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Construction of Highly Glycosylated Mucin-Type Glycopeptides Based on Microwave-Assisted Solid-Phase Syntheses and Enzymatic Modifications

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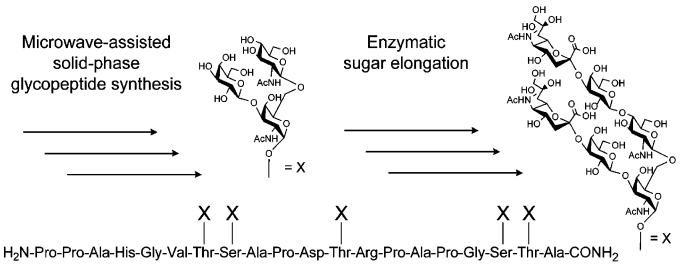
Received December 28, 2005



A MUC1-related glycopeptide having five core-2 hexasaccharide branches ($C_{330}H_{527}N_{46}O_{207}$, MW = 8450.9) was synthesized by a new strategy using a combination of microwave-assisted solid-phase synthesis (MA-SPGS) and enzymatic sugar elongation. Synthesis of a key glycopeptide intermediate was best achieved in a combination of PEGA [poly(ethylene glycol)-poly-(*N*,*N*-dimethylacrylamide) copolymer] resin and MA-SPGS using glycosylated amino acid building blocks with high speed and high purity. Deprotection of the glycopeptide intermediate and subsequent glycosyltransferase-catalyzed sugar elongations were performed for generation of the additional diversities with the sugar moieties of glycopeptides using β1,4-galactosyltransferase (β1,4-GalT) and two kinds of α2,3-sialyltransferases [ST3Gal III; α2,3-(*N*)-SiaT and ST3Gal II; α2,3-(*O*)-SiaT]. These reactions proceeded successfully in the presence of 0.2% Triton X-100 to convert the chemically synthesized trisaccharide glycans to disialylated hexasaccharide.

Introduction

Mucin-type *O*-glycans are known to exist ubiquitously on the surface of mammalian cell membrane and play various roles in biological systems. Among many types of mucin *O*-glycan motifs, core-2 *O*-glycans are one major class of the core structures having functional oligosaccharides such as sialyl-Le^x and sialyl-Le^a, and related structures are ligands for selectins and galectins in cell−cell interaction events that play important roles in T-cell development,[1] lymphocyte trafficking,[2,3] the inflammatory process,[4,5] and cancer metastasis.[6] In addition, leukocytes in the peripheral blood express a substantial amount of core-2 branched oligosaccharides in the patients with immunodeficiency syndromes such as Wiskott−Aldrich syndrome,[7,8] AIDS,[9] and leukemia,[10] while leukocytes of normal individuals do not express them. Core-2 type oligosaccharides

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10.1021/jo0526643 CCC: $33.50
Published on Web 03/15/2006

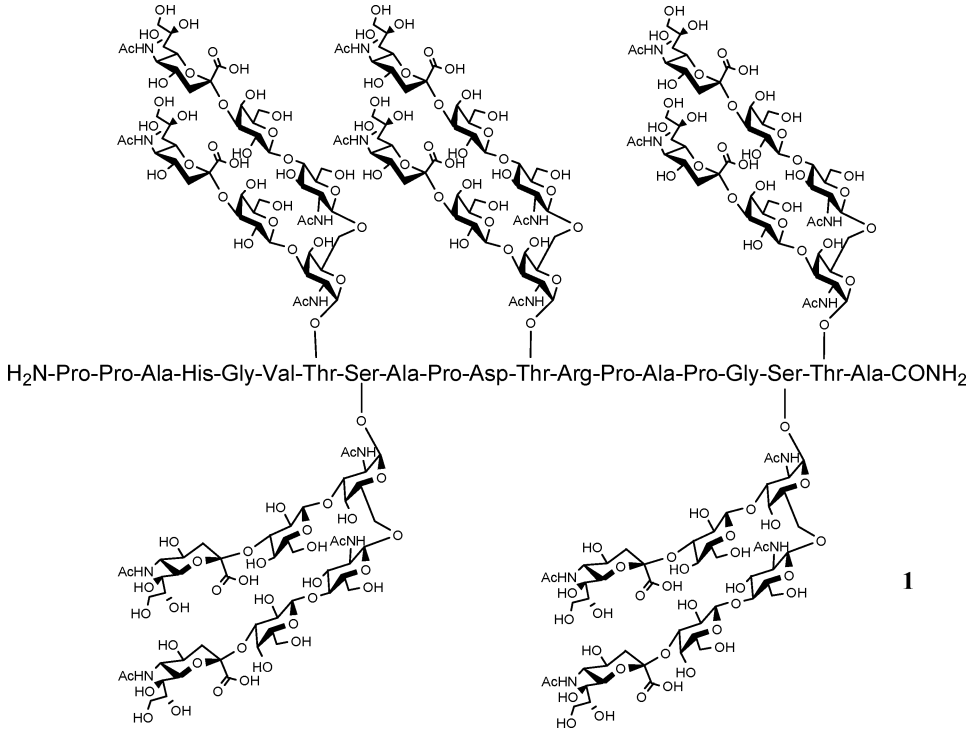

FIGURE 1. Representative synthetic target of glycopeptide related to the mucin MUC1 tandem repeating unit.

are also the basement structure for the generation of tandem repeating *N*-acetyllactosamine extensions[6,11−13] and provide the backbone structure for additional modifications of functional sugars such as sialyl-Le^x tetrasaccharide.[2,6] Therefore, glycopeptides having core-2 based *O*-glycans are attractive candidates for drug discovery because of their various biological functions and consequent therapeutic potentials. If large and diverse glycopeptide libraries can be constructed, they will become nice tools for vast areas of many biological investigations. However, syntheses of glycopeptides having complex sugars such as core-2 type branched trisaccharide are still challenging studies.[14−18] The main difficulty in glycopeptide synthesis is caused by the extremely low coupling yield due to the amino acids bearing sterically hindered sugar moiety. At present, solid-phase glycopeptide synthesis (SPGS) is needed to use large excess amounts of sugar-bound amino acid derivatives compared to those of common amino acid derivatives and require a much longer reaction time (over 10 h) for achieving a satisfactory yield in each step. We thought that the advent of novel methods for the practical solid-phase synthesis of simple glycopeptides would contribute to the construction of much more complicated glycopeptides by enzymatic modifications.[19−22]

Recently, our attention has been directed toward studying the effects of microwave irradiation on accelerating the reaction speed of a variety of chemical reactions.[23,24] In particular, highly enhanced coupling efficiency in solid-phase peptide synthesis by microwave irradiation[25,26] motivated us to apply this procedure to the synthesis of some key glycopeptide intermediates that can be employed for further enzymatic modifications. In this paper, we describe a practical synthesis of highly glycosylated glycopeptide by combined microwave irradiation[27] and enzymatic modifications. Figure 1 shows the representative target compound, a MUC1-related glycopeptide (**1**), having five hexasaccharide branches at the positions of Thr-7, Ser-8, Thr-12, Ser-18, and Thr-19.

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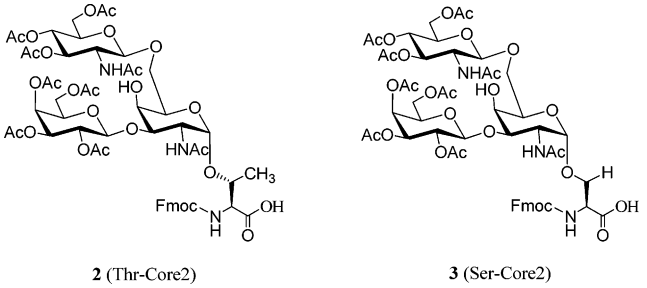
TABLE 1. Solid-Phase Synthesis of Glycopeptide 4 with/without Microwave Irradiation

SPGS condition	1	2	3	4
microwave	−	−	+	+
resin	TentaGel	TentaGel	TentaGel	PEGA
T (°C)	rt	50	50	50
coupling for Fmoc-AA-OH (3.0 equiv)	2 h	10 min	10 min	10 min
coupling for **1** or **2** (1.5 equiv)	12 h	20 min	20 min	20 min
acetyl capping		5 min, rt (entries 1−4)		
Fmoc deprotection (min)	20	3	3	3
total operating time[a] (h)	99	7	7	7
total reaction yield[b] (%)	41	15	44	67

[a] Sum total time of iteration procedures of couplings, Fmoc-deprotections, and acetyl cappings (5 min, rt). [b] Determinated by Fmoc photometric test.



2 (Thr-Core2) **3** (Ser-Core2)

FIGURE 2. Glycosylated amino acid building blocks **2** and **3** with core 2 structure.

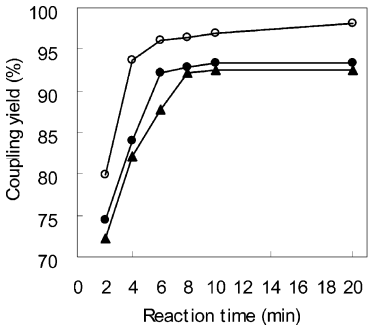


FIGURE 3. Coupling reaction of **2** with Ala-TentaGel resin at 50 °C under microwave irradiation (○), at 50 °C without microwave irradiation (●), and at room temperature without microwave irradiation (▲). The yields were calculated from Fmoc photometric tests according to the method described in the Experimental Section.

Results and Discussion

A. Microwave-Assisted Glycopeptide Synthesis on TentaGel or PEGA Resin. In our earlier communication,[27] we demonstrated microwave-assisted efficient synthesis of glycopeptides using Fmoc amino acid building blocks **2** and **3**, each having a core-2 type trisaccharide moiety[14,28] (Figure 2), with alanine preloaded TentaGel resin functionalized with Rinkamide linker[29,30] (Ala-resin). The coupling reaction was promoted by using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[31] *N*-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIEA) in DMF under microwave irradiation (2450 Hz) at 50 °C. As shown in Figure 3, coupling of **1** with Ala-resin proceeded smoothly, and over 90% yield of the reactions were obtained after 4 min with microwave irradiation and 98% yield after 20 min. These preliminary results motivated us to apply the microwave irradiation to the practical solid-phase synthesis of glycopeptides such as *O*-linked glycopeptides named MUC1-related glycopeptides as synthetic targets. MUC1 has an antigenic structure that consists of highly *O*-glycosylated tandem-repeating units of mucin-type glycoprotein which is expressed on the surfaces of epithelial cells in a variety of tissues.[32] The tandem-repeating units of MUC1 are composed of 20 amino acid residues with core-2 class *O*-glycans, and all serine and threonine residues are found in sequence. Scheme 1 shows the synthetic route to a MUC1-related glycopeptide having five core-2 branched trisaccharide **6** using building blocks **2** and **3**.

