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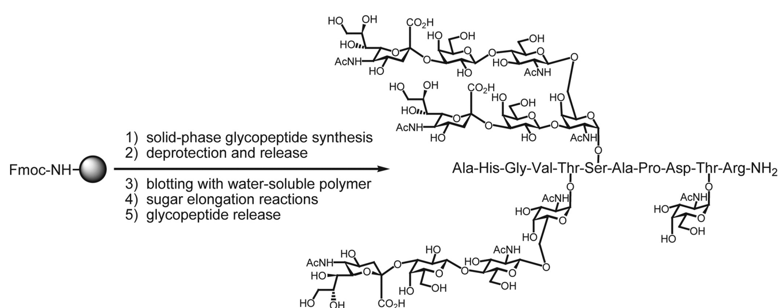


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# Combinatorial Synthesis of MUC1 Glycopeptides: Polymer Blotting Facilitates Chemical and Enzymatic Synthesis of Highly Complicated Mucin Glycopeptides

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***Abstract:*** The chemoselective polymer blotting method allows for rapid and efficient synthesis of glycopeptides based on a “catch and release” strategy between solid-phase and water-soluble polymer supports. We have developed a heterobifunctional linker sensitive to glutamic acid specific protease (BLase). The general procedure consists of five steps, namely (i) the solid-phase synthesis of glycopeptide containing BLase sensitive linker, (ii) subsequent deprotections and the release of the glycopeptide from the resin, (iii) chemoselective blotting of the glycopeptide intermediates in the presence of water-soluble polymers with oxylamino functional groups, (iv) sugar elongations using glycosyltransferases, and (v) the release of target glycopeptides from the polymer platform by selective BLase promoted hydrolysis. The combined use of the solid-phase chemical syntheses of peptides and the enzymatic syntheses of carbohydrates on water-soluble polymers would greatly contribute to the production of complicated glycopeptide libraries, thereby enhancing applicative research. We report here a high-throughput synthetic system for the various types of MUC1 glycopeptides exhibiting a variety of sugar moieties. It is our belief that this concept will become part of the entrenched repertoire for the synthesis of biologically important glycopeptides on the basis of glycosyltransferase reactions in automated and combinatorial syntheses.

## Introduction

Glycoconjugates chemically synthesized down to the last detail should prove useful in the research of unidentified sugar functions and their applications. Toward this end, many synthetic methods, both chemical and enzymatic, have been developed. Despite these efforts, in contrast to the research fields of proteins (peptides) and nucleotides, general synthetic technologies for the glycoconjugates have yet to be established. This causes difficulties in chemical synthesis and structural analysis due to the complicated structures and diversity of glycoconjugates. Polymer-supported synthesis would be one of the practical and convenient methods to overcome these problems as it can simplify purification steps and can also make combinatorial processes feasible. The fact that automated synthesizers of nucleotides (DNA/RNA) and peptides (proteins) are already commercially available[1,2] establishes them as indispensable devices for the investigation of the functional roles of genomes and proteins as well as the development of a variety of therapeutic reagents. Although recent progress has been made in the liquid-phase and solid-phase chemical syntheses allowed for making a variety of oligosaccharides and glycopeptides,[3] these methods are still limited regarding the target structures

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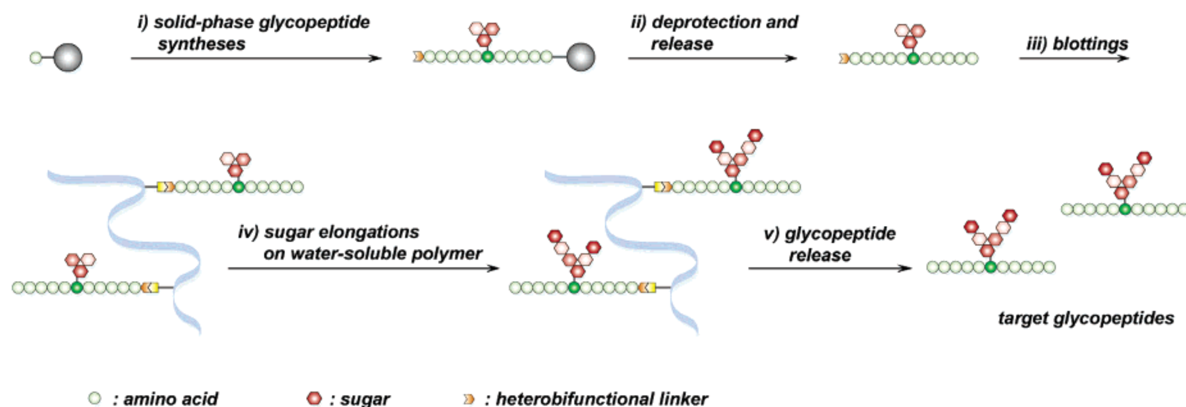


***Figure 1.*** Chemical and enzymatic glycopeptide syntheses using "polymer blotting" strategy.

and were found technically troublesome by general biochemists or medical scientists. Enzymatic synthesis is a potential alternative for the chemical syntheses of complicated oligosaccharides. They have the advantage of stereo- and regioselectivities for the glycosylations;[4] however, the solid-support procedure of immobilizing glycosyl acceptors on resins would not be well suited for these enzymatic reactions in terms of efficiency and versatility in practical syntheses.

To solve these problems, we have developed polymer-supported enzymatic syntheses of various glycoconjugates such as oligosaccharides, sugar derivatives, sphingoglycolipids, and glycopeptides using a water-soluble polymer.[5,6] In addition, we thought that the advent of an appropriate methodology combining solid-phase peptide syntheses and liquid-phase carbohydrate syntheses in a blending of chemical and enzymatic strategies would be a very powerful way to achieve efficient glycopeptide syntheses. Consequently, we have developed a rapid and efficient synthetic system for creating complex glycopeptides using a convenient "heterobifunctional linker" that shuttles product between two different support polymers.[6] As shown in Figure 1, our synthetic strategy is as follows: (i) perform the solid-phase chemical syntheses of the glycopeptides with the newly introduced heterobifunctional linker, (ii) deprotection and release of glycopeptides from the resin, (iii) chemoselective blotting of glycopeptides by a water-soluble polymer to act as a "primers",[6,7] (iv) sugar elongation by glycosyltransferase, and (v) release of the target glycopeptides.

In the course of our studies of enzymatic syntheses based on the cluster effects of sugar-attached water-soluble polymers as multivalent acceptor substrates, we developed an interest in the feasibility of the above strategy toward the efficient synthesis of MUC1 mucin glycopeptides as important molecules for in vivo cellular regulation.[8] In breast, ovary, and other carcinomas, the MUC1 mucin is aberrantly glycosylated in comparison with mucin from corresponding normal tissues.[9] Though the character of sugar moieties at the MUC1 are interesting in terms of cancer related immunogenecity,[10] the functional role of the oligosaccharide structure in the MUC1 has not been revealed yet because of its complexity and myriad glycosylation patterns. Taking this into consideration, MUC1 glycopeptides having various mucin core carbohydrates are key in the fulfillment of their essential biological roles. We describe herein the rapid and combinatorial synthesis of MUC1 glycopeptide derivatives by the tandem synthetic process of solid-phase chemical syntheses and solution phase enzymatic syntheses using a heterobifunctional linker which acts as a suspension bridge between two different polymer platforms.

## Results and Discussion

**A. Solid-Phase Chemical Synthesis of the Glycopeptide Primer Tagged by Protease-Sensitive Linker Followed by Selective Blotting by the Water-Soluble Polyacrylamide through the Oxime Formation.** A heterobifunctional linker having a protease-sensitive moiety and a reactive ketone group was designed for accelerating chemical and enzymatic synthesis of MUC1 library (Figure 2a). In the previous study,[6] a photosensitive moiety was incorporated into the linker. We selected here a peptidase-sensitive dipeptide moiety, Phe-Glu, as a useful linker that is selectively digested by *Bacillus licheniformis* glutamic acid-specific protease (BLase). BLase protease could cut off at the C-terminus of glutamic acid with high efficiency and selectively to release the target glycopeptides

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(5) For example, see: (a) Nishimura, S.-I.; Matsuoka, K.; Lee, Y. C. *Tetrahedron Lett.* **1994**, *31*, 5657. (b) Nishimura, S.-I.; Yamada, K. *J. Am. Chem. Soc.* **1997**, *119*, 10555. (c) Yamada, K.; Fujita, E.; Nishimura, S.-I. *Carbohydr. Res.* **1998**, *305*, 443.

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(8) For example, see: (a) Hanisch, F.-G.; Müller, S. *Glycobiology* **2000**, *10*, 439. (b) Baldus, S. E.; Engelmann, K.; Hanisch, F.-G. *Crit. Rev. Clin. Lab. Sci.* **2004**, *41*, 189.

(9) For example, see: (a) Lloyd, K. O.; Burchell, J.; Kudryashov, V.; Yin, B. W. T.; Taylor-Papadimitriou, J. *J. Biol. Chem.* **1996**, *271*, 33325. (b) Hanisch, F.-G.; Stadie, T. R. E.; Deutzmann, F.; Peter-Katalinic, J. *Eur. J. Biochem.* **1996**, *236*, 318. (c) Bhavanandan, V. P.; Zhu, Q.; Yamakami, K.; Dilulio, N. A.; Nair, S.; Capon, C.; Lemoine, J.; Fournet, B. *Glycoconjugate J.* **1998**, *15*, 37.

