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Construction and Structural Characterization of Versatile Lactosaminoglycan-Related Compound Library for the Synthesis of Complex Glycopeptides and Glycosphingolipids

Kentarou Naruchi,[†] Tomoki Hamamoto,[‡] Masaki Kurogochi,[†] Hiroshi Hinou,[†] Hiroki Shimizu,[§] Takahiko Matsushita,[§] Naoki Fujitani,[†] Hirosato Kondo,[¶] and Shin-Ichiro Nishimura*[,†,§]

Division of Advanced Chemical Biology, Graduate School of Advanced Life Science, Frontier Research Center for the Post-Genome Science and Technology, Hokkaido University, N21, W11, Sapporo 001-0021, Japan, YAMASA Company Choshi, Chiba 288-0056, Japan, Drug-Seeds Discovery Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Japan, and Shionogi & Company, Ltd., Osaka 541-0045, Japan

shin@glyco.sci.hokudai.ac.jp

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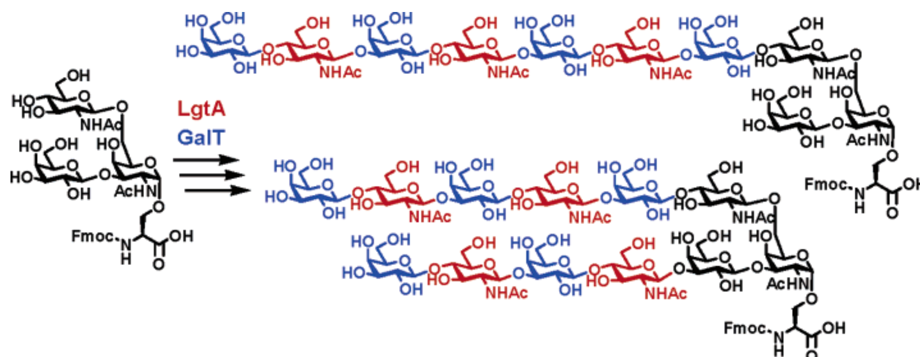

We have established a facile and efficient protocol for the preparative-scale synthesis of various compound libraries related to lactosaminoglycans: cell surface oligosaccharides composed of *N*-acetyllactosamine as a repeating disaccharide unit, based on chemical and enzymatic approaches. Substrate specificity and feasibility of a bacterial glycosyltransferase, *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase (LgtA), were investigated in order to synthesize various key intermediates suited for the construction of mammalian *O*-glycopeptides and glycosphingolipids containing poly-*N*-acetyllactosamine structures. Recombinant LgtA exhibited the highest glycosyltransferase activity with strongly basic conditions (pH = 10, glycine−NaOH buffer) and a broad range of optimal temperatures from 20 to 30 °C. Interestingly, it was found that LgtA discriminates L-serine and L-threonine and functions both as a core-1 β1,3-*N*-acetylglucosaminyltransferase and core-2 β1,3-*N*-acetylglucosaminyltransferase toward Fmoc-Ser derivatives, while LgtA showed only core-2 β1,3-*N*-acetylglucosaminyltransferase activity in the presence of Fmoc-Thr derivatives. Combined use of LgtA with human β1,4-galactosyltransferase allowed for controlled sugar extension reactions from synthetic sugar amino acids and gave synthetic lactosaminoglycans, such as a decasaccharide derivative, Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 6)[Galβ(1 → 3)]GalNAcα1 → Fmoc-Ser-OH (**6**), and a dodecasaccharide derivative, Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)-GlcNAcβ(1 → 6)[Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 3)]GalNAcα1 → Fmoc-Ser-OH (**9**). A partially protected pentasaccharide intermediate, GlcNAcβ(1 →3)Galβ(1 → 4)-GlcNAcβ(1 → 6)[Galβ(1 → 3)]GalNAcα1 → Fmoc-Thr-OH (**11**), was applied for the microwave-assisted solid-phase synthesis of a MUC1-related glycopeptide **19** (MW = 2610.1). The findings suggest that this sugar extension strategy can be employed for the modification of lactosyl ceramide mimetic polymers to afford convenient precursors for the synthesis of various glycosphingolipids.

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Introduction

Lactosaminoglycans (poly-*N*-acetyllactosamine) are cell surface carbohydrates composed of the disaccharide unit, *N*-acetyllactosamine [3Galβ(1 → 4)GlcNAcβ(1 → 3)], and function as a variety of signal molecules by altering structures in cases of cellular differentiation, cell−cell interaction, and malignant alterations. Mucin-type glycoproteins (mucins) having *O*-glycans play a fundamental role in many biological processes, including cell adhesion, signal transduction, and immune response.[1] It has been well documented that a dynamic structural change in the lactosaminoglycan motif might be essential for modulating functions of mucins in cell differentiation, aging, and malignant alteration.[2] For example, core-2 *O*-glycans exhibit terminal ligand structures such as sialyl Le^x, 6-sulfo sialyl Le^x, and sialyl Le^a through these lactosaminoglycan structures. It seems that the repeating *N*-acetyllactosamine structures can be extended from the core-2 *O*-glycans, forming poly-*N*-acetyllactosaminyl *O*-glycans as tailored linkers to display distinct ligand molecules. Overexpression of these core-2 *O*-glycans on T-lymphocyte cell surface glycoproteins has been involved in certain pathological phenomena, such as the Wiskott−Aldrich syndrome, AIDS, leukemia, and tumor invasions.[3]

