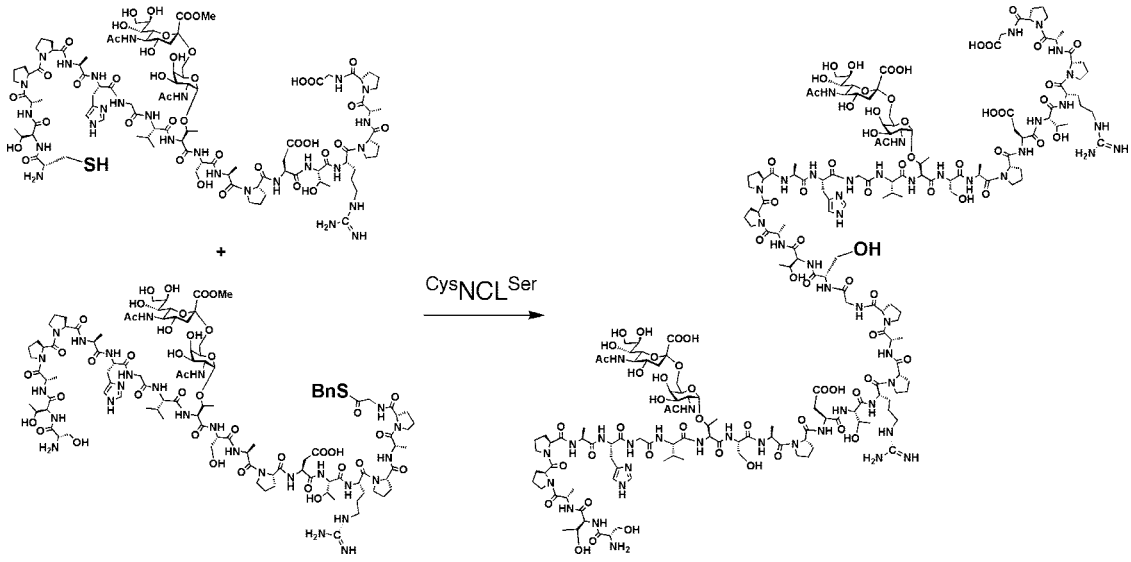

This paper reports the synthesis of a 40-residue glycopeptide having two antigenic sialyl-T_N (NeuAc-α-(2,6)-GalNAc-Thr) residues in the MUC1 sequence. This target glycopeptide is a tandem repeat form of 20-residue glycopeptides. For the synthesis of this large molecule, native chemical ligation (NCL) at the serine site was used (^{Cys}NCL^{Ser}). The concept of ^{Cys}NCL^{Ser} relies on the following: (1) conventional NCL between peptide-α-thioester and the cysteine residue of another peptide segment; (2) methylation of the thiol that was used for NCL; (3) acidic CNBr conversion of the cysteine residue to the serine residue forming an *O*-ester linkage; and (4) an *O*- to *N*-acyl shift to couple the two glycopeptides through a native amide bond. To synthesize glycopeptide having an acid-labile sugar moiety, a 20-residue glycopeptide-α-thioester and 20-residue glycopeptide having a cysteine residue at the N-terminal were synthesized by solid phase glycopeptide synthesis, and then coupled by ^{Cys}NCL^{Ser}. As the result of extensive investigation, CNBr activation with an additional acid (trifluoroacetic acid) was found to be essential to obtain good reactivity and yield, and this condition afforded a tandem repeat form of 40-residue sialylglycopeptide having two sialyl-T_N residues. In addition to this, it was demonstrated that the cysteine thiol protected by the acetoamidomethyl (Acm) group did not react with the CNBr reagent, and therefore ^{Cys}NCL^{Ser} can be used for repetitive native chemical ligation in the presence of a protecting N-terminal cysteine residue with an Acm group.

Introduction

Glycoproteins are functional on the cell surface and in body fluids.[1] To study glycoprotein function, glycoprotein and glycopeptide chemical syntheses have been extensively examined. Glycoproteins have been prepared by cell expression methods, but not in a homogeneous form due to the heterogeneous structure of oligosaccharides on the expressed protein.[2] Recently, chemical synthesis has come to be recognized as a new strategy

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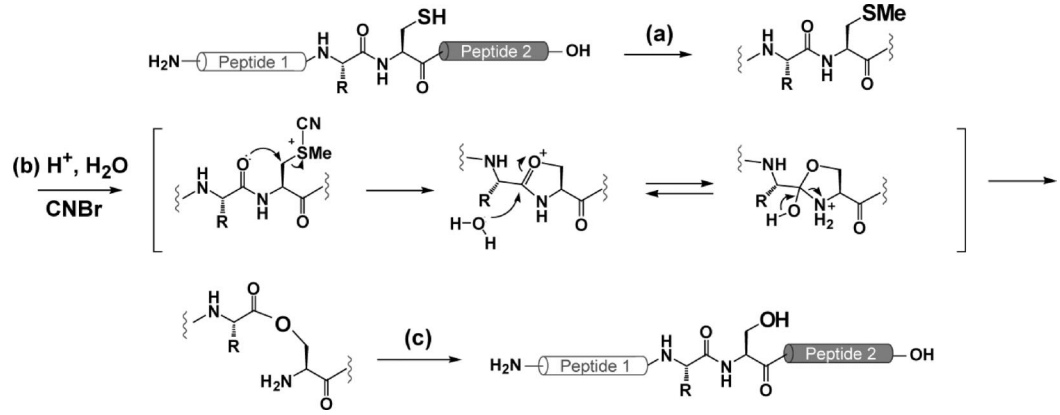

FIGURE 1. Conversion of Cys to Ser residue.