As we expected, solid-phase syntheses of compound **4** on a TentaGel resin functionalized with Rinkamide linker (0.25 mmol/g) were accelerated by employing microwave irradiation at 50 °C (entry 3 in Table 1, 44%), while the yield obtained without irradiation was found to be 15% at the same temperature (entry *2* in Table 1). To achieve a similar level of yield for the synthesis of **4** by a classical solid-phase protocol, we had to employ a much longer reaction time (99 h) as shown in entry 1 in Table 1 (41%), compared with the time required for entry 3 (44%, 7 h). Compound **5** released from the resin under general conditions [TFA/triisopropylsilane/H_2O (95/2.5/2.5), 2 h at room temperature] was characterized by reversed-phase HPLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Figure 4A shows analytical reversed-phase HPLC profiles of the crude product **5** prepared under the conditions listed in Table 1 (entries 1−3). The peaks eluted at 25.8 min (indicated by asterisks) were identified as the desired compound **5** by MALDI-TOFMS ($[M + H]^+$: *m*/*z* 6197.429) (Figure 4B). Other peaks at a−f were assigned as byproducts by MALDI-TOF/TOFMS analysis[27] (Figure 4C). The proportion of the desired product **5** in three HPLC profiles (Figure 4A) coincide with the trends in the overall yields in the synthesis of **4** (Table 1). These results also suggest that microwave irradiation significantly accelerated the speeds of the coupling reaction on the solid-phase support. Figure 4C indicates that major byproducts can be classified into two types: (1) products derived due to the difficulty in the coupling reactions between sterically hindered amino acids, namely Thr-core2, Ser-core2, Arg(Pbf), and His(Trt), and (2) compounds generated by missing *O*-acetyl

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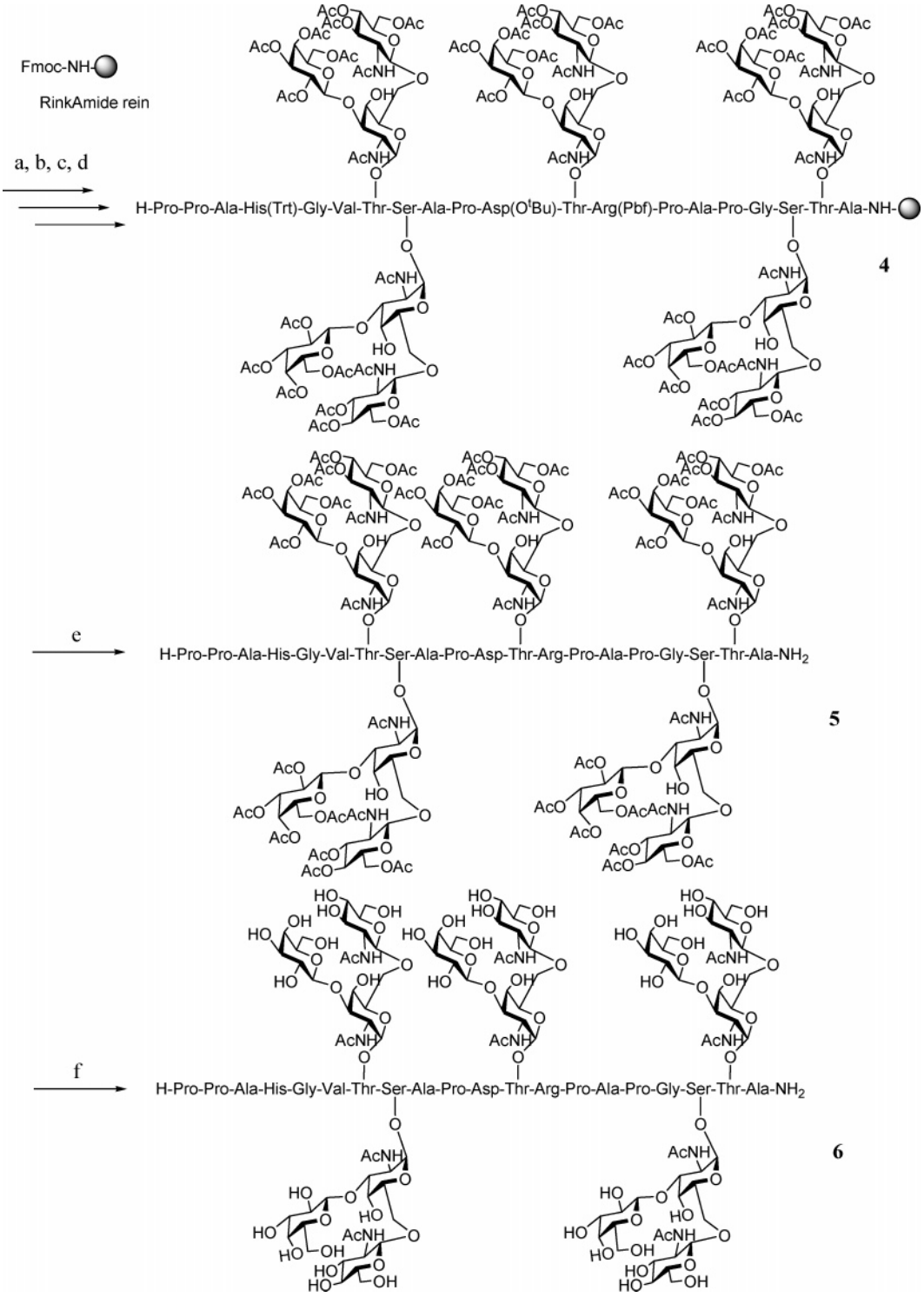
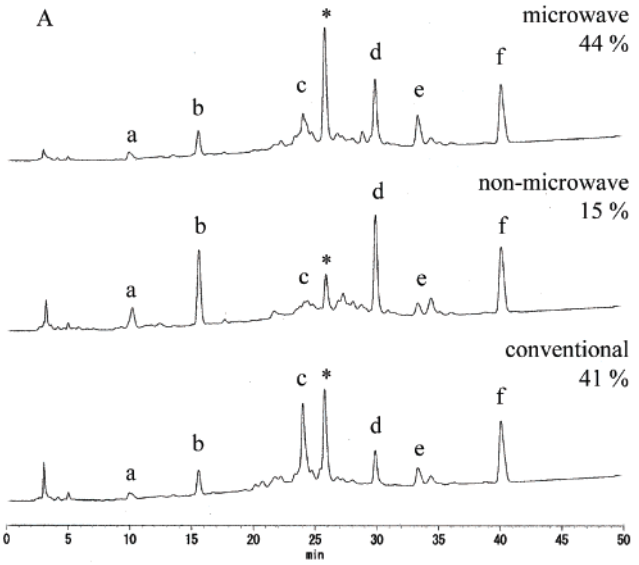
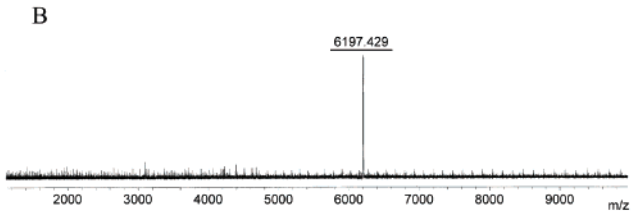
SCHEME 1. Synthetic Route to MUC1 Glycopeptide[a]



[a] Conditions: (a) **2**, **3**, or Fmoc-amino acid, HBTU, HOBt, DIEA, and DMF; (b) Ac_2O, HOBt, DIEA, and DMF; (c) 20% piperidine and DMF; (d) repeating the procedures a−c; (e) TFA/triisopropylsilane/H_2O (95:2.5:2.5, v/v/v); (f) 10 mM NaOH/MeOH.

groups owing to basic conditions during coupling and N^{α}-Fmoc removal reactions. Peak b (*m*/*z* 2522.7) was found to be the octapeptide byproduct generated during the coupling reaction at the 13th Arg(Pbf) with the 12th FmocThr-core2 (**2**). Peak a (*m*/*z* 2480.9) was identified as a de-*O*-acetylated derivative from the compound detected at peak b. It was suggested that peak c contains two kinds of byproducts such as molecular ion peaks at *m*/*z* 3771.2 corresponding to the glycopeptide having three carbohydrate chains and at *m*/*z* 6155.4 corresponding to the de-*O*-acetylated byproduct derived from **5**. Similarly, peaks d (*m*/*z* 4719.6) and e (*m*/*z* 5838.4 *m*/*z*) were also assigned as two incomplete glycopeptides. This result suggests that improvement of the efficiency in the coupling between the 7th Thr-core2 and 8th Ser-core2 may become a crucial step to enhance the overall





C

peak	found mass (m/z)	estimated by-product sequences
a	2480.9	Ac-RPAPGS**T**A-NH$_2$ - (1 × Ac)
b	2522.7	Ac-RPAPGS**T**A-NH$_2$
c	3771.2	Ac-APD**T**RPAPGS**T**A-NH$_2$
	6155.4	PPAHGV**TS**APD**T**RPAPGS**T**A-NH$_2$ - (1 × Ac)
d	4719.6	Ac-**S**APD**T**RPAPGS**T**A-NH$_2$
e	5838.4	Ac-GV**TS**APD**T**RPAPGS**T**A-NH$_2$
f	100 - 700	low weight molecular by-products

FIGURE 4. (A) HPLC profiles of a crude glycopeptide **5** synthesized under the conditions of entry 1 (bottom), entry 2 (middle), and entry 3 (top) indicated in Table 1, respectively. Peaks marked with an asterisk (*) indicate the desired glycopeptide **5**. (B) MALDI-TOF mass spectrum of the purified product **5** by HPLC. $C_{260}H_{379}N_{36}O_{137}$ $[M + H]^+$, calcd *m*/*z* 6197.380, found *m*/*z* 6197.429. (C) Byproducts detected by analytical reversed-phase HPLC (Figure 4A, a−f) were characterized by MALDI-TOF MS. Bold red letters in the sequences in the byproducts indicate the Thr or Ser residues having acetylated core-2 structure.

yield of the full-length glycopeptide on the solid phase even under microwave irradiation.