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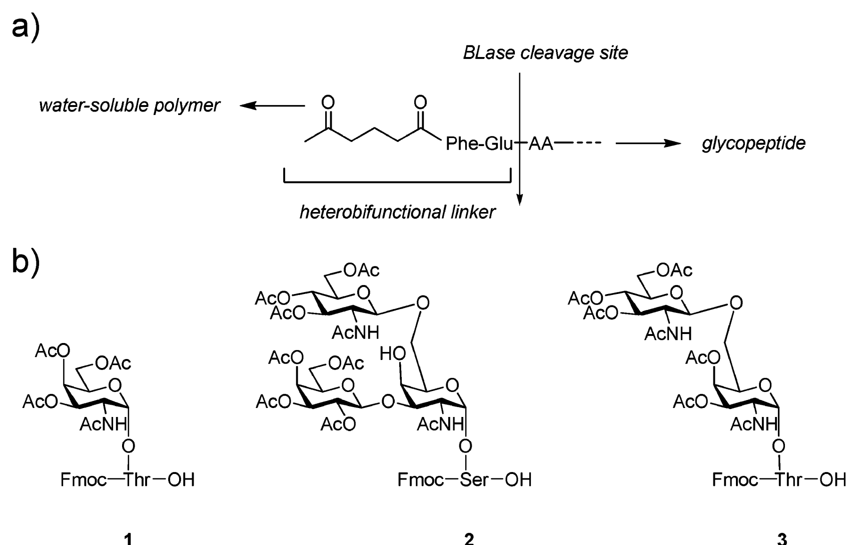


***Figure 2.*** (a) Heterobifunctional linker sensitive to BLase; (b) Fmoc protected sugar amino acids.

from the water-soluble polymer.[11] Scheme 1 shows the general synthetic route to the key primer polymer (**7**) that can be subsequently used for the parallel synthesis of MUC1 glycopeptides. The solid-phase synthesis of the glycopeptide using the linker (**4**) was performed by using TentaGel resin, which was functionalized with Rinkamide linker.[12] Reagents, such as Fmoc protected amino acids (Fmoc-AA) or sugar amino acids **1**, **2**, and **3** which have GalNAc (Tn antigen),[13] core2,[14,15] or core6[16] (Figure 2b), were correctly aligned.

It should be noted that the synthetic procedure for the new heterobifunctional linker is definitely much simpler than that of the photosensitive one, although the glycopeptides containing Glu residue are exchanged from becoming the target molecules by means of this linker. In such cases, other peptidases with different substrate specificity can be employed. Syntheses were performed by combined use of an automated peptide synthesizer and off-line microwave irradiation couplings.[15] In general, 20 equiv of Fmoc protected amino acids were charged to the automatic synthesizer (10 equiv per reaction, twice iterated) for the active functional groups on resin. However, in the case of microwave irradiation coupling, it was suggested that only 1.1−1.5 equiv of sugar amino acids were required. The BLase sensitive linker was designed to have a Phe-Glu sequence that could cut the peptides off from the water-soluble polymer taking advantage of the fact that phenylalanine group is known to accelerate peptide hydrolysis by BLase at the C-terminus of glutamic acid.[11] Therefore, extra Fmoc-Glu-OH, Fmoc-Phe-OH, and 5-oxohexanoic acid were introduced at the final step of the peptide synthesis. The protected glycopeptide on the TentaGel could then be released by treatment with 90% TFA aqueous solution.

After the deprotection of hydroxyl groups on sugar moieties by alkaline solution, the intermediate (**5**) was blotted onto the water-soluble polyacrylamide derivative (**6**) by chemoselective ligation of the ketone group of **5** with the oxylamino group of **6** (Scheme 1).[6,7] Figure 3 indicates that the blotting shows that the consumption of the intermediate glycopeptide (**5**) proceeded to completion at high efficiency and selectively to give the polymer product **7**. As expected, polymer **6** captured only the target peptide **5**, since all of the amino groups of the unreacted peptides on the polymer supported synthesis stages should have been capped by acetylations. The resulting polymeric material **7** was able to be purified using only centrifugal ultra filtration (UF), without more laborious purifications (i.e. column chromatography etc.). We would like to comment at this point that even though we selected the water-soluble polymer **6** as the most suitable platform in this case, the oxylamino group could be adapted to various supporting systems with similar strategies.

**B. Enzymatic Oligosaccharide Elongations Using the Water-Soluble Primer, and Synthesis of MUC1 Glycopeptides Having the Complicated Oligosaccharide Side Chains.** To show the advantage of this "polymer blotting" strategy, we demonstrated the enzymatic oligosaccharide elongations of **7**. Tandem galactosylation and disialylation reactions proceeded smoothly in the mixture of suitable glycosyltransferases, sugar nucleotides, and **7** (Scheme 2).[17] The reaction was monitored by MALDI-TOF/MS analysis of a separated small portion of the reaction mixture treated with BLase. After completion of the sugar elongation reaction, the product **8** was purified by the same UF method as that of the starting primer **7** to remove the excess sugar-nucleotides, byproducts, and buffer. Treatment of **8** with BLase followed by UF separation gave the glycopeptide **9** containing a GalNAc monosaccharide, core-2 hexasaccharide, and core-6 tetrasaccharide. Although no HPLC purification was carried out up to this point, this crude glycopeptide **9** showed more than 70% purity, as estimated by

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(17) Takano, Y.; Kojima, N.; Nakahara, Y.; Hojo, H.; Nakahara, Y. *Tetrahedron* **2003**, *59*, 8415.

*Scheme 1* [a]

[a] (A) (i) Piperidine, DMF, (ii) Fmoc-AA, **1**, **2**, **3**, or 5-oxohexanoic acid, HBTU, HOBt, *N*,*N*-diisopropylethylamine, NMP, DMF, (iii) $Ac_2O$, HOBt, *N*,*N*-diisopropylethylamine, DMF; (B) 90% TFA aq.; (C) NaOH in MeOH; (D) **6**, sodium acetate buffer (pH 5.0−5.5).

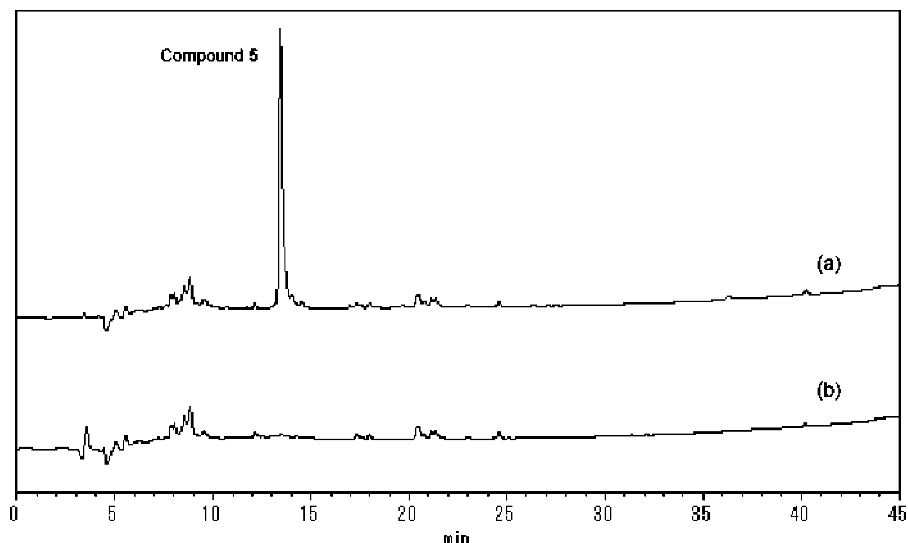


***Figure 3.*** RP-HPLC analyses of (a) crude **5** and (b) reaction mixture in the blotting step for **5** with **6**. The peak of **5** at 13−14 min disappeared, indicating the blotting reaction proceeded completely.

the HPLC profile in Figure 4a. Furthermore, just one iteration of HPLC purification of this crude **9** gave the pure **9** in 3.6% overall yield (Figure 4b).

The avoidance of HPLC purifications is of great benefit in the high-throughput and combinatorial syntheses of glycopeptides. This method has advantages similar to those of the solid-phase synthetic methodology, namely the simplification of purification strategy. In addition, it has the extra advantage of direct application to the enzymatic reaction and exhibits high reactivity based on the clustering of the sugar moieties of the water-soluble polymer. Even if we face the necessity of having to purify intermediates for the next reaction, we could achieve this simply by passing the crude through an UF unit. In the present experiment indicated in the Scheme 1 & 2, we performed the synthesis of MUC1 glycopeptide in 18 $\mu$mole scale and obtained 7.0 mg of compound **9** (8.6% overall yield from the solid-phase peptide synthesis, purity from HPLC profile ≥ 70%, Figure 4a).