for the preparation of homogeneous glycoproteins.[3] A chemical strategy is expected to solve the role of the posttranslational modification patterns made by the puzzlingly diverse oligosaccharide structures. In this strategy, preparation of the target glycosylated polypeptide chain relies on a peptide-segment coupling, including the glycopeptide segments.[3,4] Therefore this peptide segment coupling is a key reaction step to the production of a long polypeptide chain, which is subsequently folded into the desired three-dimensional protein structure. There are several methods which have been developed for peptide coupling. Of these, native chemical ligation (NCL)[5] has been widely used for the synthesis of proteins and it has also been applied to the synthesis of glycoproteins. However, NCL normally requires the use of a cysteine residue at the junction between two peptide segments, and this means that proteins without cysteine residues cannot be synthesized by means of NCL. Therefore, attempts at the improvement and further development of NCL have been conducted.[6] These can be roughly divided into two categories. The first strategy uses an auxiliary mediated type of reaction.[7] Although this method theoretically can be used for all of the amino acid residues, it is usually applied for the less sterically hindered amino acid residues such as alanine and glycine at the junction points. The most recently reported strategy is a conversion of cysteine after NCL.[8] This type uses the reduction reaction of a thiol group after conventional NCL and enables the use of alanine, phenylalanine, and valine as the ligation sites. This method overcame the site limitation problem in the NCL reaction.

However, to synthesize glycopeptides and glycoproteins, further development of this conversion reaction is essential. Ideally, NCL would be applicable to any positions in a polypeptide chain. In addition to these reactions, we also demonstrated an efficient glycopeptide ligation at serine ($^{Cys}NCL^{Ser}$) based on conversion strategy after conventional NCL.[9] This strategy includes three reaction steps, as shown in Figure 1: (a) cysteine methylation,[10] (b) CNBr conversion reaction,[11] and (c) *O*- to *N*-acyl shift.[12]

Of these reactions, CNBr conversion is one of the critical reaction steps. CNBr has conventionally been used for the C-terminal peptide cleavage of methionine or chemically methylated cysteine residues under an acidic condition such as with formic acid.[11] However, this reaction has also been shown to cause formylation toward alcohols of serines or threonines in peptides.[11,13] In terms of glycopeptide synthesis, this reaction condition has come to be considered unsuitable, because long-term reactions under acidic conditions may lead to the cleavage of labile glycosylated linkages (e.g., sialyl-linkage and fucosyl linkage) as well as the undesired formylation of sugar alcohols. To synthesize glycoproteins having acid labile sialylglycopeptides or glycoproteins by means of $^{Cys}NCL^{Ser}$, we have extensively studied $^{Cys}NCL^{Ser}$ through a synthesis of the MUC1 peptide with sialyl-T_N residues. This class of sialylglycopeptide has potentially important value as a cancer vaccine, but the synthesis has been thought to be an extremely difficult task.[3,14] Here we report a practical synthesis of MUC1 tandem repeat 40-residue sialylglycopeptide having two sialyl-T_N residues by means of $^{Cys}NCL^{Ser}$. In addition, we also report that this $^{Cys}NCL^{Ser}$ method can be used for powerfully repetitive NCL reactions, indicating that $^{Cys}NCL^{Ser}$ can be performed in the presence of methionine[9] and other cysteine residues in the peptide chain.

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SCHEME 1. CNBr Conversion Reaction with 11-Residue Peptide 1 (LFRVYC(Me)NFLRG)

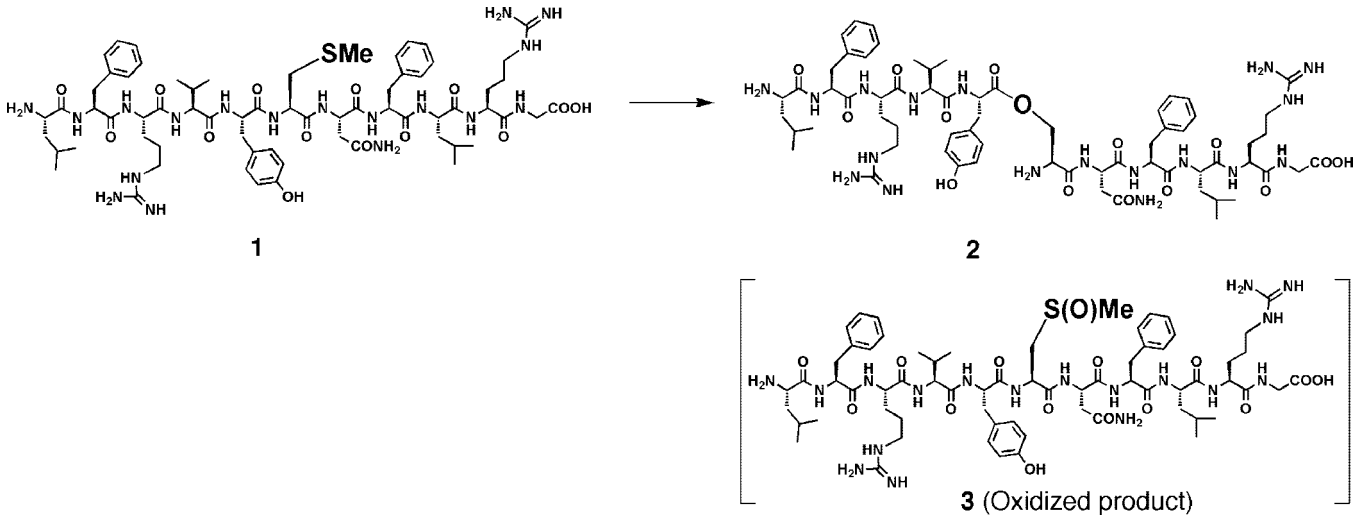

TABLE 1. Formic Acid Concentration Dependence of the CNBr Conversion Reaction[a]

		yield (%)		
entry	acid condition	**1**	**2**	**3**
1	100% HCOOH	86	13	1
2	70% HCOOH/30% H_2O	60	37	3
3	50% HCOOH/50% H_2O	48	21	31
4	20% HCOOH/80% H_2O	28	5	67

[a] Reaction condition: peptide **1** (1equiv, concentration was adjusted to 1 mM), CNBr (100 equiv). Yields were estimated by HPLC area intensity.