Since a combination of the highly bulky amino acids **2**/**3** with the hydrophobic nature of the TentaGel resin seemed to make large glycopeptide intermediates difficult, we selected PEGA resin,[14,33−36] a poly(ethylene glycol)-poly(dimethylacrylamide) copolymer, as an alternative potential solid support to the

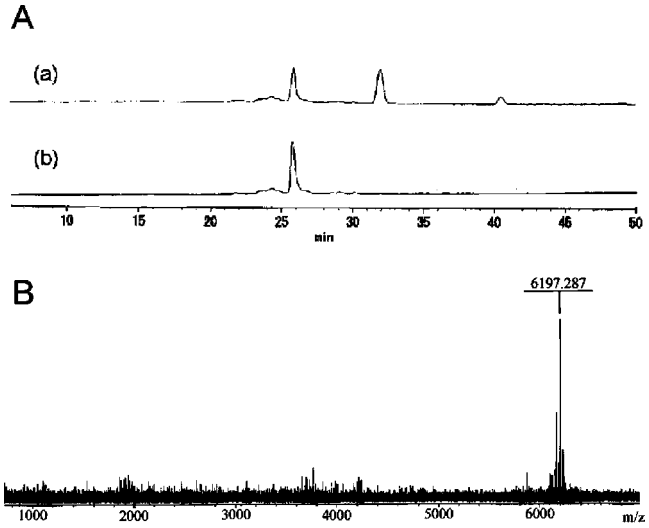


FIGURE 5. HPLC and MALDI-TOF MS analysis of the crude and purified product **5** synthesized under the conditions of entry 4 in Table 1. **(**A) HPLC profiles: (a) the reaction mixture obtained by treatment with a "cleavage cocktail" for 2 h, (b) the product obtained by addition of *tert*-butylmethyl ether. (B) MALDI-TOF mass spectrum of **5** prepared by the above purification procedure.

TentaGel resin. As anticipated, PEGA resin allowed much more efficient glycopeptide synthesis than those observed in cases of TentaGel resin. As shown in entry 4 listed in Table 1, the total yield detected by the Fmoc-photometric assay was significantly increased to be 67% by combined use of microwave irradiation and PEGA resin. This clearly suggests that DMF-swelled PEGA resin may permit the permeation both of reagents and steric building blocks **2**/**3** into the porous surfaces of the polymer particles. The resulting PEGA resin was treated with a cleavage cocktail [TFA/triisopropylsilane/H_2O (95:2.5:2.5)] for 2 h, and the mixture obtained by filtration was applied onto HPLC column chromatography (Figure 5A-a). When the mixture is simply purified by precipitation with *tert*-butylmethyl ether, much improved purity of the compound **5** compared to those of entries 1−3 (Figure 4A) can be achieved as shown in Figure 5A-b and Figure 5B. However, it was revealed that subsequent purification of this crude product by HPLC gave pure **5** in 11% isolated yield. Here, the isolated yield was determined by amino acid analysis and estimated on the basis of the first alanine residue loading on PEGA resin (the yield based on the weight of the freeze-dried material was approximately 13%). This unfavorable result means that the low isolated yield of the glycopeptide **5** may be due to the general condition for the deprotection of the peptides and cleavage of the linker moiety. Since acidic cleavage cocktails seem to cleave acid-labile glycoside bonds of the oligosaccharide branches, alternative combination of the protective groups for amino acids and the releasing reagent for the glycopeptides will be required for the totally successful glycopeptide synthesis on the solid supports. We are currently investigating a novel protection/deprotection strategy in amino acid residues suitable for solid-phase synthesis of glycopeptides. Finally, *O*-acetyl groups of the sugar moiety in **5** were removed by treating **5** with 10 mM sodium hydroxide in methanol solution at room temperature for 1 h to afford MUC1 glycopeptide **6** having five core-2 branches in 57% yield.

Figure 6B shows MALDI-TOF/TOF MS analysis of the compound **6** under the matrix-dependent selective fragmentation method in the presence of 2,5-dihydroxybenzoic acid (DHB)

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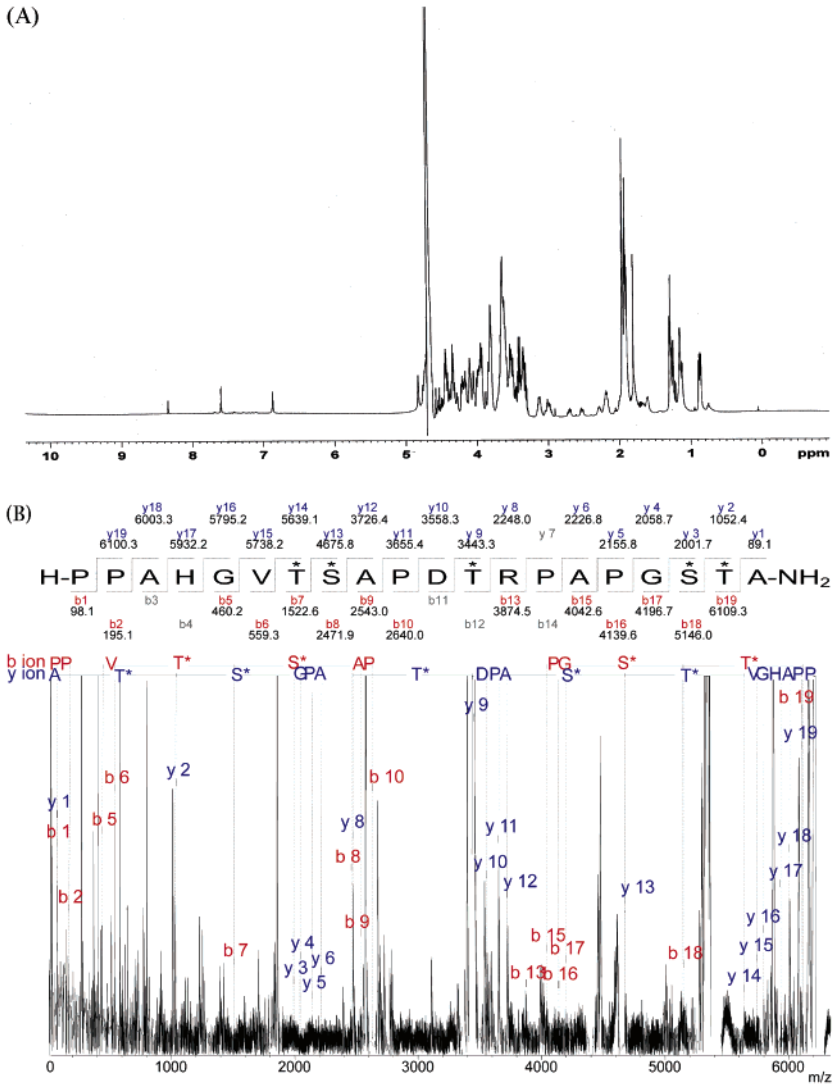

FIGURE 6. (A) 600 MHz ^{1}H NMR spectrum of **6**. The 1D spectrum was recorded on a Bruker DRX 600 Avance spectrometer equipped with a cryoprobe using a Shigemi tube at 300 K. The measurement was achieved by suppressing the water peak using the presaturation method. [Compound **6**] = 100 μM in 300 μL of 99.96% D_2O. (B) MALDI-LIFT-TOF/TOF mass spectrum of glycopeptide **5**. S* and T* indicate serine and threonine residues having a per-*O*-acetylated core-2 trisaccharide, respectively.

as a matrix.[37,38] Fragmentation by LIFT-TOF/TOF occurred successfully in the peptide linkages and gave meaningful b- and y-series product ion peaks without serious cleavage at *O*-glycoside linkages. The result suggests that important fragment ion peaks such as b6, b8, b11, b12, b17, b18, b19, y1, y2, y3, y8, y9, y12, y13, and y14 are due to the target 20-residue glycopeptide having five fully *O*-acetylated core-2 trisaccharide residues attached to the positions at Thr-7, Ser-8, Thr-12, Ser-18, and Thr-19.

B. Enzymatic Sugar Elongation. Our next interest was focused on the feasibility of enzymatic modification of highly glycosylated glycopeptide **6** to generate diversities in the synthetic libraries of MUC1-related glycopeptides. As indicated in Scheme 2, commercially available human D-glucose 4-β-D-galactosyltransferase (β1,4-GalT), rat liver β-D-galactosyl-β1,3/4-*N*-acetyl-β-D-glucosamine-α2,3-sialyltransferase[39] [α2,3-(N)-SiaT], and rat liver β-D-galactosyl-β1,3-*N*-acetyl-β-D-galactosamine-α2,3-sialyltransferase[40] [α2,3-(*O*)-SiaT] were preliminarily examined for generation of more complicated glycopeptide series (**7**, **8**, **9**, **10**, and **1**) than the starting compound **6**. First, we examined an extension of galactose residue at the C-4 position of the GlcNAc residue of compound **6** using β1,4-GalT with UDP-Gal under general conditions. However, the reaction did not proceed smoothly and gave a mixture of the partially galactosylated materials (data not shown). We thought that this may be due to a poor solubility of the macromolecular intermediate **6** in addition to the sterically hindered sugar clustering on the MUC1 polypeptide. Fortunately, it was found that enzymatic reaction toward an unusually modified peptide chain was promoted efficiently in the presence of 0.2% Triton X-100 and gave a target compound **7** in quantitative yield as shown in the analytical reversed-phase HPLC (Figure 7b). Similarly, α2,3-(*O*)-SiaT catalyzed sialylation of **6** also pro-

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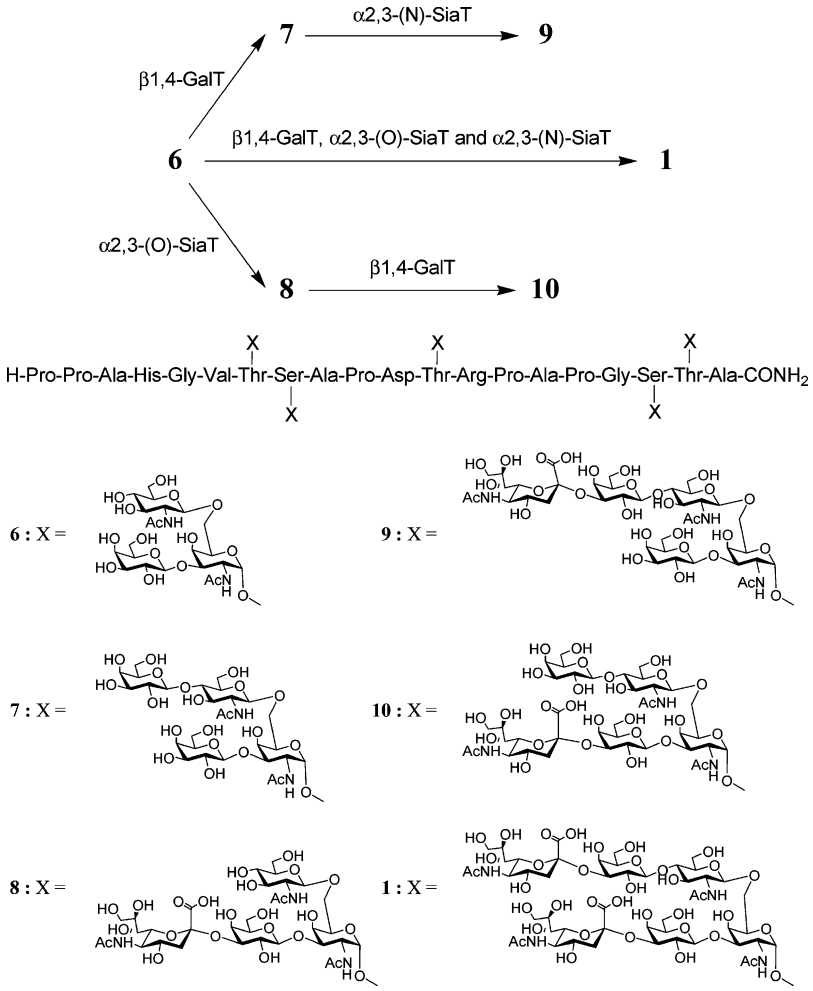
SCHEME 2. Enzymatic Glycosylation Strategies



ceeded smoothly in the presence of CMP-Neu5Ac and 0.2% Triton X-100 at 25 °C for 24 h to afford compound **8** in 77% yield (Figure 7c). As for the synthesis of the compounds **9**, **10**, and **1**, it was demonstrated that selective or sequential sialylation reactions by means of α2,3-(*O*)-SiaT and α2,3-(*N*)-SiaT made parallel syntheses of these glycopeptides possible in satisfactory yields as shown in Figure 7d−f. Figure 8 indicates MALDI-TOFMS data of the target compounds synthesized in this study.