**C. Parallel Synthesis of MUC1 Glycopeptides Using Various Combinations of Several Glycosyltransferases.** Our next topic of interest focuses on the parallel synthesis of various glycopeptides using combinations of several glycosyltransferases. We thought that this glycopeptides synthesis methodology using a water-soluble polymer support would be suitable for parallel synthesis. Scheme 3 shows parallel syntheses of six kinds of MUC1-related glycopeptides from the intermediate **7** (1.2 $\mu$mole scale, approximately 2.7 mg from solid-phase peptide synthesis) by using the $\beta$1,4-galactosyltransferase, α2,3-(*O*)-sialyltransferase, and α2,3-(*N*)-sialyltransferase.[18] All sugar elongations by glycosyltransferases except for **10** were performed as one-pot reactions. Target glycopeptides were isolated by same method as that described for the case of **9**. These processes from reactions to isolations were performed in parallel, and we successfully obtained the compounds **9**−**14** at the same time. RP-HPLC analysis for each product is shown in Figure 5, suggesting that their purities are similar to that of compound **9** indicated in Figure 4. Although in the case of the synthesis of **14** additional sialylation (oversialylation) was found to proceed at the core-2 Gal$\beta$1−3GalNAc moiety, the byproduct **9** can be separated by further, simple, iterative HPLC purification. Amino acid analyses-based overall yields were **9** (5.9%), **10** (9.7%), **11** (8.2%), **12** (8.3%), **13** (7.9%), and **14** (6.5%), respectively. MALDI-TOF/MS analyses supported complete construction of all glycopeptides (Figure 6). Figure 7 shows the NMR spectra of the iteratively purified compound **9** which yields insight into the relationship between structure and function of the mucin glycopeptides,[19] the results of which will be reported soon.

Finally, it was demonstrated that our present method was applied in the combinatorial synthesis of 36 kinds of MUC1 glycopeptides as indicated in Figure 8. The synthesis assisted by "polymer blotting" was conducted by an automatic sampler on general plastic plates. Here, six primers **15**−**20** shown in Table 1 prepared by the solid-phase synthesis and three kinds of glycosyltransferases were employed for the synthesis of the 36 possible structures (Scheme 4) using this combination. This trial was conducted in the plastic vessel by means of the automated sampler controlled by a simple program, and the mixture was left for 24 h at 25°C. All products **21**−**56** were purified and obtained by the same procedure described in the parallel synthesis of compounds **9**−**14** (MALDI-TOFMS and HPLC profiles were listed in the Supporting Information).

## Conclusion

We have achieved the rapid and combinatorial syntheses of complicated MUC1 glycopeptides in a range from 1.0 to 20 $\mu$mol (approximately from 200 $\mu$g to 4 mg) scale by using a "heterobifunctional linker" interfacing two different types of polymer platforms for chemical and enzymatic syntheses. The

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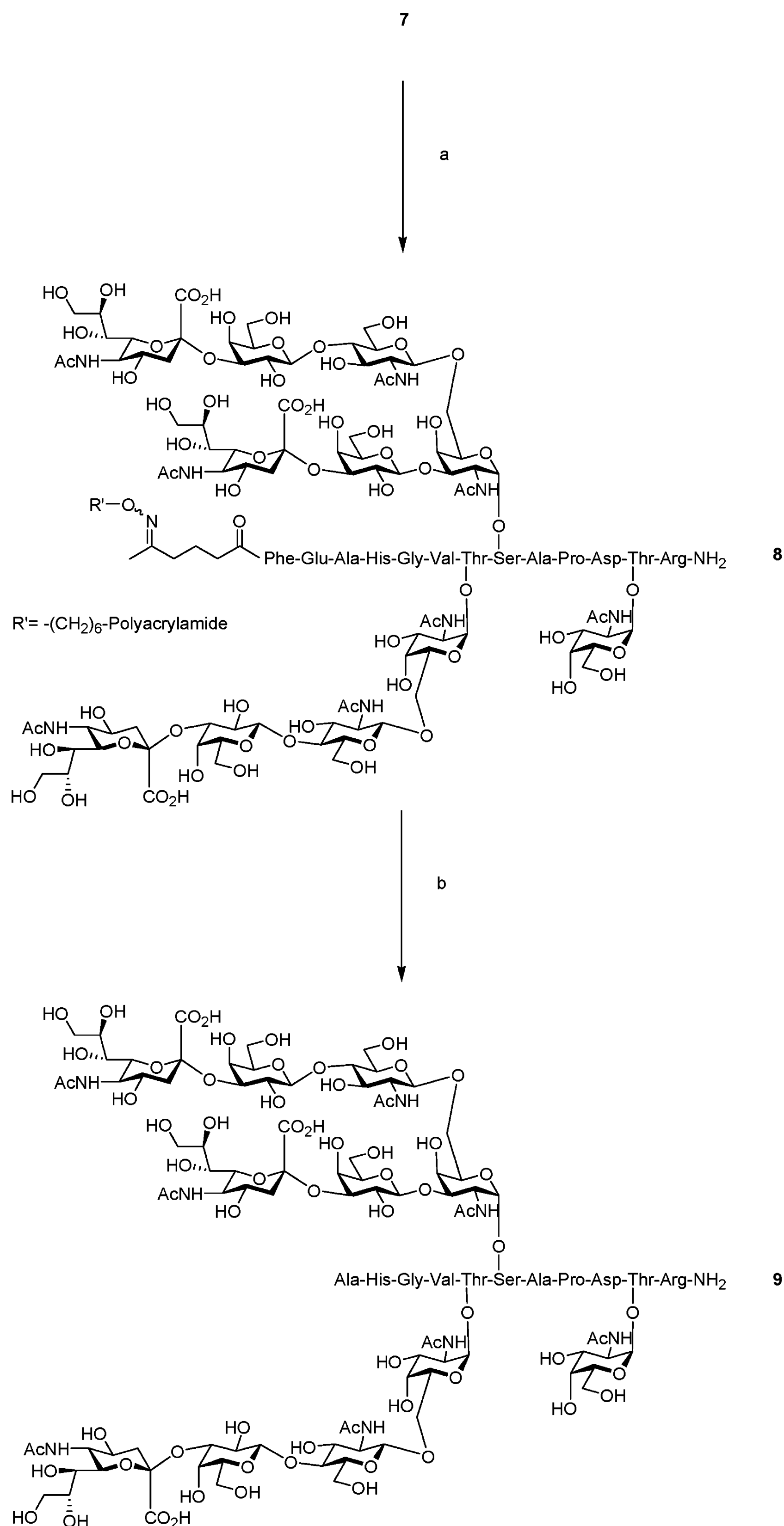
***Scheme 2*** [a]




[a] (A) UDP-Gal (5 mM), CMP-NANA (5 mM), $\beta$1,4-galactosyltransferases (100 mU/mL), α-2,3-(*O*)-sialyltransferases (17.5 mU/mL), α-2,3-(*N*)-sialyltransferases (74 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH 7.0); (B) BLase, ammonium acetate buffer (25 mM, pH 6.5).

"polymer-supported" enzymatic reaction enables conveniently simple purification, and therefore parallel (6 kinds) and combinatorial (36 kinds) synthesis of glycopeptide libraries can be easily accomplished. It was also demonstrated that the protease sensitive linker is useful for glycopeptide syntheses, because it is efficient and suitably mild enough to yield complicated and

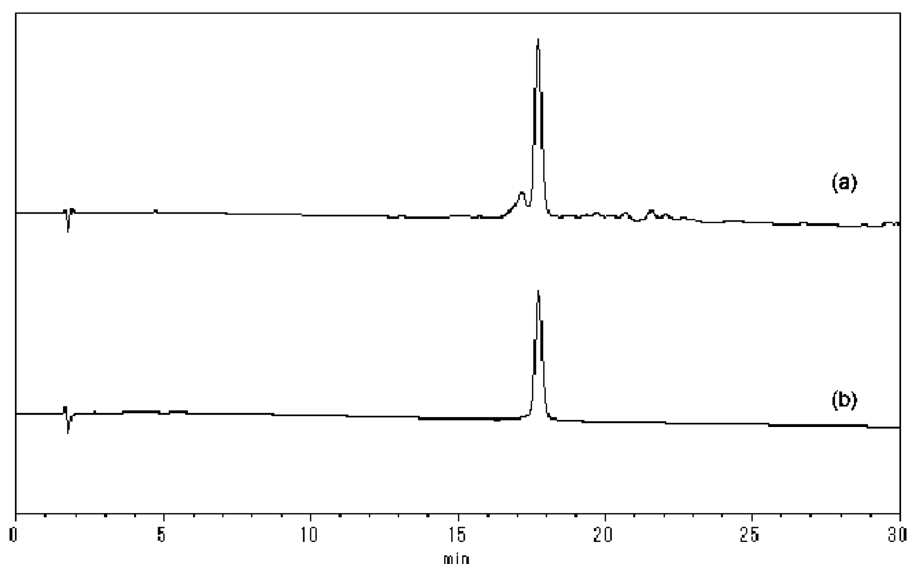



***Figure 4.*** RP-HPLC analyses of (a) crude product **9** and (b) purified product **9**.

***Scheme 3*** [a]

[a] Parallel syntheses of MUC1 glycopeptides. (A) BLase; (B) (i) $\beta$1,4-GalT, UDP-Gal, (ii) BLase; (C) (i) $\alpha$2,3-(O)-SiaT, CMP-NANA, (ii) BLase; (D) (i) $\beta$1,4-GalT, $\alpha$2,3-(*O*)-SiaT, UDP-Gal, CMP-NANA, (ii) BLase; (E) (i) $\beta$1,4-GalT, $\alpha$2,3-(*N*)-SiaT, UDP-Gal, CMP-NANA, (ii) BLase; (F) (i) $\beta$1,4-GalT, $\alpha$2,3-(*O*)-SiaT, $\alpha$2,3-(*N*)-SiaT, UDP-Gal, CMP-NANA, (ii) BLase.