TABLE 2. Varying the Acidic Condition for the CNBr Conversion Reaction[a]

		yield (%)		
entry	acid condition	**1**	**2**	**3**
1	80% HCOOH/20% H_2O	25	58	17
2	0.1 M HCl	30	33	35
3	60% H_2O/40% CH_3CN containing MsOH (1% v/v)	9	66	24
4	60% H_2O/40% CH_3CN containing TsOH (3% v/v)	6	68	22
5	60% H_2O/40% CH_3CN containing H_3PO_4 (3% v/v)	8	5	83
6	60% H_2O/40% CH_3CN containing TBA (3% v/v)	11	2	61
7	60% H_2O/40% CH_3CN containing TFA (3% v/v)	11	63	25
8	60% H_2O/40% CH_3CN containing TFA (2% v/v)	10	63	26
9	50% TFA/50% H_2O	44	34	7

[a] Reaction condition: peptide **1** (1 equiv, concentration was adjusted to 2 mM), CNBr (200 equiv). Yields were estimated by HPLC area intensity.

Results and Discussion

Protein cleavage at the methionine or methylated cysteine with CNBr has been examined with formic acid or HCl.[11] In the case of methionine, the cleavage reaction takes place at the C-terminal amide bond of methionine, while the N-terminal amide bond of methylated cysteine is cleaved by the CNBr reaction. Although this CNBr reaction under acidic conditions toward methionine has been well investigated, there are few reports which discuss the reaction conditions for the cleavage reaction at the methylated cysteine site. Therefore, we first investigated the relationship of the formic acid concentration and the reactivity of the CNBr conversion reaction toward the peptide having methylated cysteine residues. The model 11-residue peptide (LFRVYC(Me)NFLRG) was prepared by the reported method (Scheme 1).[9] We performed the CNBr conversion reaction at several HCOOH concentrations (Table 1).

As shown in Figure 1, this reaction requires H_2O, therefore the reaction shown in entry 1 (100% HCOOH) did not proceed well. In the case of entries 2−4, decrease of the formic acid concentration led to a decrease in the product amount and an increase of the sulfoxide formed by oxidation of the starting material. In addition to the formic acid condition, we performed the CNBr conversion reaction by using several different kinds of acid (Table 2). Entries 1 and 2 are the conventional reaction conditions in the case of peptide cleavage at methionine sites.[11] In contrast to these conditions, applying a low concentration of an acid such as methanesulfonic acid (MsOH), *p*-toluenesulfonic acid (TsOH), or trifluoroacetic acid (TFA) exhibited good yield, as shown in entries 3, 4, 7, and 8. On the other hand, phosphoric acid (entry 5) or tribromoacetic acid (TBA, entry 6) generated the largest amount of sulfoxide. As shown in Table 2, we found that the conditions of entry 7 or 8 are suitable for CNBr activation. In addition, because TFA can be removed from reaction mixture by evaporation, we used this condition for glycopeptides synthesis.

We have also performed the conversion reaction by another activation system, as shown in Scheme 2. In the case of the CNBr condition, it generated the sulfonium cation through the activating SMe group of the CN cation, as shown in Figure 1. We expected this generation of the sulfonium cation could be obtained directly from the sulfuhydryl group. Therefore, we used the commercially available reagent diethyl *meso*-2,5-dibromoadipate. This type of reagent afforded a five-membered-ring intermediate, as shown in Scheme 2.[15] However, as a result, this reaction afforded only a β-elimination product and did not afford any desired *O*-ester product (Scheme 2, data shown in the Supporting Information).[16] We speculate that this is because of the steric hindrance of the five-membered ring. This ring cannot apparently be placed in a position where the amide carbonyl oxygen could attack the β carbon of cysteine. On the other hand, in the case of the CNBr activation, the intermediate of the sulfonium cation may be placed in a suitable position for oxazolone formation rather than elimination, because the sulfonium cation formed by CNBr appears to be a sterically