Structural characterization of all new synthetic MUC1 glycopeptides by NMR could also be performed, indicating that the present strategy is suited for the preparative-scale (**6**, 14.7 mg; **7**, 4.2 mg; **8**, 4.7 mg; **9**, 2.4 mg; **10**, 2.9 mg; and **1**, 3.0 mg) synthesis of this class of mucin-type glycopeptides. Figure 9 exhibits a fingerprint region of 600 MHz TOCSY spectra of MUC1 glycopeptides **7** (a), **9** (b), **10** (c), and **1** (d) at pH 4.0 in 90% H_2O/10% D_2O at 300 K. It was clearly suggested that the connectivities of the intraresidue spin system of carbohydrate moieties in these glycopeptides can be well identified as indicated by the solid lines in these TOCSY spectra. In addition, the TOCSY and NOESY spectra of glycpeptide 1 revealed that overlapping cross-peaks with TOCSY spectra represent intraresidue NOEs and other cross-peaks represent interresidue NOEs (Figure 10). ^{1}H NMR chemical shifts of glycopeptides assigned by means of DQF-COSY, TOCSY, and NOESY spectra are summarized in Tables 2 and 3. Further precise structural characterization and biological evaluation are under investigation and the results will be reported as soon as possible.

Conclusion

A rapid synthesis of highly glycosylated MUC1 glycopeptides having core-2 type *O*-glycans at five *O*-glycosylation sites was carried out by combining microwave-assisted solid-phase glycopeptide synthesis (MA-SPGS) and enzymatic modifications. Coupling reactions of highly bulky sugar−amino acid building blocks on solid supports were greatly accelerated by microwave irradiation. In fact, our protocol for SPGS of the MUC1-related 20-residue glycopeptide **4** was accomplished in only 7 h using microwave heating, while a general and conventional protocol required more than 4 days to synthesize this same compound. In addition, the combination of PEGA resin and MA-SPGS gave the best conditions for achieving the synthesis of MUC1 glycopeptides with high speed and high purity. Subsequent enzymatic glycosylation using β1,4-galactosyltransferase, α2,3-(*N*)-sialyltransferase, and α2,3-(*O*)-sialyltransferase were also carried out to generate molecular diversities of the biologically interesting glycopeptides. It was also demonstrated that addition of the detergent (Triton X-100) allowed for much-improved enzymatic glycosylation of glycopeptide intermediates with poor solubility toward buffer solutions and/or relatively rigid conformation. The combination strategy of MA-SPGS and glycosyltransferase-catalyzed sugar extension should greatly contribute to high-speed parallel syntheses of useful glycopeptide libraries for further biochemical and immunological investigations.

TABLE 2. 600 MHz ^{1}H NMR Chemical Shifts (ppm) of the Amino Acid Moieties in Glycopeptides 6−10 and 1 at pH 4.0 in 90% H_2O/10% D_2O at 300 K

		glycopeptides								glycopeptides					
residues		**6**	**7**	**8**	**9**	**10**	**1**	residues		**6**	**7**	**8**	**9**	**10**	**1**
Pro[1]	αH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Asp[11]	NH	8.34	8.36	8.36	8.36	8.37	8.35
	βH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		αH	4.64	4.63	4.59	4.63	4.44	4.68
	γCH$_2$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		βCH$_2$	2.54	2.56	2.60	2.56	2.61	2.54
	δCH$_2$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			2.71	2.72	2.75	2.72	2.76	2.71
Pro[2]	αH	4.27	4.40	4.27	4.26	4.27	4.28	Thr[12]	NH	8.61	8.61	8.63	8.61	8.62	8.66
	βH	2.17	2.17	2.18	2.16	2.16	2.17		αH	4.40	4.39	4.40	4.38	4.40	4.41
	γCH$_2$	1.77	1.77	1.89	1.77	1.77	1.78		βH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		1.90	1.89	n.d.	1.90	1.90	1.90		γCH$_3$	1.11	1.09	1.11	1.13	1.11	1.07
	δCH$_2$	3.56	3.55	3.56	3.58	3.57	3.50	Arg[13]	NH	8.24	8.24	8.17	8.25	8.17	8.20
		3.67	3.67	3.66	3.70	3.63	3.63		αH	4.36	4.37	4.34	4.38	4.35	4.32
Ala[3]	NH	8.37	8.37	8.37	8.38	8.37	8.39		βH	1.64	1.64	1.65	1.62	1.65	1.67
	αH	4.44	4.42	4.44	4.43	4.44	4.42			1.74	1.73	1.73	1.73	1.74	1.72
	βCH$_3$	1.27	1.27	1.27	1.27	1.26	1.26		γCH$_2$	1.60	1.59	1.60	1.58	1.60	1.59
His[4]	NH	8.39	8.39	8.35	8.39	8.36	8.34		δCH$_2$	3.11	3.11	3.12	3.11	3.11	3.11
	αH	4.60	4.60	4.64	4.58	4.59	4.58	Pro[14]	αH	4.35	4.34	4.35	4.23	4.36	4.28
	βH	3.09	3.09	3.09	3.09	3.09	3.09		βH	2.20	2.21	2.16	2.20	2.21	2.17
		3.19	3.19	3.19	3.19	3.19	3.20		γCH$_2$	1.89	1.90	1.90	1.90	1.90	1.90
	2H	8.44	8.44	8.42	8.44	8.43	8.41		δCH$_2$	3.50	3.49	3.50	3.49	3.50	3.50
	4H	7.22	7.22	7.23	7.23	3.09	3.09			3.62	3.62	3.62	3.61	3.62	3.64
Gly[5]	NH	8.39	8.39	8.39	8.39	8.39	8.37	Ala[15]	NH	8.45	8.45	8.46	8.45	8.46	8.45
	αH	3.82	3.81	3.81	3.83	3.81	3.82		αH	4.14	4.12	4.12	4.13	4.12	4.12
		3.93	3.93	3.94	3.91	3.93	3.92		βCH$_3$	1.24	1.23	1.24	1.23	1.24	1.24
Val[6]	NH	8.04	8.04	8.07	8.03	8.05	8.04	Pro[16]	αH	4.33	4.32	4.33	4.31	4.32	4.32
	αH	4.23	4.23	4.21	4.22	4.22	4.20		βH	2.20	2.19	2.19	2.20	2.20	2.21
	βH	1.98	1.98	1.98	1.99	1.98	1.98		γCH$_2$	n.d.	n.d.	n.d.	n.d.	n.d.	1.76
	γCH$_3$	0.86	0.86	0.87	0.86	0.87	0.88			1.89	1.93	1.90	1.87	1.88	1.89
Thr[7]	NH	8.63	8.63	8.63	8.61	8.62	8.59		δCH$_2$	3.55	3.56	3.56	3.56	3.56	3.56
	αH	4.60	4.60	4.60	4.61	4.59	4.61			3.65	3.68	3.69	3.68	3.69	3.70
	βH	4.18	4.17	n.d.	n.d.	n.d.	n.d.	Gly[17]	NH	8.25	8.27	8.24	8.27	8.25	8.27
	γCH$_3$	1.12	1.13	1.09	1.11	1.12	1.12		αH	3.85	3.84	3.83	3.84	3.82	3.81
Ser[8]	NH	8.69	8.64	8.77	8.67	8.73	8.72			4.00	4.00	4.02	4.01	4.02	4.05
	αH	4.54	4.54	4.56	4.54	4.55	4.57	Ser[18]	NH	8.33	8.33	8.33	8.35	8.35	8.37
	βCH$_2$	3.68	3.68	3.65	3.71	3.67	3.70		αH	4.74	4.74	4.73	4.73	4.73	4.72
		3.80	3.80	3.82	3.79	3.82	3.81		βCH$_2$	3.78	3.78	3.78	3.77	3.79	3.79
Ala[9]	NH	8.35	8.36	8.43	8.36	8.44	8.46			3.95	3.94	3.93	3.95	3.94	3.93
	αH	4.40	4.40	4.38	4.38	4.38	4.39	Thr[19]	NH	8.79	8.79	8.85	8.81	8.83	8.82
	βCH$_3$	1.27	1.27	1.28	1.27	1.27	1.25		αH	4.51	4.50	4.52	4.50	4.52	4.51
Pro[10]	αH	4.31	4.27	4.40	4.27	4.27	4.25		βH	4.15	4.14	4.13	4.14	4.13	4.12
	βH	2.18	2.16	2.18	2.17	2.16	2.16		γCH$_3$	1.13	1.12	1.13	1.13	1.11	1.12
	γCH$_2$	1.80	1.78	1.76	1.78	1.77	1.77	Ala[20]	NH	8.37	8.37	8.37	8.37	8.35	8.34
		1.90	1.89	1.89	1.89	1.90	1.87		αH	4.17	4.17	4.16	4.16	4.16	4.15
	δCH$_2$	3.55	3.55	3.56	3.55	3.57	3.54		βCH$_3$	1.27	1.27	1.27	1.27	1.27	1.27
		3.67	3.67	3.66	3.70	3.63	3.64								

Experimental Section

General Method and Materials. All commercially available solvents and reagents were used without further purification. Fmoc amino acids of Arg, Asp, and His were employed as Arg(Pbf), Asp(O^{t}Bu), and His(Trt) according to the general synthetic protocols reported previously.[41,42] All solid-phase reactions for glycopeptide synthesis were performed manually in a polypropylene tube equipped with a filter. Swelling, washing, acetyl capping, and final cleavage procedures in the solid-phase syntheses were carried out at room temperature. The reaction vessel for solid-phase synthesis was placed inside a cavity of a microwave instrument and was stirred with a vortex mixer. Single-mode microwave was irradiated at 2450 MHz using temperature control at 50 °C. Glycopeptide syntheses without microwave irradiation at 50 °C were performed in a dry oven, and the reaction vessel was stirred. The resulting glycopeptidyl-resins were cleaved by treatment with a "cleavage cocktail" solution [TFA/triisopropylsilane/DCM (95/2.5/2.5, v/v/v)]. FAB-mass analyses were carried out using *m*-nitrobenzyl alcohol (NBA) as a matrix. In enzymatic glycosylation experiments, the prepurification in order to remove detergent was accomplished by HPLC equipped with gel permeation chromatography (GPC) column eluted with 10 mM ammonium acetate containing 10% CH_3-CN at a flow rate of 1 mL/min. Condition of the analytical reversed-phase HPLC is described as follows: elution buffer A, 10 mM ammonium acetate buffer (pH 5.8); elution buffer B, CH_3CN containing 10% buffer A; linear gradient from 0 to 60 min, (A/B) = (98/2) to (89/11); flow rate, 1 mL/min; UV monitoring, 220 nm; column temperature, 25 °C.