unstable target compounds safely. It also can be constructed in such a way that the usual solid-phase peptide syntheses can be terminated by protease specific sequences, like Phe-Glu which are hydrolyzed by BLase specifically. The present two-dimensional synthetic scheme using a versatile "polymer blotting" strategy will greatly accelerate both practical enzymatic

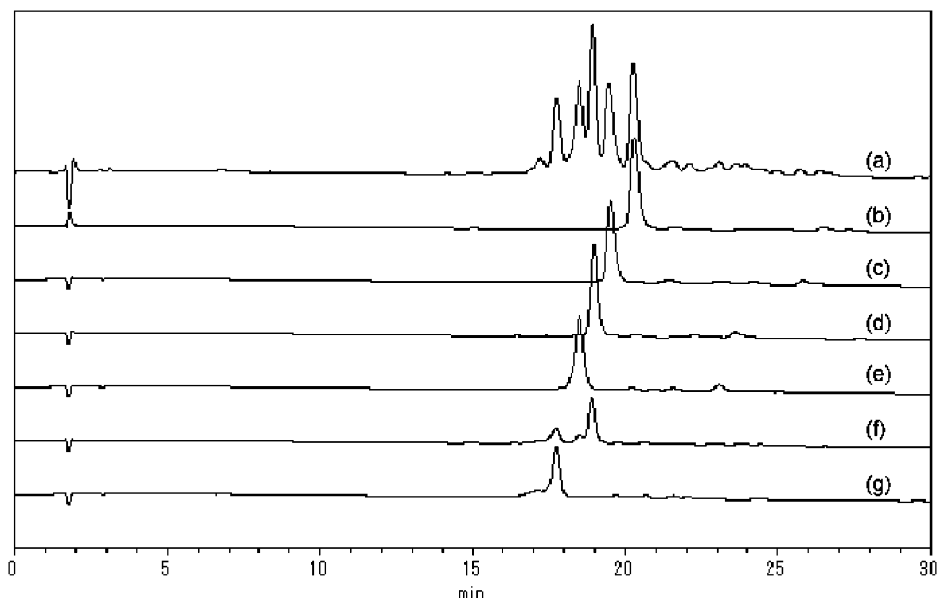



***Figure 5.*** RP-HPLC analyses of glycopeptides **9**−**14** prepared by parallel syntheses. (a) the chromatogram for the mixture of compounds **9**−**14**, (b) **10**, (c) **11**, (d) **12**, (e) **13**, (f) **14**, and (g) **9**.

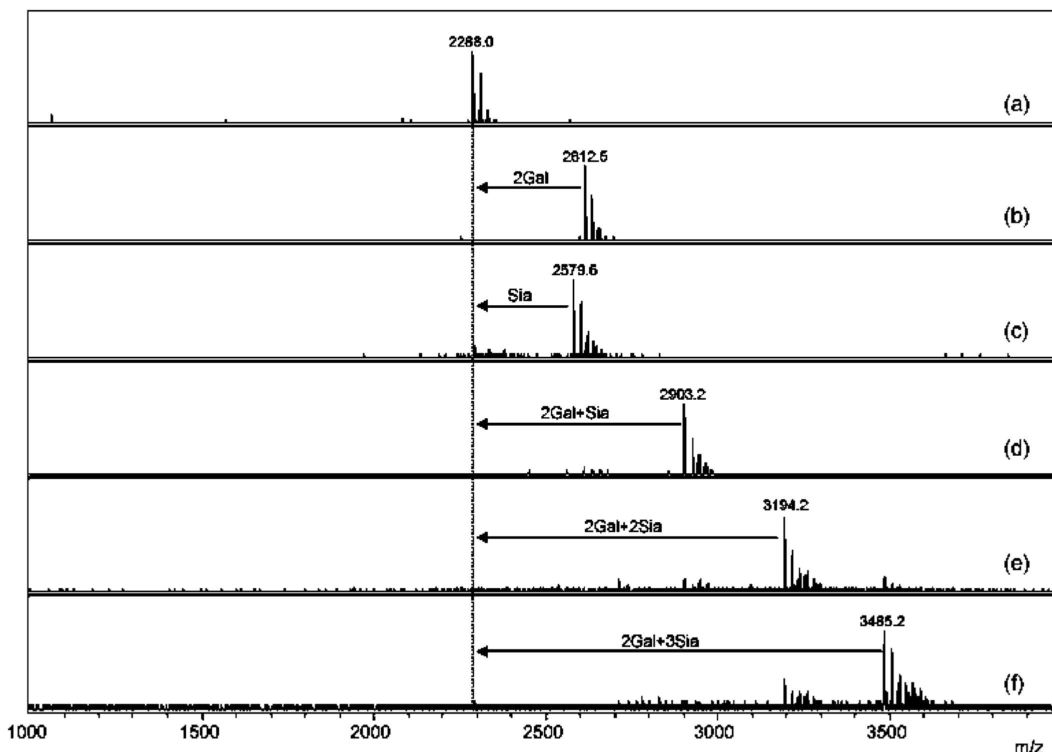



***Figure 6.*** MALDI-TOFMS analyses of (a) **10**, calcd for $C_{91}H_{151}N_{22}O_{46}$ (M + H), 2288.0; found 2288.0; (b) **11**, calcd for $C_{103}H_{171}N_{22}O_{56}$ (M + H), 2612.1; found 2612.5; (c) **12**, calcd for $C_{102}H_{168}N_{23}O_{54}$ (M + H), 2579.1; found 2579.6; (d) **13**, calcd for $C_{114}H_{188}N_{23}O_{64}$ (M + H), 2903.2; found 2903.2; (e) **14**, calcd for $C_{125}H_{205}N_{24}O_{72}$ (M + H), 3194.3; found 3194.2; and (f) **9**, calcd for $C_{136}H_{222}N_{25}O_{80}$ (M + H), 3485.4; found 3485.2.

syntheses using immobilized glycosyltransferases[20] and functional identification of biologically important glycopeptides.

## Experimental Section

**Materials and General Procedures.** Unless otherwise stated, all commercially available solvents and reagents were used without purification. TentaGel SRAM resin[21] was purchased from Hipep Laboratories, and Fmoc amino acid derivatives were purchased from NOVA Biochem Co. Ltd. $\beta$-1,4-Galactocyltransferase was purchased from TOYOBO Co. Ltd. α-2,3-(*N*)-Sialyltransferase and α-2,3-(*O*)-sialyltransferase were purchased from Calbiochem Co. Ltd. Uridine-5′-diphosphogalactose, 2Na (UDP-galactose), and cytidine-5′-monophospho-*N*-acetylneuraminic acid, 2Na (CMP-NANA), were purchased from YAMASA CO. Matrix-associated laser-desorption ionization time-

(20) (a) Nishiguchi, S.; Yamada, K.; Fuji, Y.; Shibatani, S.; Toda, A.; Nishimura, S.-I. *Chem. Commun.* **2001**, 1944. (b) Toda, A.; Yamada, K.; Nishimura, S.-I. *Adv. Synth. Catal.* **2002**, *344*, 61. (c) Nagahori, N.; Niikura, K.; Sadamoto, R.; Taniguchi, M.; Yamagishi, A.; Monde, K.; Nishimura, S.-I. *Adv. Synth. Catal.* **2003**, *345*, 729.

(21) Bayer, E.; Rapp, W. In *Chemistry of Peptides and Proteins*; Voelter, W., Bayer, E., Ovchinnikov, Y. A., Ivanov, V. T., Eds.; Waltler de Gruyter & Co.: Berlin, New York, 1986; Vol. 3, pp 3−8.