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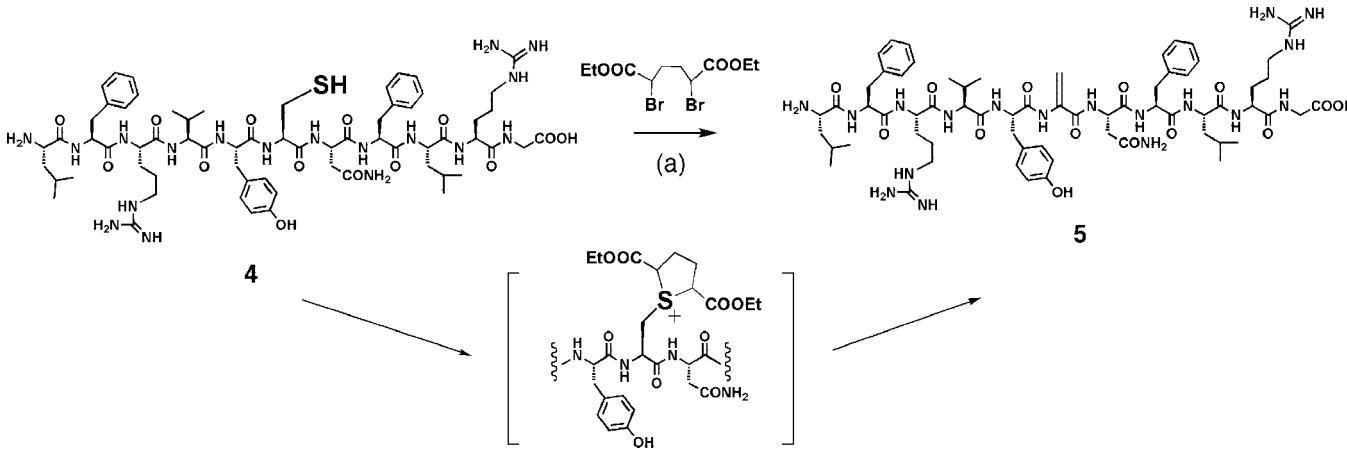
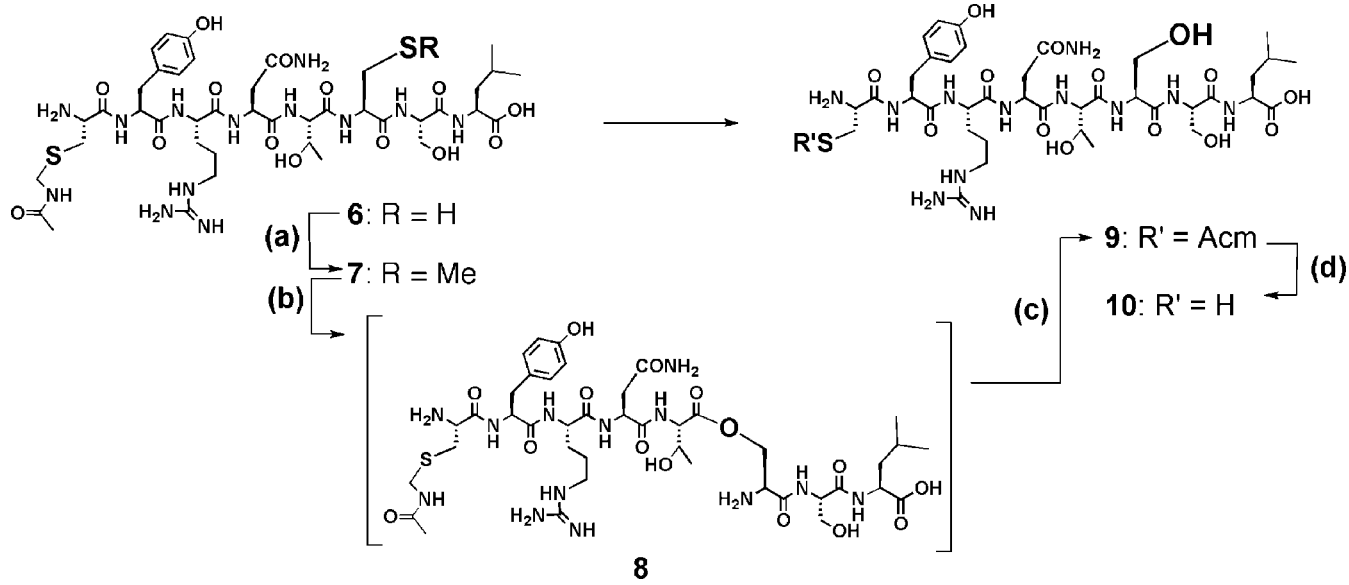
SCHEME 2. Activation of Sulfuhydryl Group Directly through a Five-Membered-Ring-Type Intermediate[a]



[a] Reagents and conditions: (a) peptide **4**, diethyl *meso*-2,5-dibromoadipate, CH_3CN, 0.2 M phosphate buffer containing 6 M guanidine (pH 6.3).

SCHEME 3. Cys to Ser Conversion Reaction with 8-Residue Peptide 6[a]



[a] Reagents and conditions: (a) methyl 4-nitrobenzenesulfonate, CH_3CN, 0.25 M tris buffer (pH 8.6) containing 6 M guanidine and 3.3 mM EDTA2Na; (b) CNBr, 80% HCOOH solution or CNBr, 40% CH_3CN solution, TFA (2% v/v); (c) 5% hydrazine solution, then TFA, NH_4I, SMe_2; (d) 50% CH_3CN solution containing 0.1% TFA (v/v), AgOAc.

smaller reaction species than that activated by *meso*-2,5-dibromoadipate. Therefore, we concluded that CNBr would be a suitable reagent for the conversion reaction.

We also expected that a new acidic condition with use of TFA for the CNBr conversion reaction would not cause any acylation of the hydroxyl groups. In terms of formylation during the CNBr activation, we speculated that a simple formylation would be caused under the acidic condition (HCOOH). In the case of the TFA/CNBr condition, we suppose that the same acidic trifluoroacetylation may be caused. However, it is known that a trifluoroacetyl group is easily hydrolyzed in a solution, meaning that the TFA/CNBr condition may avoid unexpected esterification of the hydroxyl groups. In addition to overcoming the formylation problem, we have had an interest in repetitive NCL by the use of ^{Cys}NCL^{Ser}. In this case, a cysteine at the N-terminal should not react with CNBr during the CNBr conversion reaction. In our experiments, since the free thiol group of cysteine potentially reacts with CNBr, we introduced the N-terminal cysteine in an acetoamidomethyl (Acm)-protected form. We expected that the Acm-protected cysteine would not react with CNBr. To determine whether the TFA/CNBr condition would avoid both esterification of the hydroxyl groups and activation of the protected cysteine at the N-terminal, we applied this condition for the model 8-residue peptide **6** having three hydroxyl groups, a guanidine group, an N-terminal amino group, a free cysteine group, and a Acm-protected cysteine group at the N-terminal (Scheme 3). If the conversion reaction of Cys at the 6 position to a serine residue acts selectively, the N-terminal cysteine can be used in the second NCL.