Synthetic glycopeptides containing core-2-type poly-*N*-acetyllactosaminyl *O*-glycans could become nice tools both for investigating the basic structure−function relationship of mucins and for drug discovery research. Considering the occurrence of the poly-*N*-acetyllactosaminyl motif on cell surface glycosphingolipids[4] as well as *N*-glycans of glycoproteins,[5] we hypothesized that a wide range of synthetic analogues of naturally occurring lactosaminoglycans, namely, a lactosaminoglycans compound library, will become a key indispensable resource. Although extensive efforts have been paid toward the chemical synthesis of partial oligosaccharide structures related to lactosaminoglycan moieties,[6] chemical synthesis of complex oligosaccharides still requires extremely laborious and time-consuming procedures that entail multistep protections/deprotections and silica gel chromatographic purification.

Chemical and enzymatic (enzyme-assisted chemical) synthesis of glycoconjugates is a promising method due to its high stereo- and regioselectivity in protection-free glycosidation and its compatibility with high-throughput/combinatorial protocols.[7] Enzyme-assisted chemical processes allow for automated and parallel synthesis of complicated compound libraries of MUC1-related glycopeptides bearing core-2-type *O*-glycans.[8] To expand this strategy toward much more complex glycopeptides/glycolipids having poly-*N*-acetyllactosamine structures, our attention has been focused on the feasibility of using *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase (LgtA), which catalyzes glycoside bond formation between GlcNAc and a terminal Gal residue to afford the [GlcNAcβ(1 → 3)Galβ(1 → 3)] unit in the lactosaminoglycans. Pioneering work[9] on genomic and biochemical assignment of LgtA in the biosynthetic pathway of lipopolysaccharide in *Neisseria meningitidis* allowed for its high-level production and further application of this enzyme to the synthesis of a variety of poly-*N*-acetyllactosamine derivatives,[10] including complex core-2 *O*-glycan-linked peptides related to P-selectin glycoprotein ligand-1 (PSGL-1) and high affinity ligands of galectin-1.[10b,e] On the other hand, we have reported that microwave irradiation at 50 °C facilitates rapid and efficient solid-phase chemical synthesis of glycopeptide intermediates as key primers suited for further enzymatic

* To whom correspondence should be addressed. Tel: +81-(0)11-706-9043. Fax: +81-(0)11-706-9042. E-mail: shin@glyco.sci.hokudai.ac.jp or tiger.nishimura@
aist.go.jp.
† Hokkaido University.
‡ YAMASA Company.
§ AIST.
¶ Shionogi & Company, Ltd.

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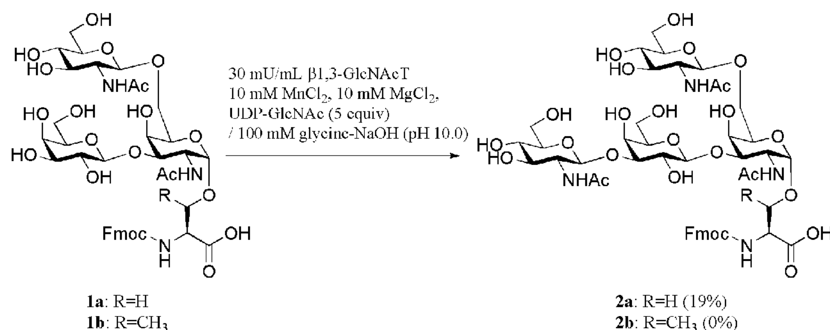
SCHEME 1. Substrate Specificity of LgtA



modification study.[8b,11] It is noteworthy that the microwave-assisted protocol greatly accelerated coupling reactions of bulky glycosylated amino acid building blocks having per-*O*-acetylated neutral mono-, di-, and trisaccharides such as GalNAcα1 → Fmoc-Ser/Thr-OH, GlcNAc*β*(1 → 6)GalNAcα1 → Fmoc-Ser/Thr-OH, and GlcNAc*β*(1 → 6)[Gal*β*(1 → 3)]GalNAcα1 → Fmoc-Ser/Thr-OH. Therefore, we thought that Fmoc-Ser/Thr-OH derivatives having lactosaminoglycans with different size and/or branching structures could become a new class of convenient building blocks for the construction of highly complicated mucin-type glycopeptides. In the present study, we describe a systematic synthesis and structural characterization of a versatile compound library of lactosaminoglycan derivatives based on the substrate specificity of LgtA and human *β*1,4-galactosyltransferase (*β*1,4-GalT) toward various synthetic glycosyl acceptors.