Preparation of Crystals for MALDI-TOF and MALDI-LIFT-TOF/TOF MS. Sinnamic acid (CHCA) or 2,5-dihyroxybenzoic acid (DHB) was used as matrix. DHB (10 mg) was dissolved in water (1 mL), and CHCA was prepared as a saturated solution in 3:1 (v/v) of acetonitrile/water. Stock solutions of glycopeptides were prepared by dissolving them in pure water. A matrix solution (0.5 μL) was applied on the target spot of a MALDI-TOF plate and the sample solution (1.0 μL) was added to the matrix droplet, and then dried at room temperature. We employed these samples (about 1−10 pmol) with both MALDI-TOF and MALDI-LIFT-TOF/TOF mode using the above preparation procedure.

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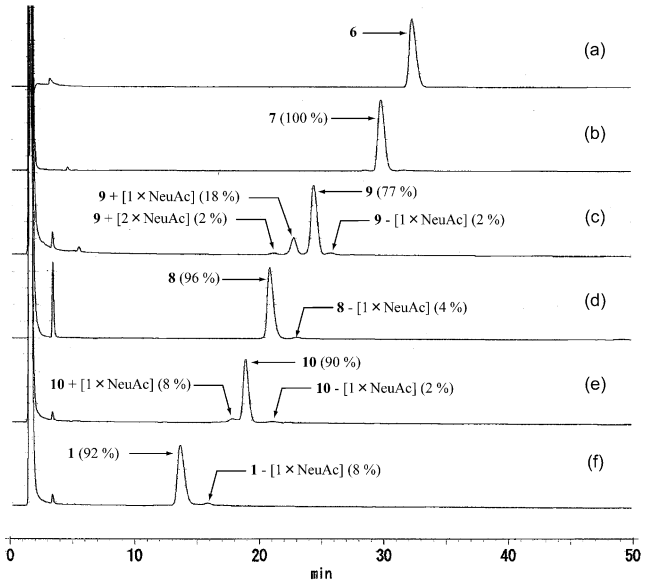

FIGURE 7. HPLC profiles of a key intermediate **6** (a) and crude products **7** (b), **9** (c), **8** (d), **10** (e), and **1** (f) obtained by the modifications with glycosyltransferases. All materials were purified by using GPC−HPLC to remove the detergent before applying to HPLC analyses.

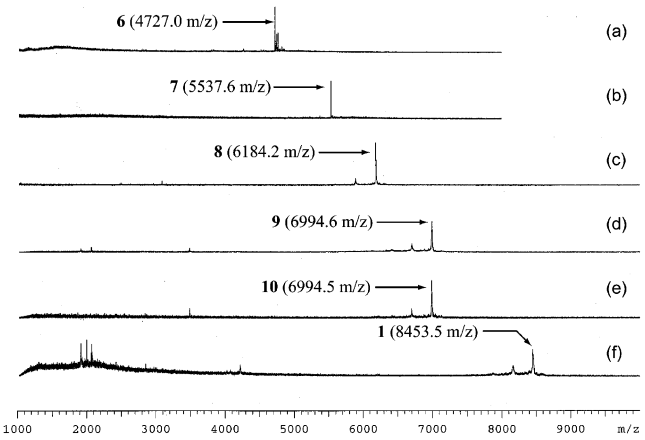

FIGURE 8. Positive-ion-mode MALDI-TOF mass spectra of **6** (a) and **7** (b) and negative-ion-mode MALDI-TOF mass spectra of **8** (c), **9** (d), **10** (e), and **1** (f), respectively. All materials were purified by HPLC before the measurements.

MALDI-TOF Mass Spectrometry. The MALDI-TOF mass spectrometer was equipped with a reflector and controlled by the Flexcontrol 1.2 software package according to the general protocols reported in the previous papers.[37,38] In MALDI-TOF MS reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas.

In MALDI-LIFT-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell (LIFT means "lifting" the potential energy for the second acceleration of ion source), and their masses were analyzed after the ion reflector passage. Masses were automatically annotated by using FlexAnalysis 2.0 software package. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (*m*/*z* 1046.542), bombesin (*m*/*z* 1619.823), ACTH (18−39) (*m*/*z* 2465.199), and somatostatin 28 (*m*/*z* 3147.472). The mixture of these peptides was measured on the central spot of a 3 × 3 square by using external calibration. To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically by utilizing a customized macro command of the FlexControl 2.1 software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the above-mentioned peptides. TOF/TOF spectra were annotated with the BioTools 2.1.

TABLE 3. 600 MHz ^{1}H NMR Chemical Shifts of the Carbohydrate Moieties in MUC1 Glycopeptides 6−10 and 1 at pH 4.0 in 90% H_2O/10% D_2O at 300 K

		glycopeptides					
residues		**6**	**7**	**8**	**9**	**10**	**1**
GalNAc **I** (Thr7)	H-1	4.81	4.83	4.81	4.83	4.83	4.83
	H-2	4.09	4.09	4.10	4.10	4.10	4.10
	H-3	3.92	3.92	3.91	3.92	3.90	3.91
	H-4	n.d.	n.d.	4.03	4.03	4.05	4.03
	^{CH}3	1.90	1.90	1.90	1.89	1.89	1.89
	NH	7.35	7.36	7.26	7.32	7.27	7.24
GalNAc **I** (Ser8)	H-1	4.75	4.75	4.74	4.76	4.77	4.75
	H-2	4.20	4.10	4.10	4.20	4.18	4.18
	H-3	3.93	3.92	3.90	3.97	3.94	3.96
	H-4	n.d.	n.d.	4.08	4.08	4.07	4.06
	^{CH}3	1.94	1.89	1.90	1.88	1.88	1.88
	NH	7.88	7.88	7.93	7.83	7.84	7.79
GalNAc **I** (Thr12)	H-1	4.75	4.76	4.76	4.65	4.71	4.71
	H-2	4.10	4.20	4.18	4.11	4.11	4.10
	H-3	3.92	3.95	3.94	3.91	3.90	3.91
	H-4	n.d.	n.d.	4.07	4.01	4.04	4.01
	CH_3	1.94	1.89	1.88	1.89	1.89	$^{1.8}$9
	NH	7.87	7.86	7.81	7.87	7.94	7.99
GalNAc **I** (Ser18)	H-1	4.73	4.81	4.65	4.79	n.d.	n.d.
	H-2	4.15	4.14	4.12	4.14	4.13	4.12
	H-3	3.92	3.92	3.92	3.91	3.91	3.91
	H-4	n.d.	n.d.	n.d.	4.08	4.04	4.07
	^{CH}3	1.88	1.88	1.86	1.87	1.87	1.88
	NH	7.59	7.58	7.54	7.56	7.53	7.51
GalNAc **I** (Thr19)	H-1	4.81	4.81	4.80	4.80	4.80	4.79
	H-2	4.14	4.12	4.13	4.14	4.13	4.13
	H-3	3.96	3.93	3.95	3.93	3.91	3.91
	H-4	n.d.	n.d.	4.05	4.06	4.05	4.04
	^{CH}3	1.91	1.88	1.89	1.89	1.90	1.90
	NH	7.57	7.57	7.49	7.59	7.57	7.61
GlcNAc **IIIa**	H-1	4.43	4.44	4.42	4.43	4.44	4.43
	H-2	3.58	3.60	3.59	3.62	3.60	3.59
	H-3	3.40	3.48	3.33	3.48	3.48	3.47
	H-4	n.d.	3.88	3.40	3.56	n.d.	3.57
	^{CH}3	$^{1.9}$4	1.93	1.93	1.94	1.92	1.93
	NH	8.11	8.13	8.08	8.13	8.12	8.12
GlcNAc **IIIb**	H-1	4.43	4.44	4.43	4.44	4.44	4.44
	H-2	3.59	3.57	3.52	3.63	3.61	3.61
	H-3	3.42	3.48	3.33	3.48	3.48	3.47
	H-4	n.d.	3.88	3.44	3.57	n.d.	3.56
	^{C}H3	1.94	1.94	1.96	1.93	1.93	1.93
	NH	8.08	8.10	8.03	8.10	8.09	8.09
Neu5Ac **V**	H-3a			1.67	1.68		1.67
	H-3e			2.64	2.64		2.63
	^{CH}3			1.90	1.91		1.91
	NH			7.96	7.96		7.95
Neu5Ac **VI**	H-3a					1.66	1.67
	H-3e					2.63	2.63
	CH_3					1.90	1.91
	NH					7.95	7.95

NMR Spectroscopy. The glycopeptides **6**−**10** and **1** were dissolved at a final concentration of 1.4−2.0 mM in 300 μL of 90% H_2O/10% D_2O at pH 4.0. The pH was adjusted to 4.0 by addition of HCl and NaOH. All 1D and 2D ^{1}H NMR spectra were recorded with an operating frequency of 600 MHz at a temperature of 300 K. Standard NMR pulse sequences were used for 2D DQF-COSY, TOCSY, and NOESY experiments. TOCSY spectra with MLEV-17 sequence[43,44] were collected with a spin-lock time of