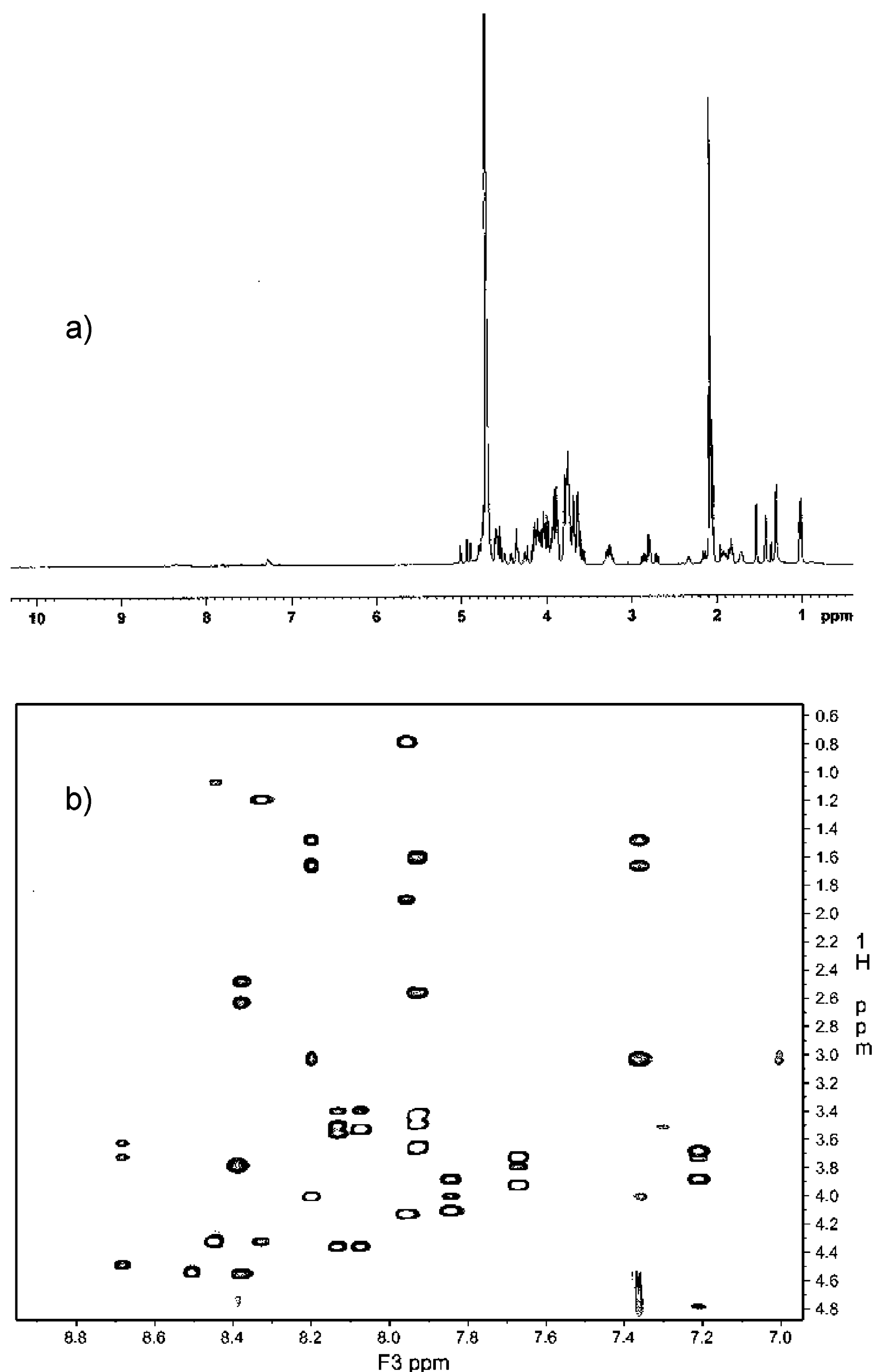
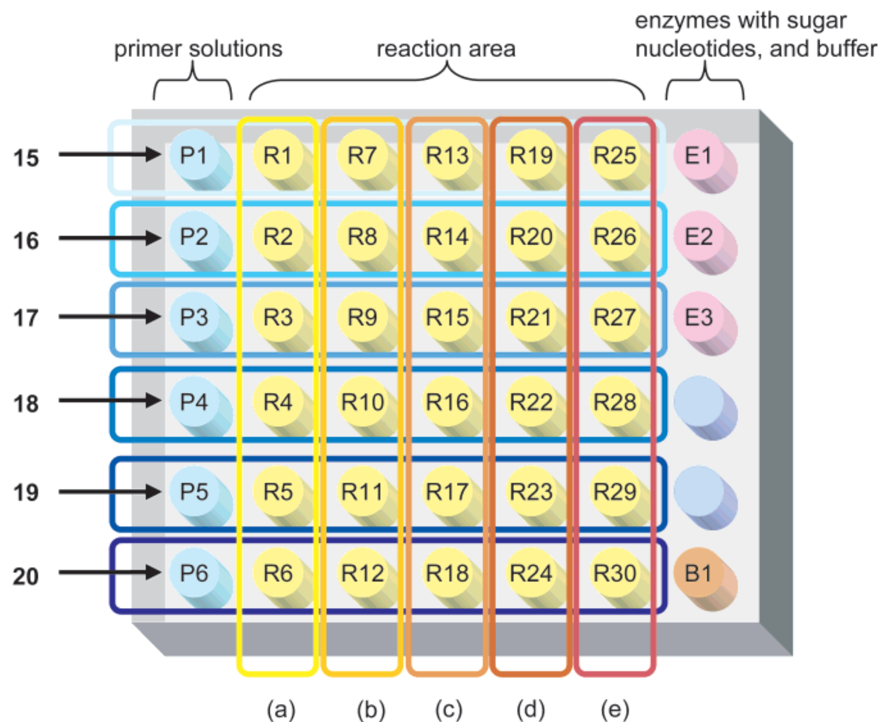



***Figure 7.*** (a) Proton NMR spectrum of the compound **9** in $D_2O$ at 313 K (600 MHz). b) Proton−proton TOCSY NMR spectrum of the compound **9** measured in 10% $D_2O$ in $H_2O$ at 300 K (600 MHz).

of-fright mass spectrometry (MALDI-TOF-MS) data were recorded by Bruker REFLEXIII and Ultraflex machines. Analytical HPLC was performed on a Hitachi HPLC system equipped with an L-2130 intelligent pump and an L-2420 UV detector or with an L-7100 intelligent pump and an L-7405 UV detector, using a reversed-phase (RP) C18 column, Inertsil ODS-3, 4.6 × 250 $mm^2$ (GL Sciences Inc.) or Mightysil RP-18, 4.6 × 150 $mm^2$ (KANTO CHEMICAL CO., INC.) at a flow rate of 1.0 mL $min^{-1}$. The chromatographies were monitored by using UV absorption at 220 nm. Electrospray ionization mass spectrometry (ESI-MS: JEOL JMS-700TZ), amino acid analyses (JEOL JLC-500V), and peptide sequence analyses (Procise491 cLC, Applied Biosystems) were performed in Center of Instrumental Analysis at Hokkaido University.

**Preparation of the Water-Soluble Polymer Carrying MUC1 Glycopeptide (7). (A). Solid-Phase Synthesis of BLase-Sensitive Glycopeptide Having GalNAc, Core-2, and Core-6 Sugar Moieties 4:** Syntheses of the BLase-sensitive glycopeptide having GalNAc, Core-2, and Core-6 (**4**) were performed on TentaGel S RAM resin (0.25 mmol/g, 120 mg, 30 $\mu$mol) using an automated peptide synthesizer (Advanced ChemTech, APEX396) and off-line microwave irradiation coupling reactions in the cases of precious sugar amino acids, namely Fmoc-Thr(GalNAcα)-OH (**1**), Fmoc-Ser(Core2α)-OH (**2**), and Fmoc-




Reaction program

| | a | b | c | d | e |
|---|---|---|---|---|---|
| E1 | + | - | + | + | + |
| E2 | - | + | + | - | + |
| E3 | - | - | - | + | + |

Stock solutions composition

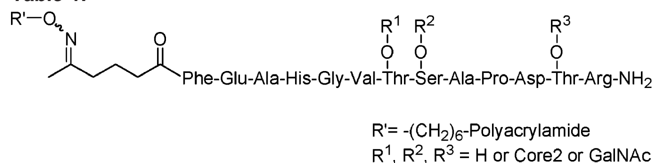
| | |
|---|---|
| P1-P6 | 6.67 mM primer polymer **15**-**20** (theoretical concentration based on the TentaGel), 16.7 mM $MnCl_2$, 0.1 % BSA, and 50 mM HEPES buffer (pH7.0) |
| E1 | 20 mM UDP-galactose, 1 U / ml $\beta$1,4-galactosyltransferase, 0.1 % BSA, and 50 mM HEPES buffer (pH7.0) |
| E2 | 20 mM CMP-NANA, 0.175 U / ml $\alpha$2,3-(O)-sialyltransferase, 0.1 % BSA, and 50 mM HEPES buffer (pH7.0) |
| E3 | 20 mM CMP-NANA, 0.185 U / ml $\alpha$2,3-(N)-sialyltransferase, 0.1 % BSA, and 50 mM HEPES buffer (pH7.0) |
| B1 | 0.1 % BSA and 50 mM HEPES buffer (pH7.0) |

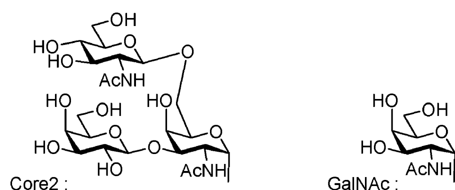
***Figure 8.*** Combinatorial synthesis of 36 kinds of MUC1 related glycopeptides performed by means of autosampler. Reaction conditions: (a) primer polymer **15**−**20** (4 mM, theoretical concentration of glycopeptide based on the TentaGel), UDP-Gal (2 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH 7.0); (b) primer polymer **15**−**20** (4 mM, theoretical concentration of glycopeptide based on the TentaGel), CMP-NANA (2 mM), $\alpha$2,3-(*O*)-sialyltransferase (17.5 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH7.0); (c) primer polymer **15**-**20** (4 mM, theoretical concentration of glycopeptide based on the TentaGel), UDP-Gal (2mM), CMP-NANA (2 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), $\alpha$2,3-(*O*)-sialyltransferase (17.5 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH7.0); (d) primer polymer **15**−**20** (4 mM, theoretical concentration of glycopeptide based on the TentaGel), UDP-Gal (2 mM), CMP-NANA (2 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), $\alpha$2,3-(*N*)-sialyltransferase (18.5 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH 7.0); (e) primer polymer **15**−**20** (4 mM, theoretical concentration of glycopeptide based on the TentaGel), UDP-Gal (2 mM), CMP-NANA (4 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), $\alpha$2,3-(*O*)-sialyltransferase (17.5 mU/mL), $\alpha$2,3-(*N*)-sialyltransferase (18.5 mU/mL), $MnCl_2$ (10 mM), BSA (0.1%), HEPES buffer (50 mM, pH 7.0). All procedures were carried out at 25°C for 24 h.