Results and Discussion

A. Preparation and Characterization of LgtA. Recombinant LgtA was produced in *Escherichia coli* according to the method reported by Blixt et al.[10a] with some modifications. The effects of pH and temperature on the LgtA activity were investigated by means of UDP-GlcNAc and lactose in the presence of $MgCl_2$ and $MnCl_2$ (Figure S-1 in Supporting Information). Although LgtA has been known to catalyze the transfer reaction of GlcNAc most efficiently at pH = 7.5−8.0 and 20−30 °C,[10a] careful evaluation using buffer solutions revealed that the recombinant LgtA has two distinct optimal conditions at around pH = 7.0 (Tris-HCl buffer) and pH = 10.0 (glycine−NaOH buffer). Interestingly, LgtA showed higher activity under strongly basic conditions (150 U/mL) than that at neutral pH (100 U/mL), while general glycosyltransferases function under neutral conditions. As a result, we concluded that this enzyme can be used as a GlcNAc transferring catalyst at broad pH (pH = 6.5−10.5) and at a wide temperature range from 20 to 30 °C.

We initially investigated basic substrate specificity of LgtA toward Fmoc amino acids carrying a typical core-2-type trisaccharide, GlcNAc*β*(1 → 6)[Gal*β*(1 → 3)]GalNAcα1 → Fmoc-Ser-OH (**1a**) and GlcNAc*β*(1 → 6)[Gal*β*(1 → 3)]-GalNAcα1 → Fmoc-Thr-OH (**1b**) as indicated in Scheme 1. Herein, our interest was focused on the substrate specificity of LgtA against core-2 trisaccharide structure that might become acceptor substrates on route to *O*-glycans with biantennary lactosaminoglycans. It was clearly demonstrated that LgtA can discriminate the structural difference between Ser and Thr (Figure S-2 in Supporting Information). RP-HPLC and MALDI-TOF MS analyses revealed that LgtA transferred GlcNAc residue only to the Fmoc-Ser derivative **1a**[12] to give **2a**, while **1b**[13] was not a substrate for this enzyme, suggesting that LgtA can function as a *β*1,3-*N*-acetylglucosaminyltransferase in the presence of non-reducing terminal Gal residue of core-2-type glycans linked with serine residue. In the course of the enzyme reaction, no detectable precipitation due to oxidation of $MnCl_2$ was observed. Chemical shifts of α and *β* protons of the L-serine residue of compound **2a** were not changed after the reaction, indicating that the basic media used in this reaction did not cause any isomerization of the amino acid residue. Although it seems simply that the threonine-type substrate does not fit to the enzyme binding pocket, this result may suggest that the *γ*-methyl group of the Thr residue induces specific but unfitted conformational change of the core-2 trisaccharide moiety against the active site of LgtA. Our previous study[14] on the synthetic antifreeze glycoproteins (*sy*AFGPs) also indicated that the *γ*-methyl group of the Thr residue is indispensable for the formation of a specific poly-L-proline type II helix structure (a 3-fold left-handed helix) in order to achieve unique freezing point depression activity. However, this essential conformation was completely disrupted by the replacement of Thr with Ser residues, and the resulted Ser-substituted AFGP lost antifreeze activity.[14d]

B. Synthesis of Fmoc-Ser Derivatives Having Mono- and Lactosaminoglycan Chains. On the basis of above results, we synthesized a series of Fmoc-Ser derivatives having single (**4**−