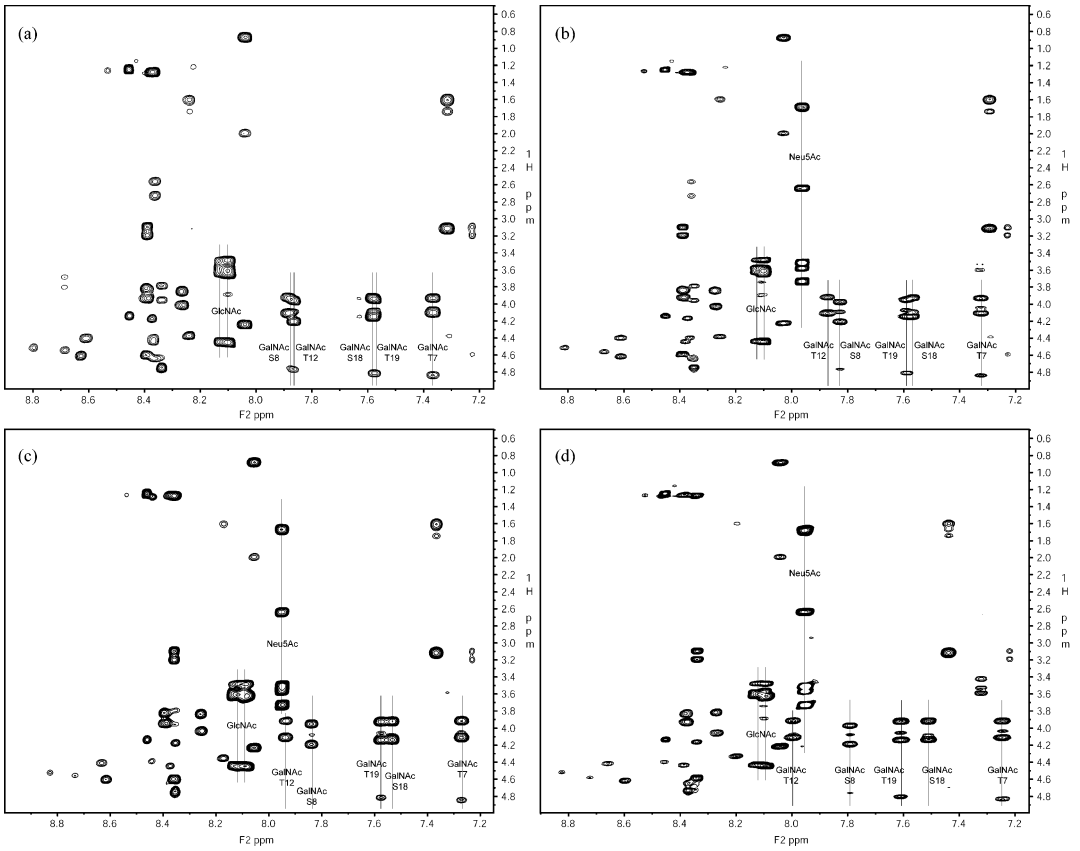

FIGURE 9. Fingerprint region of 600 MHz TOCSY spectra of MUC1 glycopeptides **7** (a), **9** (b), **10** (c), and **1** (d). The two-dimensional TOCSY spectra were recorded on a Bruker DRX 600 Avance spectrometer equipped with cryoprobe at pH 4.0 in 90% H_2O/10% D_2O at 300 K. Solid lines represent the connectivities of the intraresidue spin system of carbohydrate moieties in glycopeptides.

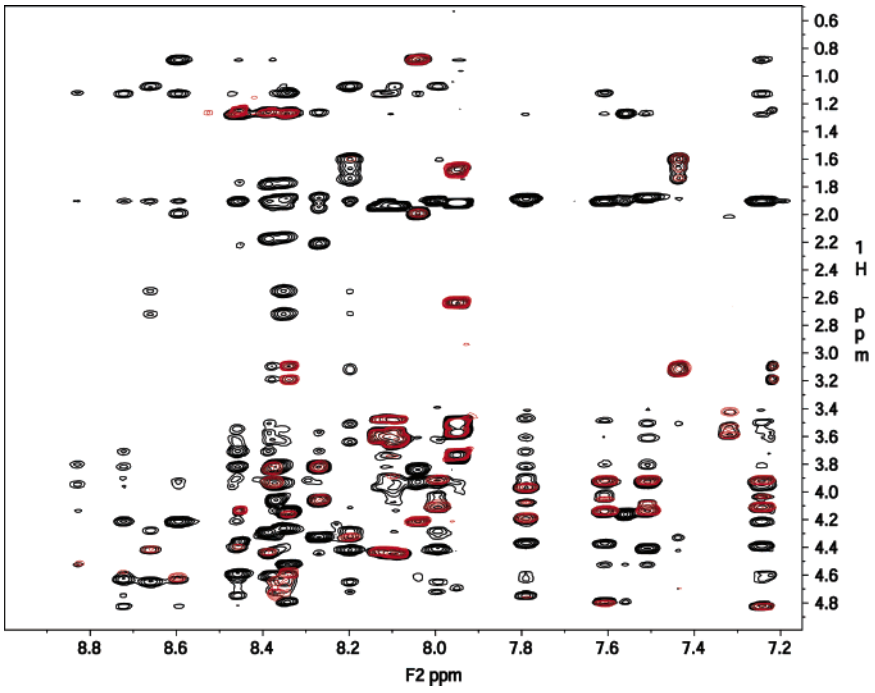

FIGURE 10. Overlay of TOCSY (red) and 400 ms-NOESY spectra (black) of glycpeptide **1**. Overlapping cross-peaks with TOCSY spectra represent intraresidue NOEs, and other cross-peaks represent interresidue NOEs.

100 ms. NOESY spectra[45] were acquired with a mixing time of 100, 200, and 400 ms. All 2D spectra were processed using NMRPipe software.[46] Time domain data in both dimensions were multiplied by a 90° phase shift sine bell window function prior to Fourier transformation. Proton resonance assignments were achieved using XEASY software.[47]

Synthesis of Sugar Amino Acids. *O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1−6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1−3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine (2). *O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1−6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1−3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine *tert*-butyl ester was synthesized by modifying the procedures reported previously:[14,28] ^{1}H NMR (400 MHz, $CDCl_3$; Me_4Si) δ 7.80−7.32 (8 H, m, Ar*H*), 6.02 (1 H, d, $J_{2,NH}$ 9.6, N*H*), 5.80 (1 H, d, $J_{2'',NH''}$ 8.9, N*H*″), 5.56 (1 H, d, $J_{CH\alpha,NH}$ 8.9, N*H* Thr), 5.37 (1 H, $J_{3',4'}$ 2.9, 4′-H), 5.19−5.13 (2 H, m, 2′-H and 3″-H), 5.09−5.05 (1 H, dd, 4″-H), 4.98−4.96 (1 H, dd, 3′-H), 4.79 (1 H, d, $J_{1,2}$ 3.4, 1-H), 4.59 (2 H, 2 d, $J_{1'},_{2'}$ 8.0, 1′-H and $J_{1'',2''}$ 8.0, 1″-H), 4.55−4.45 (3 H, m, Fmoc CH_2 and 2-H), 4.28−4.23 (2 H, m, Fmoc CH and 6″-H^a), (10 H, m, 6″-H^b, Thr CH$^{\alpha}$, 2″-H, 4-H, 6′-H^{a,b}, 5-H, 6-H^{a,b}, and Thr CH$^{\beta}$), 3.77 (1 H, dd, 5-H), 3.69−3.66 (2 H, m, 3-H and 5″-H), 2.80 (1 H, br s, 4-OH), 2.16, 2.08, 2.07, 2.02, 1.99, 1.98, 1.90 and 1.78 (27 H, 8 s, 9 × $COCH_3$), 1.46 (9 H, s, Bu^t) and 1.27 (3 H, d, Thr CH$^{\gamma}$) (the primes (′) and (″) mean Gal and GlcNAc residues, respectively); HRMS-FAB (*m*/*z*) calcd for $C_{59}H_{78}N_3O_{27}$ $[M + H]^+$ 1260.4823, found 1260.4850.

The *tert*-butyl group of the above precursor (101 mg, 80 μmol) was cleaved by treatment with a solution of 95% TFA in DCM at room temperature for 60 min. The mixture was coevaporated with toluene. The residue was dissolved in 30% aq CH_3CN and lyophilized to give **2** (97 mg, 85%): HRMS-FAB (*m*/*z*) calcd for $C_{55}H_{70}N_3O_{27}$ $[M + H]^+$ 1204.4197, found 1204.4194.

***O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1−6)]-2-deoxy-O-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1−3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-serine (3).** *O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1−6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1−3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-serine *tert*-butyl ester was also synthesized by modifying the procedures in the previous papers:[14,28] ^{1}H NMR (400 MHz, $CDCl_3$; Me_4Si) δ 7.80−7.31 (8 H, m, Ar*H*), 6.07 (1 H, d, $J_{CH\alpha,NH}$ 7.4, N*H* Ser), 6.00 (1 H, d, $J_{2'',NH''}$ 8.9, N*H*″), 5.70 (1 H, d, $J_{2,NH}$ 8.8, N*H*), 5.37−5.32 (2 H, m, 4′-H and 3″-H), 5.22−5.15 (1 H, m, 2′-H), 5.05−4.95 (2 H, m, 3′-H and 4″-H), 4.77 (1 H, d, $J_{1,2}$ 3.4, 1-H), 4.69 (1 H, d, $J_{1'',2''}$ 7.6, 1″-H), 4.56−4.40 (5 H, m, 1′-H, 2-H, Fmoc CH_2, Ser CH^{α}), 4.27−4.21 (2 H, m, 6″-H^a, Fmoc C*H*), 4.20−3.59 (13 H, m, 3-, 4-, 5-H, 6-H^{a,b}, 5′-H, 6′-H^{a,b}, 2″-, 5″-H, 6″-H^b and Ser CH^{β}, β'), 2.72 (1 H, br s, 4-OH), 2.16, 2.06, 2.05, 2.02, 1.98, 1.95, 1.89 and 1.81 (27 H, 8 s, 9 × COCH_3) and 1.50 (9 H, s, Bu^t); HRMS-FAB *m*/*z* calcd for $C_{58}H_{76}N_3O_{27}$ $[M + H]^+$ 1246.4666, found 1246.4642.

The *tert*-butyl group of the above precursor (80 mg, 64 μmol) was cleaved by treatment with a solution of 95% TFA in DCM at room temperature for 60 min. The mixture was coevaporated with toluene. The residue was dissolved in 30% aq CH_3CN and lyophilized to give **3** (48 mg, 63%): HRMS-FAB *m*/*z* calcd for $C_{54}H_{68}N_3O_{27}$ (M + H) 1190.4040, found 1190.4048.