Thr(Core6$\alpha$)-OH (**3**). A cycle of an automated peptide synthesizer is defined as followed; the resin was mixed with 20% (v/v) piperidine in DMF, and the reaction mixture was stirred for 5 min at ambient temperature. This process was repeated again but stirred for 15 min. The resin was filtered and washed with NMP (*N*-methylpyrrolidone) and DMF and then added to a solution of 10 equiv of Fmoc (*N*-fluoren-9-ylmethoxycarbonyl) amino acid (0.3 mmol), 10 equiv of HBTU[22] (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophos-

(22) Carpino, L. A.; Imazumi, H.; El-Faham, A.; Ferrer, F. J.; Zhang, C.; Lee, Y.; Foxman, B. M.; Henklein, P.; Hanay, C.; Mugge, C.; Wenschuh, H.; Klose, J.; Beyermann, M.; Bienert, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 442.

*Table 1.*




| primer | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **15** | GalNAc | H | Core 2 |
| **16** | Core 2 | H | GalNAc |
| **17** | GalNAc | Core 2 | H |
| **18** | H | GalNAc | Core 2 |
| **19** | Core 2 | GalNAc | H |
| **20** | H | Core 2 | GalNAc |

phate, 0.3 mmol), 10 equiv of HOBt (*N*-hydroxybenzotriazole, 0.3 mmol), and 15 equiv of DIEA (diisopropylethylamine, 0.44 mmol) in NMP and DMF. The reaction mixture was then stirred for 30 min at ambient temperature. This coupling process was performed again but stirred for 45 min. The reaction mixture was filtered, and the residual resin was washed with NMP and DMF. The unreacted amino groups on the resin were acetylated with a solution of $Ac_2O$ (4.75% as v/v), DIEA (2.25% as v/v), and HOBt (13 mM) in NMP. The resin was washed with NMP and subjected to deprotection of Fmoc group in the same manner as described before. In the case of **1**, **2**, or **3**, couplings were performed off-line with the solution of **1** (31 mg, 46 $\mu$mol), **2** (39 mg, 33 $\mu$mol), or **3** (33 mg, 34 $\mu$mol) in DMF with HBTU (1.0 equiv for sugar amino acids), HOBt (1.0 equiv for sugar amino acids), and DIEA (2.0 equiv for sugar amino acids). The reaction mixtures were treated for 20 min under microwave irradiation (0−40 W) at 50 °C. By the combination of these two types of assembly methods, the peptides were constructed with Fmoc-Arg(Pbf)-OH, **1**, Fmoc-Asp-(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, **2**, **3**, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, and Fmoc-Phe-OH in this order. At the final step, 5-oxohexanoic acid was coupled at the *N*-terminus of the peptide by the automated peptide synthesizer. The resin was washed with NMP and $CH_2Cl_2$, dried in vacuo, and treated with 90% TFA aq. for 2 h at ambient temperature to release the peptide from the resin and for concurrent deprotections of *t*Bu, Trt (trityl), and Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) groups. The mixture containing compound **4** was filtrated, and the resin was washed with TFA. The combined filtrate was evaporated, and the resulting syrup was precipitated from dry diethyl ether to give crude **4** as an amorphous solid.

**B. De-*O*-acetylation of Compound 4.** The crude **4** in methanol (6 mL) was neutralized with 1 N NaOH aq. and 30 $\mu$L of 1 N NaOH aq. were added. The mixture was shaken for 2 h, 15 $\mu$L of additional 1 N NaOH aq. were added, the mixture was further shaken for 1 h, another 15 $\mu$L of 1 N NaOH aq. were added, and the mixture was stirred finally for 30 min. The reaction mixture was then neutralized by Dowex 50-X8 ($H^+$) and was evaporated. The resulting residue was stocked as a 3 mL solution of 50 mM sodium acetate buffer (pH 5.5) which contained the glycopeptide **5** (10 mM theoretical concentration based on the TentaGel).

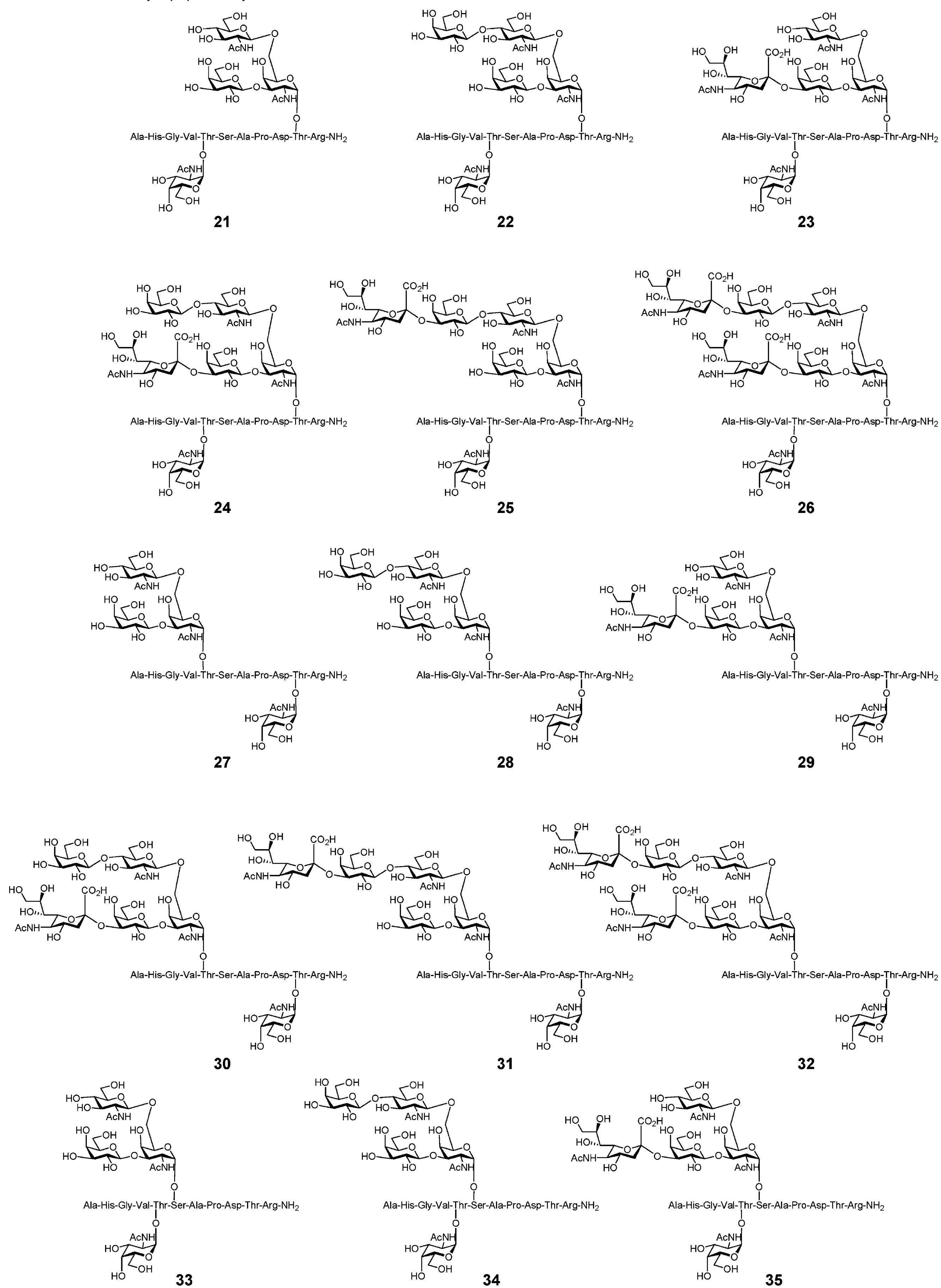
**C. Chemoselective Blotting of the Glycopeptide (5) with the Water-Soluble Polymer (6) through the Oxime Bond Formation:** An above solution of the crude glycopeptide **5** in 50 mM sodium acetate buffer (pH 5.5, 3 mL) was mixed with 3 mL of 10 mM polymer (**6**) solution in water (30 $\mu$mol for oxylamino group). The reaction mixture was stirred for 14 h at ambient temperature and passed through a centrifugal ultrafiltration (UF) unit (Orbital Biosciences, LLC; 10K Apollo 20 mL, high-performance centrifugal concentrators). The retentate was washed with 25 mM HEPES buffer (pH 7.0) three times, collected, and stocked as a 1.5 mL solution of 25 mM s HEPES buffer (pH 7.0).