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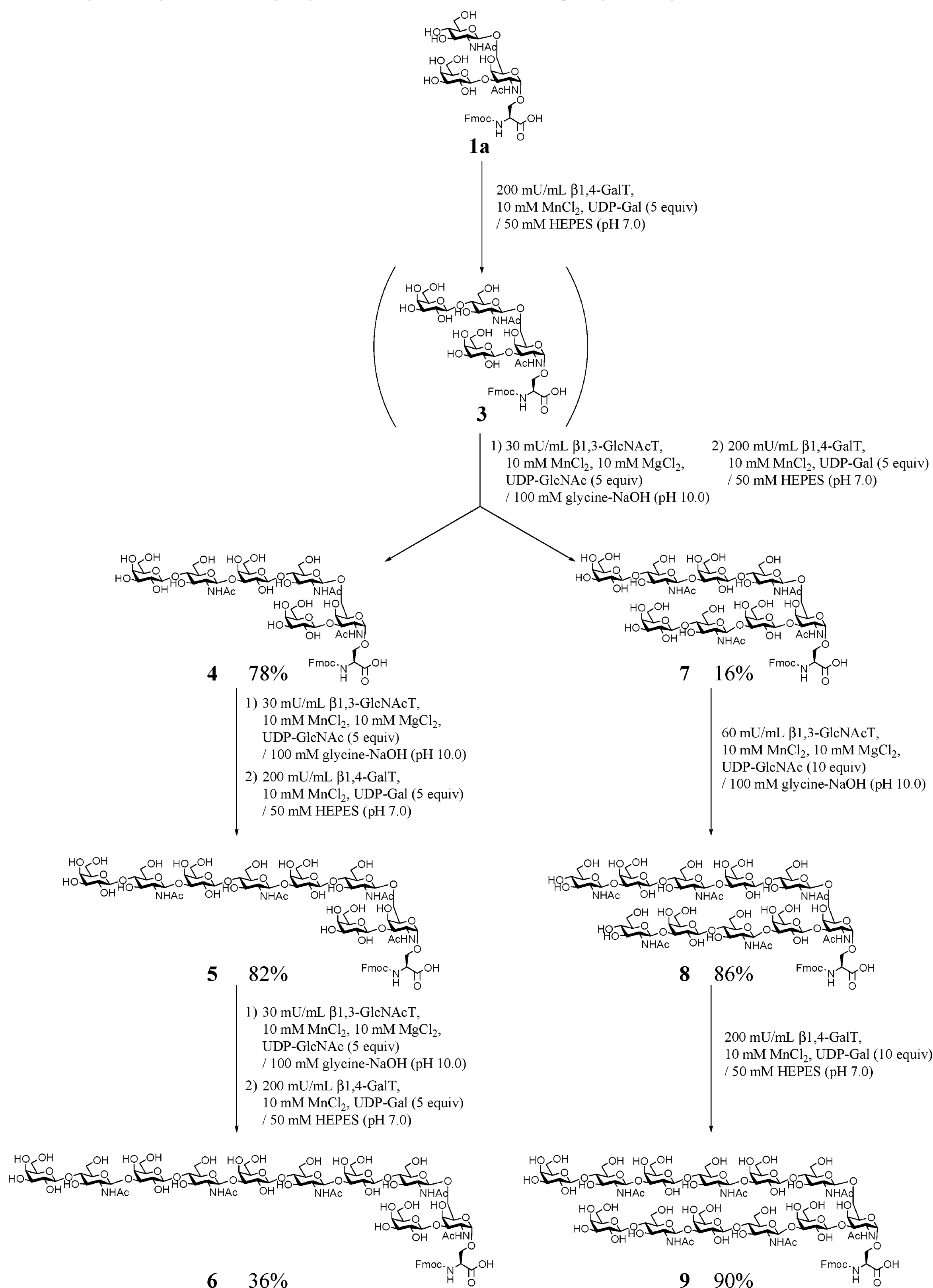
SCHEME 2. Enzymatic Synthesis of Glycosylated Amino Acids Containing Poly-*N*-acetyllactosamine



6) and double lactosaminoglycan chains (**7**−**9**) from compound **1a** as a key starting material (Scheme 2). Intermediate **3** was readily prepared from **1a** by employing commercially available recombinant human β1,4-galactosyltransferase (β1,4GalT) in the presence of UDP-Gal in quantitative yield and used directly for further stepwise sugar elongation by means of LgtA and β1,4GalT. Crude **3** (10 μmol scale) was subjected to sequential treatment with LgtA [30 mU/mL, UDP-GlcNAc (5 equiv) in 100 mM glycine−NaOH buffer (8 mL, pH 10.0) containing $MnCl_2$ and $MgCl_2$] and β1,4GalT [200 mU/mL, UDP-Gal (5 equiv) in 50 mM HEPES buffer (8 mL, pH 7.0)] to give monosubstituted **4** (78%) and disubstituted **7** (16%). As