Preparation of Preloaded Ala-Rinkamide-TentaGel Resin (Ala-resin). To Fmoc-TentaGel resin functionalized with Rinkamide linker (0.25 mmol/g, 548 mg, 137 μmol) swollen in DMF (10 mL) for 30 min was added 20% piperidine in DMF (10 mL), and the mixture was stirred for 20 min. The resin was filtered and washed with DMF (5 × 10 mL). Fmoc-Ala-OH (128 mg, 411 μmol) was assembled onto the resin by treatment with 0.4 M HBTU−HOBt in DMF solution (1.03 mL) and DIEA (141 μl, 822 μmol), and the resin was stirred for 2 h at room temperature. Quantification of Fmoc-Ala residue attached onto the resin was confirmed by measuring UV absorption at 290 nm corresponding to the dibenzofulvene−piperidine adduct. Deprotection of Fmoc group was achieved with 20% piperidine in DMF (10 mL) for 20 min. Finally, the Ala-resin was filtered, washed with DMF (5 × 10 mL) and DCM (5 × 10 mL), and then dried in vacuo for 24 h.

Preparation of Ala-PEGA Resin. PL-PEGA resin (Polymer Laboratories, Inc.) functionalized with Rinkamide linker was employed for the solid-phase synthesis using a manual procedure in polypropylene column. PL-PEGA resin (0.055 mmol/g of wet resin, 347 mg, 6.5 μmol) was washed with DMF and CH_2Cl_2 and dried in vacuo. First, Fmoc-Rink linker (35 mg, 65 μmol) was incorporated into the resin in the presence of 0.4 M HBTU−HOBt in DMF solution (164 μL, 65 μmol) and DIEA (22 μL, 130 μmol). The mixture was stirred at room temperature by using a vortex mixer. After 2 h, the resin was washed with DMF and CH_2Cl_2 and then dried in vacuo. Fmoc-Rinkamide linker introduced onto the original PL-PEGA resin was estimated to be 7.41 μmol by Fmoc-photometric test. This resin was swollen with DMF and used for the coupling with Fmoc-Ala-OH (6.92 mg, 22 μmol) under a procedure similar to that described above. Unreacted amino groups of the resin were blocked by acetyl capping. Fmoc-Ala residues substituted on the resin were quantified by photometric titration of the Fmoc group released (6.7 μmol).

Coupling Reaction of Compound 2 with the Ala-Rinkamide-TentaGel Resin. Coupling of **2** (7.0 mg, 5.85 μmol) with Ala-Rinkamide-TentaGel resin (0.26 mmol/g) (15 mg, 3.90 μmol) was performed in the presence of 0.4 M HBTU−HOBt in DMF (14.6 μL) and DIEA (2.0 μl, 11.7 μmol) and DMF (83.4 μL). The mixture was stirred by using a vortex mixer under suitable conditions (either microwave at 50 °C, nonmicrowave at 50 °C, or nonmicrowave at room temperature) for an appropriate time. The coupling reaction was stopped by filtration, and the resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL). The resulting resin was dried in vacuo.

Determination of Coupling Yields by Fmoc Photometric Tests. The amount of Fmoc groups on the resin was determined as substitution level of **2** on the Ala-resin. To the resulting resin (1.0 mg) was added 20% piperidine in DMF (1 mL) and the mixture was stirred at room temperature for 20 min. The absorbance of the sample solution was read at 290 nm (ϵ = 5800 $M^{-1}cm^{-1}$) in a quartz cell. Duplicate measurements were carried out and the average was substituted in the following *equation 1* to obtain experimental Fmoc loading.

$$\text{experimental Fmoc loading (mmol/g)} = \frac{OD_{290} \times 1000}{5800(\text{mg of resin})} \quad (1)$$

Coupling yields were calculated by comparison of experimental Fmoc loading with the theoretical loading. The theoretical loading corresponding to 100% of reaction proceeding was calculated by eq 2.

$$\text{theoretical loading (mmol/g)} = = \frac{A \times 1000}{1000 + A(B - 239)} \quad (2)$$

where *A* is the loading of starting resin (mmol/g) and *B* is the molecular weight of the target glycopeptide involved in all protecting groups.

The amount of Fmoc groups existing on the resulting resin during glycopeptide synthesis under the conditions (entries 1−4) listed in Table 1 was determined by the above-mentioned procedure, and the overall yields of the full length glycopeptide **4** on the solid phase were estimated by using these data.

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Solid-Phase Synthesis of Glycopeptide 4 Using a Standard Protocol at Room Temperature (Entry 1 in Table 1). All coupling and N^a-Fmoc removal reactions were conducted at room temperature. Fmoc-Rinkamide resin (50 mg, 12.5 μmol) swollen in DMF (1 mL) for 30 min was treated with 20% piperidine in DMF (1 mL). The reaction mixture was stirred at room temperature for 20 min and then filtered, and the resin was washed with DMF (5 × 1 mL). The appropriate Fmoc-amino acids (3.0 equiv) were incorporated to the resin with HBTU (3.0 equiv), HOBt (3.0 equiv), and DIEA (6.0 equiv) in DMF (660 μL) at room temperature for 2 h. Coupling reactions of **2** (1.5 equiv) or **3** (1.5 equiv) were carried out with HBTU (1.5 equiv), HOBt (1.5 equiv), and DIEA (3.0 equiv) in DMF (330 μL) at room temperature for 12 h. After these coupling reactions, the resin was washed with DMF (5 × 1 mL). Unreacted amino groups were capped by acetylation with 13 mM HOBt in Ac_2O/DIEA/DMF solution (4.75:2.25:93.0, v/v/v, 1 mL) for 5 min at room temperature, and the resin was washed with DMF. Removal of the N^α-Fmoc group was performed by treatment with 20% piperidine in DMF (1 mL) at room temperature for 20 min, and the resulting resin was washed with DMF (5 × 1 mL). The glycopeptidyl-resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL) and dried over in vacuo for 24 h.

Solid-Phase Synthesis of Glycopeptide 3 at 50 °C without Microwave Irradiation (Entry *2* in Table 1). All coupling and N^a-Fmoc removal reactions were conducted under heating at 50 °C in a dry oven. Fmoc-Rinkamide resin (50 mg, 12.5 μmol) swollen in DMF (1 mL) for 30 min was treated with 20% piperidine in DMF (1 mL). The reaction mixture was stirred at room temperature for 20 min and then filtered, and the resin was washed with DMF (5 × 1 mL). The appropriate Fmoc-amino acids (3.0 equiv) were incorporated to the resin with HBTU (3.0 equiv), HOBt (3.0 equiv), and DIEA (6.0 equiv) in DMF (660 μL) for 10 min. Coupling reactions of **1** (1.5 equiv) or **2** (1.5 equiv) were carried out with HBTU (1.5 equiv), HOBt (1.5 equiv), and DIEA (3.0 equiv) in DMF (330 μL) for 20 min. The resin was washed with DMF (5 × 1 mL), the unreacted amino groups were capped by acetylation with 13 mM HOBt in Ac_2O/DIEA/DMF solution (4.75:2.25:93.0, v/v/v, 1 mL) for 5 min, and the resin was washed with DMF. N^a-Fmoc removal were performed by treatment with 20% piperidine in DMF (1 mL) for 3 min and the resin was washed with DMF (5 × 1 mL). The glycopeptidyl-resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL) and dried over in vacuo for 24 h.

Solid-Phase Synthesis of Glycopeptide 3 under Microwave Irradiation (Entry 3 in Table 1). Coupling and N^α-Fmoc removal reactions were conducted under microwave irradiation at 50 °C (0−40 W). Fmoc-Rinkamide resin (50 mg, 12.5 μmol) swollen in DMF (1 mL) for 30 min was treated with 20% piperidine in DMF (1 mL). The reaction mixture was stirred for 3 min and then filtered, and the resin was washed with DMF (5 × 1 mL). The appropriate Fmoc-amino acids (3.0 equiv) were incorporated to the resin with HBTU (3.0 equiv), HOBt (3.0 equiv), and DIEA (6.0 equiv) in DMF (660 μL) for 10 min. Coupling reactions of **2** (1.5 equiv) or **3** (1.5 equiv) were carried out with HBTU (1.5 equiv), HOBT (1.5 equiv), and DIEA (3.0 equiv) in DMF (330 μL) for 20 min, and the resin was washed with DMF (5 × 1 mL). Unreacted amino groups were capped by acetylation with 13 mM HOBt in an Ac_2O/DIEA/DMF solution (4.75:2.25:93.0, v/v/v, 1 mL) for 5 min, and the resin was washed with DMF. N^α-Fmoc removal was performed by treatment with 20% piperidine in DMF (1 mL) for 3 min and washed with DMF (5 × 1 mL). The glycopeptidyl-resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL) and dried over in vacuo for 24 h.

Solid-Phase Synthesis of Glycopeptide 3 on the PEGA Resin under Microwave Irradiation (Entry 4 in Table 1). Coupling and N^α-Fmoc removal reactions were conducted with microwave irradiation at 50 °C (0−40 W). Ala-PEGA resin (50 mg, 12.5 μmol) swollen in DMF (1 mL) for 30 min was treated with 20% piperidine in DMF (1 mL). The reaction mixture was stirred for 3 min and then filtered, and the resin was washed with DMF (5 × 1 mL). The appropriate Fmoc-amino acids (3.0 equiv) were incorporated to the resin with HBTU (3.0 equiv), HOBt (3.0 equiv), and DIEA (6.0 equiv) in DMF (660 μL) for 10 min. Coupling reactions of **2** (1.5 equiv) or **3** (1.5 equiv) were carried out with HBTU (1.5 equiv), HOBt (1.5 equiv), and DIEA (3.0 equiv) in DMF (330 μL) for 20 min, and the resin was washed with DMF (5 × 1 mL). Unreacted amino groups were capped by acetylation with 13 mM HOBt in Ac_2O/DIEA/DMF solution (4.75:2.25:93.0, v/v/v, 1 mL) for 5 min, and the resin was washed with DMF. Removal of N^α-Fmoc group was performed by treatment with 20% piperidine in DMF (1 mL) for 3 min and washed with DMF (5 × 1 mL). The glycopeptidyl-resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL) and dried over in vacuo for 24 h.