**One-Pot Enzymatic Sugar Elongations Using Glycosyltransferases.** A mixture of the above solution of the crude **7** in 25 mM HEPES buffer (pH 7.0, 1.0 mL, 20 $\mu$mol theoretically based on the starting TentaGel), UDP-galactose (5 mM), CMP-NANA (5 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), α2,3-(*N*)-sialyltransferase (74 mU/mL) and α2,3-(*O*)-sialyltransferase (17.5 mU/mL) was adjusted as a 50 mM HEPES buffer solution (total volume 2.5 mL, 10 mM $MnCl_2$, 0.1% BSA (bovine serum albumin), pH 7.0), and incubated at 25 °C for 24 h. The reaction mixture was concentration by a centrifugal UF unit (10K Apollo 20 mL). The retentate was washed with 25 mM ammonium acetate buffer (pH 6.5) three times, collected, and stocked as a 2.5 mL solution of 25 mM ammonium acetate buffer (pH 6.5).

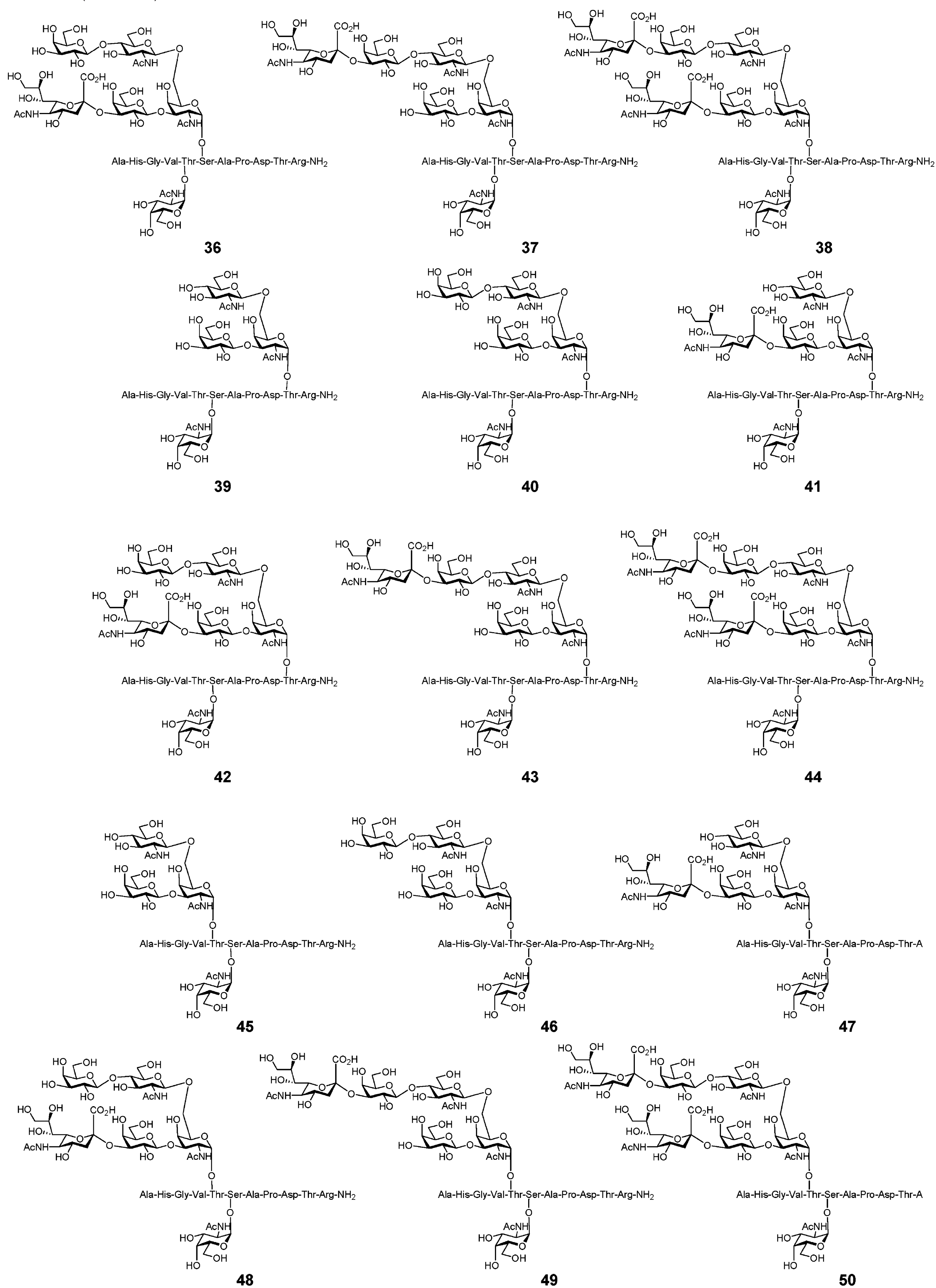
**BLase Promoted Hydrolysis and Isolation of Compound 9 from the Polymer Support.** To an above solution of the crude **8** in 25 mM ammonium acetate buffer (pH 6.5, 2.25 mL, 18 $\mu$mol) was added 11.3 $\mu$L of BLase solution (1.74 mg/mL), the reaction mixture was incubated at 25 °C for 2 h, and passed through a centrifugal UF unit (10 K Apollo 20 mL). Retentate polymers were washed with 25 mM ammonium acetate buffer (pH6.5), and the filtrate was lyophilized to give the crude glycopeptide **9** (7.0 mg) which was estimated to be more than 70% pure by the RP-HPLC (Figure 4a) and was also estimated to be 8.6% in overall yield calculated by amino acid analysis. The preparative RP-HPLC of crude material (0.7 mg) was carried out and gave the pure glycopeptide **9** (223 $\mu$g) in 3.6% overall yield calculated from the first step in the solid-phase peptide synthesis. ESI-MS (neg): 3483.3945 (M−H), calcd 3483.3916. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.1 (1), His 1.0 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.8 (2), Val 1.0 (1). 600 MHz proton NMR spectra of pure compound **9** (0.65 mM, 0.8 mg/0.35 mL) in $D_2O$ at 313 K was indicated in Figure 7.

**Parallel Syntheses of MUC1 Glycopeptides by Various Combinations of Glycosyltransferases.** Five reactions had settled independently as follows: (1) a solution of the polymer **7** (1.2 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (5 mM), and $\beta$1,4-galactosyltransferase (100 mU/mL) in 50 mM HEPES buffer (total volume 150 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0), (2) a solution of the polymer **7** (1.2 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), CMP-NANA (5 mM), and α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 150 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0), (3) a solution of the polymer **7** (1.2 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (5 mM), CMP-NANA (5 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), and α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 150 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0), (4) a solution of the polymer **7** (1.2 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (5 mM), CMP-NANA (5 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), and α2,3-(*N*)-sialyltransferase (74 mU/mL) in 50 mM HEPES buffer (total volume 150 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0), and (5) a solution of the polymer **7** (1.2 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel) UDP-galactose (5 mM), CMP-NANA (5 mM), $\beta$1,4-galactosyltransferase (100 mU/ml), α2,3-(*N*)-sialyltransferase (74 mU/mL), α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 150 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0). All reactions were performed at 25 °C for 24 h. Each reaction mixture was concentrated by centrifugal UF units (ULTRAFREE-MC 10 000 NMWL Filter Unit). The retentates were washed with 25 mM ammonium acetate buffer (pH 6.5) three times,