The 8-residue peptide **7** having a methyl cysteine residue (C(Acm)YRNTC(Me)SL) was synthesized by the selective methylation of **6**, which was synthesized by SPPS. We performed the CNBr conversion reaction using both the conventional and new TFA condition. As shown in Figure 2a, a conventional reaction condition with HCOOH generated multiple products. Mass analysis of these products suggested that byproducts seemed to be the result of formylation toward alcohols. In contrast, in the case of the new reaction condition (Figure 2b), there was no side reaction except for slight oxidation of the starting material. A one-pot reaction of the *O*- to *N*-acyl

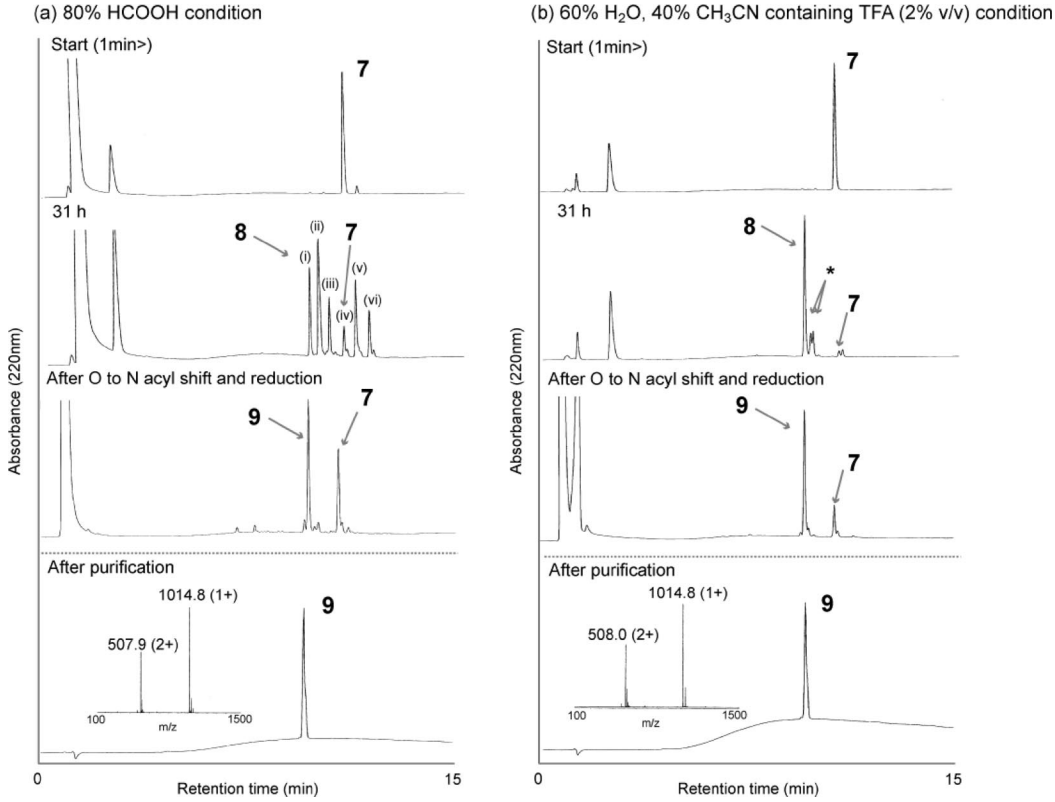


FIGURE 2. CNBr conversion reaction with 8-residue peptide **7**. The HPLC chart of 80% HCOOH condition reaction shows a multiple peak pattern at 31 h ((i) peptide **8**, (ii) monoformylated peptide of **8**, (iii) diformylated peptide of **8**, (iv) peptide **9**, (v) monoformylated peptide of **9**, and (vi) diformylated peptide of **9**). Each inset of purified product was observed from ESI-MS spectra (calcd for $[M + H]^+$ 1014.5, $[M + 2H]^{2+}$ 507.6). The asterisk indicates the diastereomer of sulfoxides.

shift afforded the desired peptide **9**. To recover the starting material, a reduction process of sulfoxide was included in this one-pot *O*- to *N*-acyl shift process. This result proved that the reaction yield under the new condition was better than that under the conventional condition (54% yield in HCOOH condition (Figure 2a) and 75% yield in the 2% TFA condition (Figure 2b); these yields were estimated by HPLC area intensities), and the recovered starting material could be used again to accumulate target peptide **9**. In addition, as expected, the N-terminal protected cysteine did not react with CNBr. Deprotection of the Acm group by the conventional condition[17] afforded the peptide having a cysteine residue at the N-terminal **10** (data shown in the Supporting Information). This means that ^{Cys}NCL^{Ser} can be applied to repetitive NCL.

Since the suitable condition had been determined, we then examined the synthesis of glycopeptides having acid labile sialyloligosaccharides. We selected 40-residue glycopeptide in a MUC1 variable number tandem repeat (VNTR) region having two sialyloligosaccharides.[18] This region consists of a 20-residue glycopeptide unit, in which the polypeptide chain possesses abundant serines, threonine, and prolines, in tandem. It is known that the sialyloligosaccharides are expressed in the MUC1 VNTR region on endothelial cancer cells.[18,19] Such special glycopeptides having multiple sialyloligosaccharides are expected to have potential as a cancer vaccine. However, the repeating sialylglycopeptides of the VNTR region had not been synthesized. Therefore we targeted the synthesis of repeated MUC1 glycopeptides having two sialyl-T_N antigens.

To synthesize such a sialylglycopeptide, we prepared 20-residue sialylglycopeptide-α-thioester **11** and 20-residue sialylglycopeptide **12**. The sialyl-T_N methyl ester threonine unit for sialylglycopeptide segments was synthesized by the reported method (Scheme 4, data shown in the Supporting Information).[20]

To couple these glycopeptides, NCL was performed under the reported condition,[20] and then selective methylation of the cysteine thiol[10] afforded the desired 40-residue glycopeptide having the two sialyl-T_N **14** in good yield (81% and 90% yield, respectively). Following this step, the CNBr conversion reaction was performed in acetonitrile−H_2O−TFA solution (Table 2, entry 8 condition). As expected, there was no acylation in the reaction. Hence, slight oxidation of the starting material was observed. To recover the starting material, reduction of sulfoxide was performed by the use of TFA/NH_4I/SMe_2 at −10 °C, and this afforded *O*-ester linked 40-residue sialylglycopeptide **15** in 38% yield. In addition, the recovered substrate was found to be repeatedly useful for this CNBr conversion reaction. Subsequently, the *O*- to *N*-acyl shift reaction and deprotection of two methyl ester groups of **15** under a basic condition was undertaken. Although this treatment afforded a small amount of cleaved peptide (ca. 30%, data shown in the Supporting Information), a subsequent deprotection step of the methyl ester afforded sialylglycopeptide in moderate yield (ca. 50% isolated