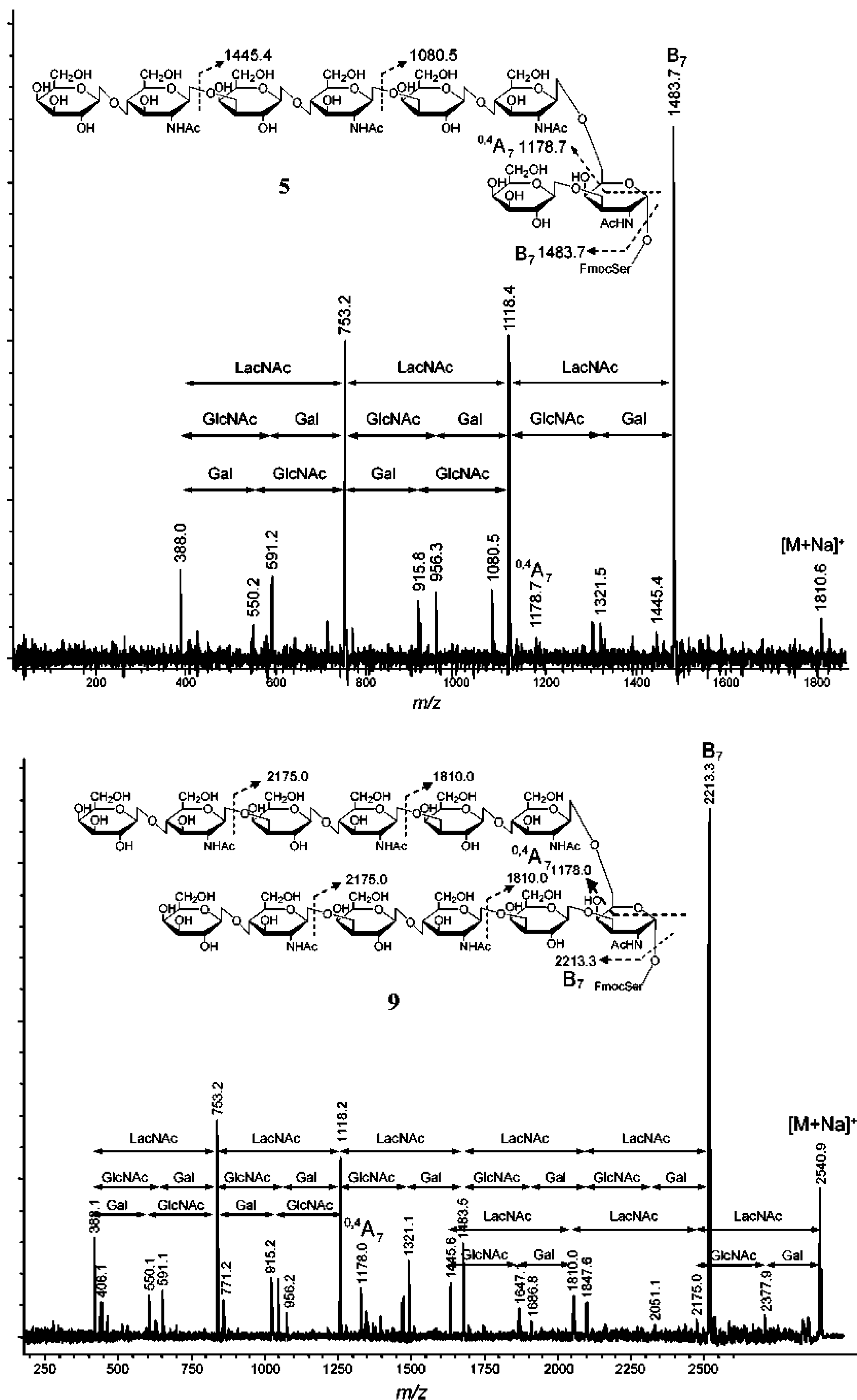


FIGURE 1. (a) MALDI-LIFT TOF/TOF MS analysis of compound **5**. (b) MALDI-LIFT TOF/TOF MS analysis of compound **9**.

indicated by the RP-HPLC profile (Figure S-3 in Supporting Information), LgtA showed much higher core-2 β1,3-*N*-acetylglucosaminyltransferase activity than core-1 extension ability when Fmoc-Ser derivative **3** was selected as a substrate displaying two Gal residues at both non-reducing terminals. Next, compounds **4** and **7** were independently employed for further extension to afford compounds **5** (82%), **6** (36%), **8** (86%), and **9** (90%) carrying single/double lactosaminoglycans, respectively. Interestingly, compound **7** could be converted into doubly GlcNAcylated **8** in high yield (86%), suggesting that LgtA cannot discriminate the two terminal Gal residues attached as core-1 and core-2 branches with the acceptor **7**. Compared to the results observed for the reaction with **4**, it is likely that the effect of aglycon structure on the acceptability of LgtA-mediated GlcNAc extension reaction seems to descend when the terminal Gal residue is far from the aglycon moiety. Since the yield in the conversion of **5** (three LacNAc repeats) into **6** (four LacNAc repeats) is drastically reduced to 36%, there might be a significant limitation of the length in GlcNAc extension capacity by LgtA. In fact, we could not detect any extension of GlcNAc residue toward compound **6** by using LgtA under the same conditions. Structural characterization of all isolated products was performed by MALDI-TOF/TOFMS (Figure 1 and Supporting Information) and high-resolution NMR (1D and HSQC in Supporting Information). Fragmentation by LIFT-TOF/TOF[15] in the presence of 2,5-dihydroxybenzoic acid (DHB) occurred successfully both at the reducing GalNAc residue and the glycoside linkage with the Fmoc-Ser moiety to give meaningful ion peaks generated by characteristic cleavage patterns represented as $^{0,4}A_7$, $^{0,4}A_9$, B_7, or B_9. In addition to the precursor ion peaks of compounds **5**, **6**, and **9** observed at m/z 1810.6, 2175.7, and 2540.9, typical product ion peaks due to the above cleavage patterns such as $^{0,4}A_7$, $^{0,4}A_9$, B_7, and B_9 allowed for precise structural identification of single and double lactosaminoglycan chains. For example, it was clearly suggested that the mass difference between B_7 (m/z 2213.3) and $^{0,4}A_7$ (m/z 1178.0) derived from compound **9** with biantennary lactosaminoglycans is becoming a key to determine the size of the LacNAc repeat due to core-2 extension as indicated in Figure 1b, while product ion peaks of B_7 (m/z 1483.7) and $^{0,4}A_7$ (m/z 1178.0) observed in the case of compound **5** indicate the existence of only single core-2-type LacNAc extension (Figure 1a).