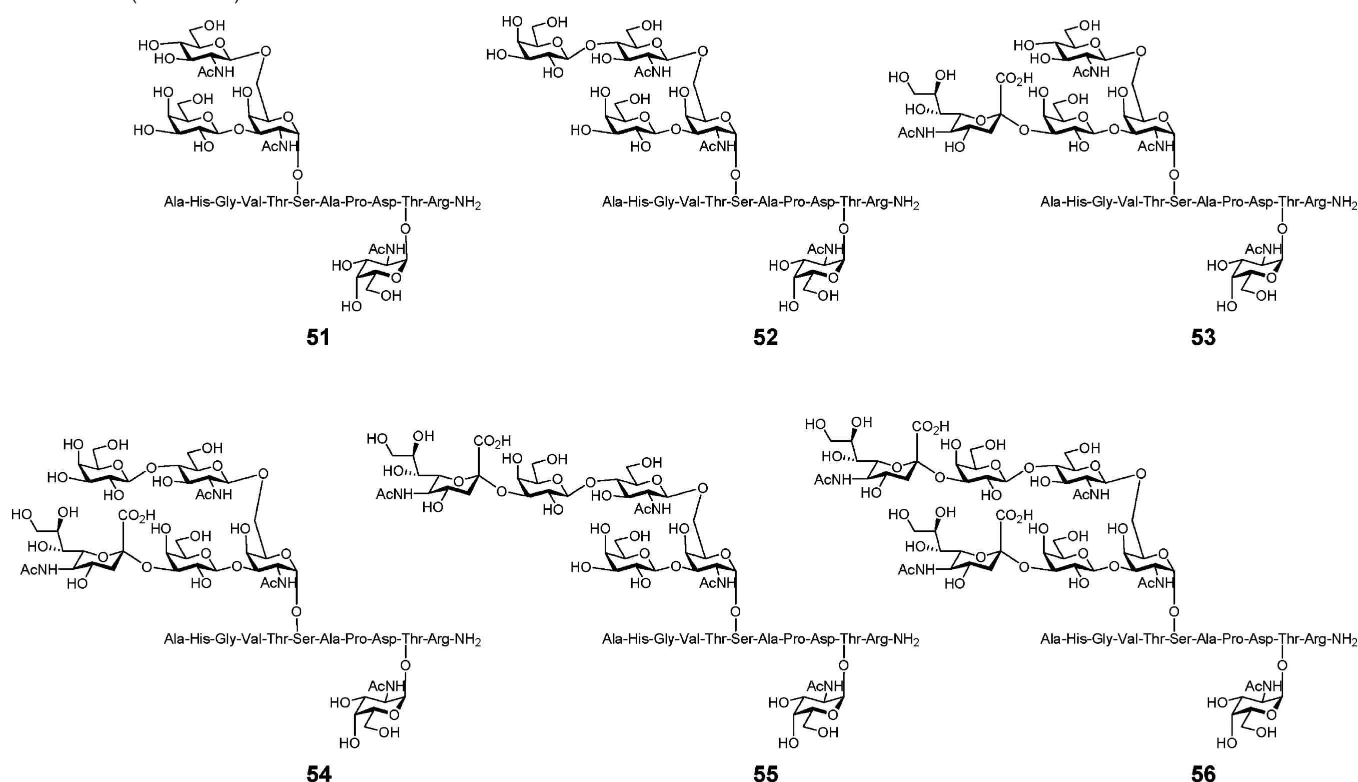
***Scheme 4.*** MUC1 Glycopeptides Synthesized in Combinatorial Manner



*Scheme 4.* (Continued)



***Scheme 4.*** (Continued)




collected, and stocked as 150 $\mu$L solutions of 25 mM ammonium acetate buffer (pH 6.5).

To each of these solutions were added the starting material **7** in 25 mM ammonium acetate buffer (pH 6.5, 150 $\mu$L) and 0.75 $\mu$L of BLase solution (1.74 mg/mL), and each reaction mixture was incubated at 25 °C for 2 h and passed through the centrifugal UF units (ULTRAFREE-MC 10 000 NMWL Filter Unit). Retentate polymers were washed by 25 mM ammonium acetate buffer (pH6.5), and the filtrates were lyophilized to give the crude glycopeptides **9** (5.9%), **10** (9.7%), **11** (8.2%), **12** (8.3%), **13** (7.9%), and **14** (6.5%), respectively. (Compounds **9**) ESI-MS (neg): 3483.3945 (M−H), calcd 3483.3916. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.0 (1), His 1.1 (1), Pro 1.0 (1), Ser 0.8 (1), Thr 1.7 (2), Val 1.0 (1). (Compound **10**) ESI-MS (neg): 2286.0044 (M−H), calcd 2285.9996. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.0 (1), His 1.1 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.8 (2), Val 1.0 (1). (Compound **11**) ESI-MS (neg): 2610.1086 (M−H), calcd 2610.1053. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.0 (1), His 1.0 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.8 (2), Val 1.0 (1). (Compound **12**) ESI-MS (neg): 2577.0920 (M−H), calcd 2577.0951. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.0 (1), His 1.1 (1), Pro 1.0 (1), Ser 0.8 (1), Thr 1.8 (2), Val 0.9 (1). (Compound **13**) ESI-MS (neg): 2901.2012 (M−H), calcd 2901.2007. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 1.0 (1), Gly 1.0 (1), His 1.1 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.8 (2), Val 0.9 (1). (Compound **14**) ESI-MS (neg): 3192.2981 (M−H), calcd 3192.2961. Amino acid analysis (theoretical ratio): Ala 2.0 (2), Asp 1.0 (1), Arg 0.9 (1), Gly 1.0 (1), His 1.1 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.7 (2), Val 0.9 (1). MALDI-TOFMS data of compounds **9**−**14** were listed in Figure 6.

**Automated Parallel Syntheses of MUC1 Glycopeptides by Various Combinations of Primers and Glycosyltransferases. Syntheses of Compounds 15**−**20.** Syntheses of compounds **15**−**20** were performed according to the same method described for the synthesis of compound **7**, using Fmoc-Thr(GalNAcα)-OH (**1**), Fmoc-Ser(GalNAcα)-OH, Fmoc-Ser(Core-2α)-OH (**2**), and Fmoc-Thr(Core-2α)-OH for solid-phase glycopeptides syntheses. Resulting polymer products were stocked as 10 mM solutions (theoretical concentration based on the TentaGel) of 25 mM HEPES buffer (pH 7.0). Chemical structures of these primers were listed in Table 1.

**Automated Parallel Syntheses of MUC1 Glycopeptides 21**−**56:** The following solutions were prepared and set to a Hitachi Programmable Autosampler L-7250 as indicated in Figure 8. **Primer solutions (P1**−**P6)**: 6.67 mM primer polymer **15**-**20** (theoretical concentration based on the TentaGel), 16.7 mM $MnCl_2$, 0.1% BSA, and 50 mM HEPES buffer (pH7.0). **Enzyme and sugar nucleotide solutions (E1**−**E3)**: (E1) 20 mM UDP-galactose, 1 U/mL $\beta$1,4-galactosyltransferase, 0.1% BSA, and 50 mM HEPES buffer (pH7.0); (E2) 20 mM CMP-NANA, 0.175 U/mL α2,3-(*O*)-sialyltransferase, 0.1% BSA, and 50 mM HEPES buffer (pH 7.0); (E3) 20 mM CMP-NANA, 0.185 U/mL α2,3-(*N*)-sialyltransferase, 0.1% BSA, and 50 mM HEPES buffer (pH 7.0). **Buffer solution (B1)**: 0.1% BSA and 50 mM HEPES buffer (pH 7.0). The following five types of reactions **(a**−**e)** for each of the primers **15**−**20** had settled independently using a program of the Hitachi D-7000 HPLC system as shown in Figure 8: (a) a solution of the primer polymer (1.0 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (2 mM), and $\beta$1,4-galactosyltransferase (100 mU/ml) in 50 mM HEPES buffer (total volume 250 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0); (b) a solution of the primer polymer (1.0 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), CMP-NANA (2 mM), and α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 250 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0); (c) a solution of the primer polymer (1.0 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (2 mM), CMP-NANA (2 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), and α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 250 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0); (d) a solution of the primer polymer (1.0 $\mu$mol of a theoretical molar quantity based on the synthesis supported TentaGel), UDP-galactose (2 mM), CMP-NANA (2 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), and α2,3-(*N*)-sialyltransferase (18.5 mU/mL) in 50 mM HEPES buffer (total volume 250 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0); (e) a solution of the primer polymer (1.0 $\mu$mol of

a theoretical molar quantity based on the synthesis supported TentaGel) UDP-galactose (2 mM), CMP-NANA (4 mM), $\beta$1,4-galactosyltransferase (100 mU/mL), α2,3-(*N*)-sialyltransferase (18.5 mU/mL), α2,3-(*O*)-sialyltransferase (17.5 mU/mL) in 50 mM HEPES buffer (total volume 250 $\mu$L, 10 mM $MnCl_2$, 0.1% BSA, pH 7.0). All reactions were conducted at 25 °C for 24 h. Each reaction mixture was concentrated by centrifugal UF units (ULTRAFREE-MC 10 000 NMWL Filter Unit). The retentates were washed with 25 mM ammonium acetate buffer (pH 6.5) for three times, collected, and stocked as 150 $\mu$L solutions of 25 mM ammonium acetate buffer (pH 6.5).

To these solutions was added 1.0 $\mu$L of BLase solution (0.174 mg/mL), and each reaction mixture was incubated at 25 °C for 2 h and passed through the centrifugal UF units (ULTRAFREE-MC 10,000 NMWL Filter Unit). In addition, the starting materials **15**−**20** in 25 mM ammonium acetate buffer (pH 6.5, 150 $\mu$L) were also subjected to the above BLase treatment without further glycosylation to obtain compounds **21**, **27**, **33**, **39**, **45**, and **51**. Retentate polymers were washed with 25 mM ammonium acetate buffer (pH 6.5), and the filtrates were lyophilized to give the glycopeptides **21**−**56**. Characterization of these glycopeptides was performed by employing MALDI-TOFMS and RP-HPLC analyses (see Supporting Information).

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**Supporting Information Available:** RP-HPLC analyses and MALDI-TOFMS spectra for compounds **21**−**56** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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