Reversed-Phase HPLC Analyses of Crude Glycopeptide 5 Released from TentaGel Resin. Dried glycopeptidyl-resins obtained by entries 1−3 in Table 1 (each 3.0 mg) were treated with a cleavage cocktail (400 μL) under vigorous stirring for 2 h at room temperature. The mixture was concentrated by streaming of nitrogen gas. Then, 50% aq CH_3CN (1 mL) was added to the residue, and the solution was lyophilized to give 0.97 mg, 0.92 mg, and 0.93 mg of crude glycopeptides in entries 1, 2, and 3, respectively. Each crude glycopeptide was dissolved in 30% aq CH_3CN containing 0.1% TFA (75 μL). These solutions (20 μL) were directly loaded onto HPLC column. HPLC analyses were performed with GL sciences C18 reversed-phase column (Inertsil ODS-3, ϕ 4.6 × 250 mm). The flow rate was 1 mL/min, and elution conditions were described as follows: elution buffer A, H_2O containing 0.1% TFA; buffer B, CH_3CN containing 0.1% TFA; buffer contents, 0−5 min in constant flow (A/B) = (70/30), 5−45 min in linear gradient flow (A/B) = (70/30) to (10/90), and 45−50 min, and constant flow (A/B) = (10/90). UV absorbance was measured at 220 nm and column temperature was 25 °C.

Reversed-Phase HPLC Analysis of Crude Glycopeptide 5 Cleaved from PEGA Resin.. A small portion (3.8 mg) of the dried glycopeptidyl-PEGA resin (compound **4** synthesized by the condition of the entry 4 in Table 1) was treated with 100 μL of the cleavage cocktail. After being stirred for 2 h at room temperature, the mixture was filtered and the solution was diluted with 30% aq CH_3CN (200 μL). A part of this solution (20 μL) was directly applied to HPLC column and analyzed under the same conditions as described above.

Glycopeptide 5. Glycopeptidyl-resin **4** was synthesized using PEGA resin (900 mg of wet resin, 49.5 μmol) by the conditions of entry 4 in Table 1 and treated with the same cleavage cocktail (10 mL) as described above. After being stirred for 2 h at room temperature, the mixture was filtered, and the resin was washed with 50% TFA in DCM (10 mL) and then filtered. The solution was concentrated by streaming of nitrogen gas to approximately 1 mL. To a solution was added *tert*-butylmethyl ether (10 mL) at 0 °C, and the mixture was kept for 10 min. The resulting precipitates were collected by centrifugation for 10 min (5000 rpm, 4 °C). The supernatant was carefully removed, and the residue was dried using stream of nitrogen gas. The residue was dissolved in 30% aq CH_3CN containing 0.1% TFA solution (4 mL) and directly loaded onto preparative reversed-phase HPLC [column, Inertsil ODS-3 (ϕ 20 × 250 mm); flow rate, 7.5 mL/min; elution buffer A, H_2O containing 0.1% TFA; elution buffer B, CH_3CN containing 0.1% TFA; composition of the solvent, 0−5 min in constant flow with (A/B) = (70/30), 5−60 min in linear gradient flow from (A/B) = (70/30) to (55/45); column temperature, 40 °C; UV monitoring, 220 nm]. The pure fraction was lyophilized to afford glycopeptide **5** as an amorphous solid (33.9 mg, 5.5 μmol, 11% isolated overall yield calculated from the first Ala residue introduced to the solid-phase support. The isolated yield was estimated by amino acid composition analysis): MALDI-TOFMS $C_{260}H_{378}N_{36}O_{137}$ $[M + H]^+$ calcd *m/z* 6197.380, found *m/z* 6197.429; ESI-HRMS $C_{260}H_{375}N_{36}O_{137}$ $[M - 2H]^{2-}$ calcd *m/z* 3097.186, found *m/z* 3097.186.

Glycopeptide 6. A purified glycopeptide **5** (33.9 mg, 5.5 μmol) was dissolved in a solution of 10 mM sodium hydroxide in MeOH (10 mL), and the mixture was stirred at room temperature for 1 h. The reaction mixture was then neutralized with acetic acid, and the solvent was removed by streaming of nitrogen gas. The residue was dissolved in 50% aqueous CH_3CN and lyophilized. The crude product was subjected to the purification by semipreparative reversed-phase HPLC [column, Inertsil ODS-3 (ϕ 20 × 250 mm); flow rate, 7.5 mL/min; elution buffer A, H_2O containing 0.1% TFA; elution buffer B, CH_3CN containing 0.1% TFA; composition of the solvent, 0−5 min in a constant flow with (A/B) = (98/2), 5−40 min in a linear gradient flow from (A/B) = (98/2) to (86.5/13.5); column temperature, 25 °C; UV monitoring, 220 nm]. Glycopeptide **6** as an amorphous solid (14.7 mg, 3.1 mmol, yield 57%): MALDI-TOFMS $C_{190}H_{309}N_{36}O_{102}$ $[M - H]^-$ calcd *m*/*z* 4727.0, found *m*/*z* 4727.0; ESI-HRMS $C_{190}H_{306}N_{36}O_{102}$ $[M - 2H]^{2-}$ calcd *m*/*z* 2361.993, found *m*/*z* 2361.994. Amino acid analysis: Asp (1) 1.0, Thr (3) 2.9, Ser (2) 1.8, Pro (5) 5.1, Gly (2) 2.0, Ala (4) 4.0, Val (1) 1.0, His (1) 1.0, $GlcNH_2$ (5) 3.7, and $GalNH_2$ (5) 4.1.

Glycopeptide 7. Compound **6** (5.6 mg, 1.2 μmol) was galactosylated by using 600 mU of β1,4-GalT and UDP-Gal (11 mg, 18.0 μmol) in a total volume of 1.2 mL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.2% Triton X-100, and 0.1% NaN_3. After incubation for 24 h at 25 °C, the reaction mixture was purified by GPC−HPLC, and the fraction containing compound **7** was analyzed by an analytical RP-HPLC (t_R = 29.9 min, Figure 7b). Further purification was performed by semipreparative RP-HPLC to give **7** (4.2 mg, 0.76 μmol, yield 64%): MALDI-TOF MS $C_{220}H_{359}N_{36}O_{127}$ $[M + H]^+$ calcd *m*/*z* 5537.274, found *m*/*z* 5537.623; ESI-HRMS $C_{220}H_{356}N_{36}O_{127}$ $[M - 2H]^{2-}$ calcd *m*/*z* 2767.1257, found *m*/*z* 2767.1253.

Glycopeptide 8. Compound **6** (5.6 mg, 1.2 μmol) was sialylated by using 130 mU of α2,3-(*O*)-SiaT and CMP-Neu5Ac (11 mg, 17.8 μmol) in a total volume of 1.2 mL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.1% Triton X-100 and 0.1% NaN_3. After incubation for 24 h at 25 °C, the reaction mixture was purified by GPC-HPLC, and the fraction containing compound **8** was analyzed by analytical RP-HPLC (t_R = 20.2 min, Figure 7d). Further purification was performed by a semipreparative RP-HPLC to give compound **8** (4.7 mg, 0.76 μmol, yield 64%): MALDI-TOF MS $C_{245}H_{392}N_{41}O_{142}$ $[M - H]^-$ calcd *m*/*z* 6183.9, found *m*/*z* 6184.2; ESI-HRMS $C_{245}H_{391}N_{41}O_{142}$ $[M - 2H]^{2-}$ calcd *m*/*z* 3089.7318, found *m*/*z* 3089.7144.

Glycopeptide 9. Compound **7** (4.2 mg, 0.76 μmol) was sialylated by using 15 mU of α2,3-(*N*)-SiaT and CMP-Neu5Ac (7.2 mg, 11.4 mmol) in a total volume of 760 μL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.2% Triton X-100 and 0.1% NaN_3. After incubation for 24 h at 25 °C, the reaction mixture was purified by GPC−HPLC and the fraction containing compound **9** was analyzed by an analytical RP-HPLC (t_R = 24.4 min, Figure 7c). Further purification was performed by a semipreparative RP-HPLC to give **9** (2.6 mg, 0.38 μmol, yield 50%): MALDI-TOF MS $C_{275}H_{442}N_{41}O_{167}$ $[M - H]^-$ calcd *m*/*z* 6994.7, found *m*/*z* 6994.6; ESI-HRMS $C_{275}H_{439}N_{41}O_{167}$ $[M - 4H]^{4-}$ calcd *m*/*z* 1746.9280, found *m*/*z* 1746.9346.

Glycopeptide 10. Compound **8** (4.6 mg, 0.65 μmol) was galactosylated by using 325 mU of β1,4-GalT and UDP-Gal (6.0 mg, 9.8 μmol) in a total volume of 650 μL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.2% Triton X-100, and 0.1% NaN_3. After incubation for 24 h at 25 °C. The reaction mixture was purified by GPC-HPLC and the fraction containing compound **10** was analyzed by an analytical RP-HPLC (t_R = 18.9 min, Figure 7e). Further purification was performed by a semipreparative RP-HPLC to give **10** (2.9 mg, 0.41 μmol, 63%): MALDI-TOF MS $C_{275}H_{442}N_{41}O_{167}$ $[M - H]^-$ calcd *m*/*z* 6994.7, found *m*/*z* 6994.5; ESI-HRMS $C_{275}H_{439}N_{41}O_{167}$ $[M - 4H]^{4-}$ calcd *m*/*z* 1746.9280, found *m*/*z* 1746.9336.

Glycopeptide 1. Compound **6** (3.5 mg, 0.74 μmol) was galactosylated by 370 mU of β1,4-GalT and UDP-Gal (6.7 mg, 11.1 μmol) in a total volume of 740 μL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.2% Triton X-100, and 0.1% NaN_3. After incubation for 24 h at 25 °C, subsequent sialylation was performed by addition of 875 mU mL^{-1} α2,3-(O)-SiaT (59 μL), 3.7 U mL^{-1} α2,3-(*N*)-SiaT (15 μL), and 200 mM CMP-Neu5Ac (111 μL) for 24 h at 25 °C. The reaction mixture was purified by GPC−HPLC and the fraction containing compound **1** was analyzed by an analytical RP-HPLC (t_R = 13.8 min, Figure 7f). Further purification was performed by a semipreparative RP-HPLC to give **1** (3.0 mg, 0.35 μmol, yield 48%): MALDI-TOF MS $C_{330}H_{527}N_{46}O_{207}$ $[M - H]^-$ calcd *m*/*z* 8450.9, found *m*/*z* 8453.5; ESI-HRMS $C_{330}H_{528}N_{46}O_{207}$ $[M - 4H]^{4-}$ calcd *m*/*z* 2110.7973, found *m*/*z* 2110.7988.

Acknowledgment. This work was supported by a grant for "Development of methodologies and databases for structural glycoproteomics" from the New Energy and Industrial Technology Development Organization (NEDO). We appreciate useful discussions of microwave instruments with Dr. H. Kaga of Research Institute of Genome-based Biofactory of AIST, Hokkaido. We also thank Ms. M. Kiuchi, Ms. S. Oka, and Mr. T. Hirose at the Center for Instrumental Analysis, Hokkaido University, for FAB-MS and ESI-MS measurement and amino acid composition analysis.

Supporting Information Available: ^{1}H NMR and TOCSY spectra for compound **1** and **6**−**10** and HSQC spectra for compound **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0526643