In addition, the present preparative-scale synthesis of lactosaminoglycans by means of LgtA and human β1,4GalT made a systematic high-resolution NMR study possible. The 600 MHz NMR (HSQC) spectra of compounds **5** and **9** yield complete assignments of ^{1}H and ^{13}C chemical shifts as summarized in tables in the Supporting Information. These fundamental data of MALDI-TOF/TOFMS and NMR will greatly contribute to high-throughput glycomics as well as synthetic studies of a variety of complex glycoconjugates involving lactosaminoglycans (LacNAc repeating unit).

C. Approaches for the Synthesis of Mucin-type Glycopeptides and Sphingoglycolipids by Means of LgtA-Mediated GlcNAc Extension. Our attention was next directed to the potential of LgtA-mediated GlcNAc extension in the synthesis of complex glycopeptides, such as MUC1-related *O*-glycopeptides having lactosaminoglycan structures. It was revealed that

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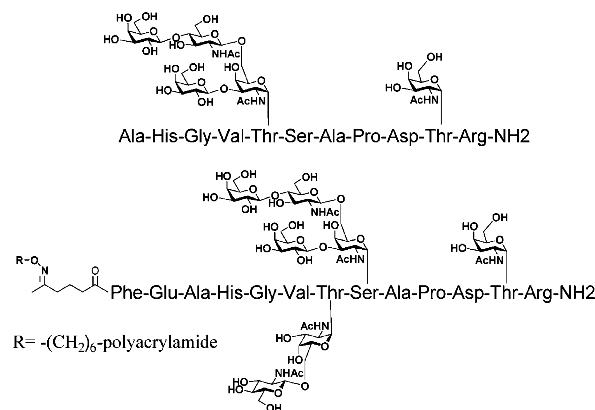


FIGURE 2. Evaluation of accessibility by LgtA for the modification of the known synthetic glycopeptides.

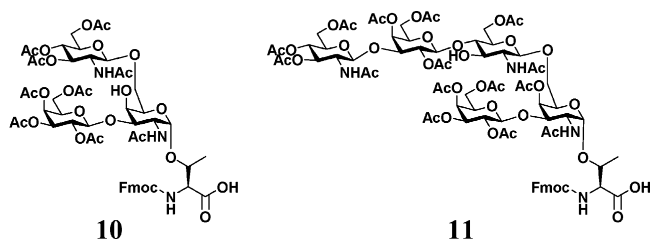
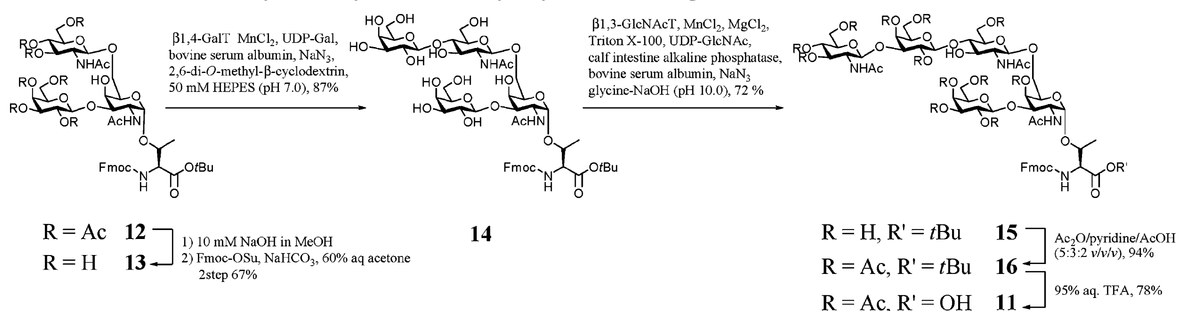


FIGURE 3. Fmoc-threonines carrying core-2-based trisaccharide (**10**) and pentasaccharide (**11**) structures.

LgtA did not show any GlcNAc transferring activity toward synthetic model glycopeptides[8] displaying some non-reducing Gal residues expected to be potential modification sites by this enzyme, as illustrated in Figure 2. Considering the results reported by Cummings et al.[10d] for the synthesis of glycosulfopeptides related to PSGL-1 in which the core-2-type GlcNAc extension using LgtA was achieved by using an acceptor substrate bearing three sulfated tyrosine residues (Tyr-SO_3^-), clustered negative charges due to Tyr-SO_3^- residues may be crucial for this successful synthetic protocol.