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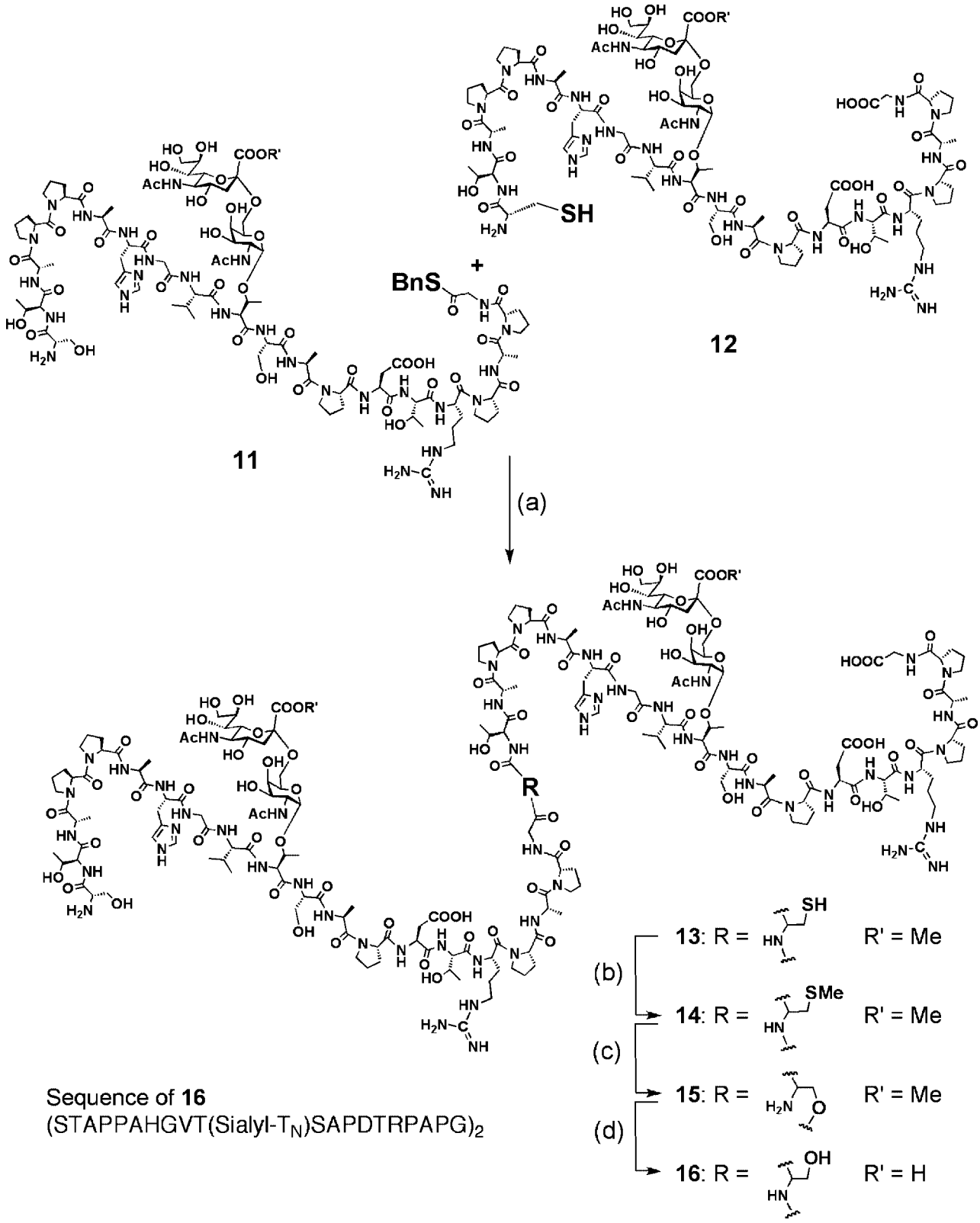
SCHEME 4. Synthesis of MUC1 Repeated Glycopeptide Having Two Sialyl-T_N[a]



[a] Reagents and conditions: (a) 0.2 M phosphate buffer (pH 7.2) containing 6 M guanidine, 20 mM tris(2-carboxylethyl)phosphine and 4-mercaptophenylacetic acid (1% v/v); (b) methyl 4-nitrobenzenesulfonate, CH_3CN, 0.25 M tris buffer (pH 8.6) containing 6 M guanidine and 3.3 mM EDTA2Na; (c) CNBr, 40% CH_3CN solution, TFA (2% v/v), then TFA, NH_4I, SMe_2;. (d) 5 mM NaOH, then 50 mM NaOH.

yield, 70% conversion yield estimated by HPLC area intensity: see the Supporting Information). In these reaction steps, we did not observe the cleavage of the NeuAc residue in sialyl-T_N moiety under acidic CNBr reaction or β-elimination of the sialyl-T_N moiety under the basic condition. The ^{1}H NMR of the MUC1 repeating glycopeptide having two sialyl-T_N **16** is shown in Figure 3. As shown in this NMR spectrum, ^{Cys}NCL^{Ser} can afford glycopeptides in moderate yield.