Therefore, we had to establish an alternate synthetic scheme to the above direct glycosylation strategy toward glycopeptide-type substrates. We thought that microwave irradiation[11] might accelerate the coupling reactions of sterically hindered Fmoc amino acid derivatives carrying large oligosaccharides and allow for solid-phase synthesis of lactosaminoglycan-related glycopeptides. In order to evaluate the feasibility of this protocol, Fmoc-Thr derivatives having suitably *O*-acetylated core-2 trisaccharide **10**[11a] and pentasaccharide **11** were prepared as tentative intermediates for further evaluation study (Figure 3). Compound **11** was readily derived from known **12**[11a] by sequential modifications with β1,4GalT and LgtA according to the conditions described in the synthesis of a series of Fmoc-Ser-based lactosaminoglycans (Scheme 3). Scheme 4 illustrates a synthetic route of a target MUC1-related glycopeptide **19** on the basis of microwave-assisted protocol.[11b] It was demonstrated that stepwise coupling reactions of Fmoc amino acid derivatives, including **10** and **11**, proceeded smoothly under microwave irradiation (0−40 W) at 50 °C for 10−20 min on a Rink amide PEGA resin, a poly(ethylene glycol)−poly(dimethylacrylamide) copolymer, and standard procedures for release and purification gave pure **19** in 5.3% isolated overall yield from the first arginine residue loading onto PEGA resin. As anticipated, this

SCHEME 3. Chemical and Enzymatic Synthesis of Glycosylated Building Block 11



SCHEME 4. Synthesis of a Glycopeptide 19

Rink Amide PEGA resin

1) Fmoc-amino acid, HBTU,HOBt, DIEA/ DMF
2) Ac_2O, HOBt, DIEA/ DMF
3) 20% piperidine/ DMF
4) repeating the procedures 1-3

17

TFA/TIS/H_2O (95:2.5:2.5, *v*/*v*/*v*)

18

10 mM NaOH/MeOH

19

satisfactory result indicates the versatility of the present approach for the synthesis of complex glycopeptides in assistance with LgtA-mediated GlcNAc extension, and this class of glycopeptides will become useful intermediates for the construction of

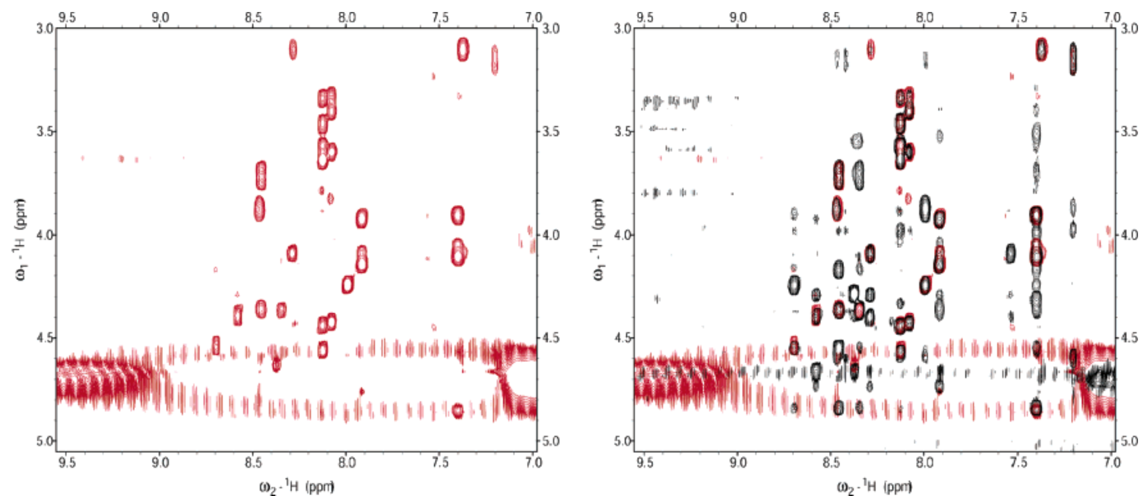


FIGURE 4. Overlay of TOCSY (red) and 300 ms NOESY spectra (black) of glycopeptide **19**.

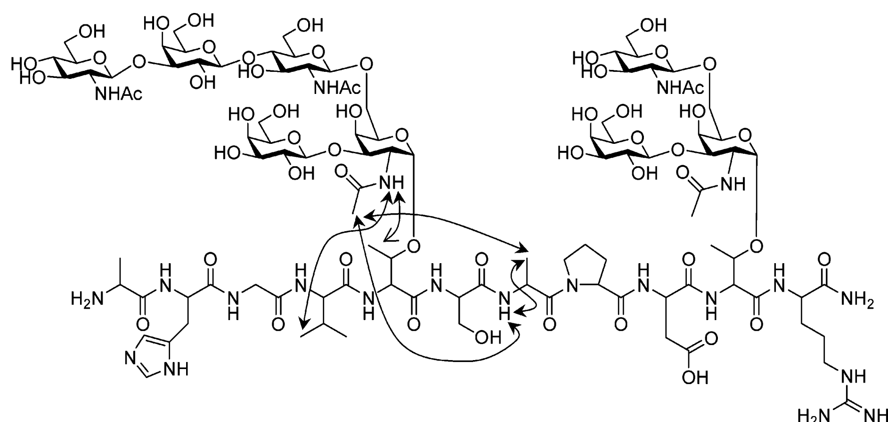


FIGURE 5. NOEs observed in the linker moiety between GalNAc and the MUC1 peptide.

much more complicated and larger glycopeptide libraries by polymer-assisted parallel synthesis as reported previously.[8b] High-quality HSQC spectra of compound **19** made complete assignment of all protons and carbons corresponding to individual sugar and amino acid residues possible as summarized in Table S-4 (Supporting Information).