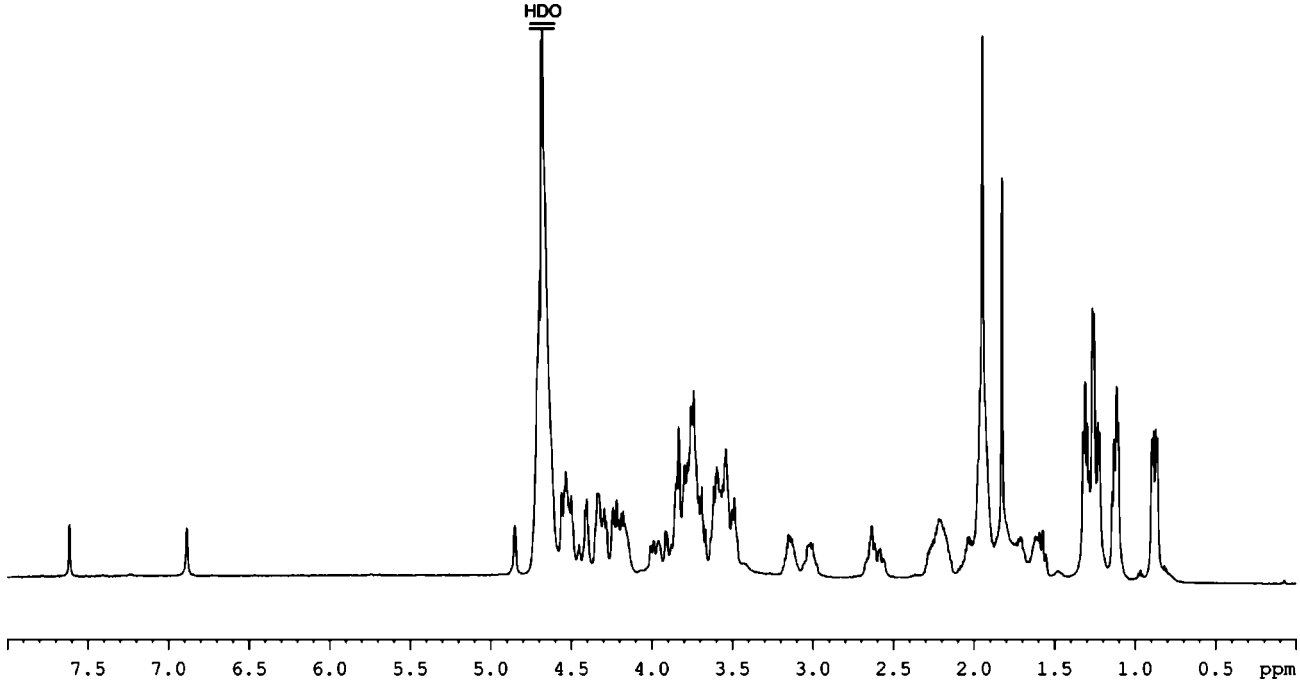


FIGURE 3. ^{1}H NMR of synthesized 40-residue sialylglycopeptide having two sialyl-T_N **16**.

In conclusion, we report the synthesis of a 40-residue MUC1 sialylglycopeptide having two sialyl-T_N. To synthesize such a large sialylglycopeptide, we used ^{Cys}NCL^{Ser}. We have demonstrated that this condition has afforded acid labile sialylglycopeptides and avoided activation of the protected cysteine at the N-terminal of the peptide. This means that ^{Cys}NCL^{Ser} can be used not only for the synthesis of acid labile sialylglycopeptides, but also for repetitive NCL. Investigation of the synthesis of several glycoproteins by means of both conventional NCL and ^{Cys}NCL^{Ser} is currently in progress.

Experimental Section

Synthesis of 7 (*S*-Methylation of Peptide 6). Compound **6** (3.3 mg, 3.21 μmol) was dissolved in 0.25 M tris buffer (pH 8.6, 3.2 mL) containing 6 M guanidine and 3.3 mM EDTA-2Na. To this mixture was added 2-mercaptoethanol (2.3 μL), then the solution was stirred for 15 min. Next a solution of methyl 4-nitrobenzenesulfonate (21.0 mg, 66.3 μmol) in acetonitrile (1.0 mL) was added to the mixture, which was then stirred for 40 min. The mixture was neutralized by 10% TFA solution (0.4 mL) and washed with Et_2O (3 mL, 3 times). The solution was then concentrated in vacuo to remove organic solvent. Purification of the mixture by RP-HPLC (HPLC condition: C-18 column, 5 μm, 250 × 4.5 mm, linear gradient of 13.5→31.5% CH_3CN containing 0.1% TFA in 0.1% TFA aqueous over 60 min at a flow rate of 1.0 mL/min) afforded the desired product **7** (2.7 mg, 83% isolated yield). ESIMS: calcd for $C_{42}H_{70}N_{13}O_{14}S_2$ $[M + H]^+$ 1044.5; found $[M + H]^+$ 1044.9.

Typical Procedure of the Synthesis of 9 (CNBr Conversion Reaction of 7). Peptide **7** (4 mg, 3.83 μmol) was dissolved in acid solution (80% formic acid or 40% CH_3CN solution containing 2% v/v TFA, 1.9 mL). To the solution was added CNBr (81 mg) and the mixture was stirred for 31 h in the dark at room temperature. Then the mixture was lyophilized. Subsequently, the residue was treated as in the following condition to reduce oxidized starting material and to perform *O*- to *N*-acyl shift reaction under slight basic condition (in the case of formic acid condition, this basic condition removed the formyl group). The residue was dissolved in 5% hydrazine hydrate solution (380 μL) and this solution was stirred for 25 min. Then, to this solution was added TFA (1.5 mL). To the solution was then added NH_4I (11 mg) and dimethyl sulfide (5.6 μL). The mixture was stirred for 50 min at room temperature and ice cooled H_2O (2.0 mL) was added. This mixture was washed with CCl_4. The water phase was concentrated in vacuo. Purification of the residue by reverse-phase HPLC (condition: C-18 column, 5 μm, 250 × 4.5 mm, Isocratic of 0.1% TFA solution for 5 min, followed by the linear gradient of 0→54% CH_3CN containing 0.1% TFA in 0.1% TFA solution over 30 min at a flow rate 1.0 mL/min) afforded peptide **9**. ESIMS: calcd for $C_{41}H_{68}N_{13}O_{15}S$ $[M + H]^+$ 1014.5; found $[M + H]^+$ 1014.8.

Synthesis of 13 (Native Chemical Ligation of Sialylglycopeptide Thioester 11 and Sialylglycopeptide 12). Sialylglycopeptide α-thioester **11** (5.0 mg, 2.0 μmol) and sialyl glycopeptide **12** (4.8 mg, 2.0 μmol) were dissolved in 0.2 M sodium phosphate buffer (pH 7.2, 1.0 mL) containing 6 M guanidine, 20 mM tris(2-carboxylethyl)phosphine, and 1.0% v/v 4-mercaptophenylacetic acid. This mixture was stirred for 12 h. Purification of the mixture by RP-HPLC (condition: C-8 column, 5 μm, 250 × 10 mm linear gradient of 9→36% CH_3CN containing 0.1% TFA in 0.1% TFA aqueous over 30 min at a flow rate of 4.0 mL/min) afforded MUC1 glycopeptide having two sialyl-T_N **13** (7.6 mg, 81%). ESIMS: calcd for $C_{200}H_{317}N_{54}O_{80}S$ $[M + 3H]^{3+} = 1597.3$, $[M + 4H]^{4+}$ 1198.3, $[M + 5H]^{5+}$ 958.8; found $[M + 3H]^{3+}$ 1597.3, $[M + 4H]^{4+}$ 1198.1, $[M + 5H]^{5+}$ 958.7.

Synthesis of 14 (*S*-Methylation of MUC1 Repeated Glycopeptide 13). Compound **13** (6 mg, 1.25 μmol) was dissolved in 0.25 M tris buffer (pH 8.6, 1.25 mL) containing 6 M guanidine and 3.3 mM EDTA-2Na. To the mixture was added a solution of methyl 4-nitrobenzenesulfonate (8.1 mg, 67.5 μmol) in acetonitrile (416 μL) and the mixture was stirred for 40 min. Then the mixture was neutralized by 10% TFA solution (0.10 mL) and the solution was concentrated in vacuo to remove organic solvent. Purification of the residue to remove excess salt by RP-HPLC (condition: C18 column, 5 μm, 250 × 4.5 mm Isocratic of 0.1% TFA aqueous for 10 min, then linear gradient of 9→36% CH_3CN containing 0.1% TFA in 0.1% TFA aqueous over 30 min at a flow rate of 1.0 mL/min) afforded product **14** (5.5 mg, 90% isolated yield). In this step, this material was used for the next step without further purification. ESIMS: calcd for $C_{201}H_{319}N_{54}O_{80}S$ $[M + 3H]^{3+}$ 1602.0, $[M + 4H]^{4+}$

1201.8, $[M + 5H]^{5+}$ 961.6; found $[M + 3H]^{3+}$ 1601.5, $[M + 4H]^{4+}$ 1201.6, $[M + 5H]^{5+}$ 961.6.

Synthesis of 15 (CNBr Conversion Reaction of Sialylglycopeptide 14). Glycopeptide **14** (5.8 mg) was dissolved in 40% CH_3CN solution (600 μL) containing 2% v/v TFA (12 μL). To this solution was added CNBr (64 mg) and the mixtuer was stirred for 30.5 h in the dark at room temperature. Then H_2O (1.0 mL) was added and the mixture was lyophilized. Subsequently, the residue was treated as in the following condition to reduce oxidized starting material. The residue was dissolved in cooled (at −10 °C) TFA (2.9 mL). To this solution was added NH_4I (7.8 mg) and dimethyl sulfide (2.6 μL) at −10 °C. The mixture was stirred for 30 min at room temperature and then ice cooled H_2O (1.2 mL) was added. This mixture was washed by ice cooled CCl_4. The water phase was concentrated in vacuo at 10 °C. Purification of the residue by reverse-phase HPLC (condition: C18 column, linear gradient of 4.5→36% CH_3CN containing 0.1% TFA in 0.1% TFA solution over 30 min at flow rate 1.2 mL/min) afforded MUC1 repeated sialylglycopeptide **15** (2.2 mg, 38% isolated yield). ESIMS: calcd for $C_{200}H_{317}N_{54}O_{81}$ $[M + 3H]^{3+}$ 1592.0, $[M + 4H]^{4+}$ 1194.2, $[M + 5H]^{5+}$ 955.6; found $[M + 3H]^{3+}$ 1592.0, $[M + 4H]^{4+}$ 1194.4, $[M + 5H]^{5+}$ 955.8.

Synthesis of 16 (O to N Intramolecular Acyl Shift and Removing Methylester of Sialyl-T_N Moiety). Sialylglycopeptide **15** (2.0 mg, 0.42 μmol) was dissolved in 5 mM NaOH solution (1.67 mL) at 0 °C and the mixture was stirred for 25 min. To the mixture was added 50 mM NaOH solution (1.3 mL) and the reaction mixture was allowed to gradually warm to room temperature and stirred for 28 h. Then, the solution was neutralized by AcOH (6 μL). Purification of the mixture by RP-HPLC (condition: C18 column, 5 μm, 250 × 4.5 mm, linear gradient of 9→27% CH_3CN in 50 mM NH_4OAc solution over 60 min at a flow rate of 1.0 mL/min) afforded MUC1 repeated segment **16** (ca. 1.0 mg, ca. 50% isolated yield, 70% yield estimated by HPLC peak area intensity). ESIMS: calcd for $C_{198}H_{313}N_{54}O_{81}$ $[M + 3H]^{3+}$ 1582.6, $[M + 4H]^{4+}$ 1187.2, $[M + 5H]^{5+}$ 950.0; found $[M + 3H]^{3+}$ 1582.8, $[M + 4H]^{4+}$ 1187.5, $[M + 5H]^{5+}$ 950.2.

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Supporting Information Available: Full experimental details for the CNBr conversion of **1** and the synthesis of **5**, **9**, **10**, and **16**, and NMR spectra of all new compounds and coupling compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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