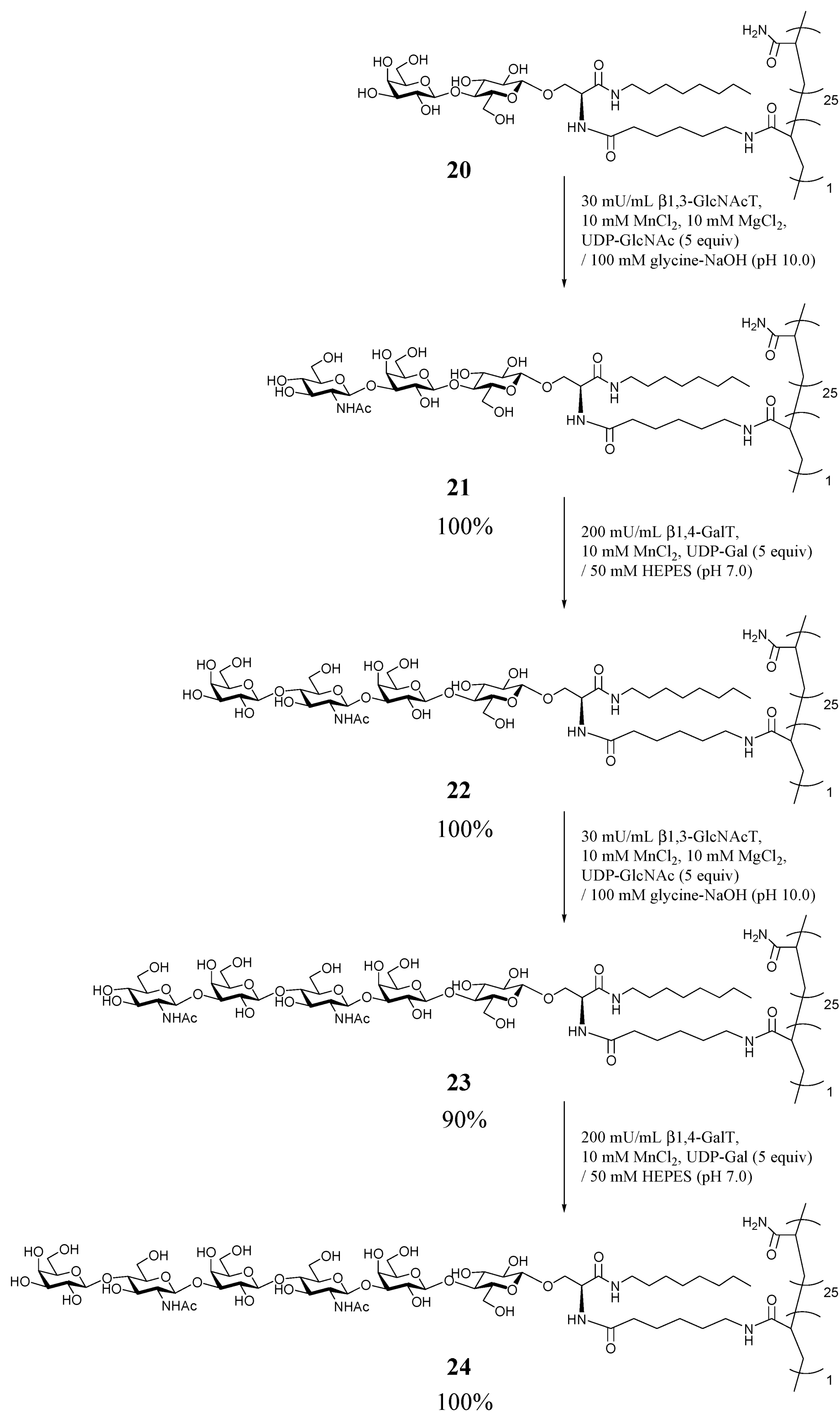
Figure 4 exhibits a fingerprint region of the 600 MHz TOCSY spectra of compound **19** at pH 4.8 in 90% H_2O/10% D_2O at 300 K. In addition to the connectivities of the intraresidue spin system of carbohydrate moieties attached with Thr5 or Thr10 residue, the TOCSY and NOESY spectra revealed that overlapping cross-peaks with TOCSY spectra represent intraresidue NOEs and other cross-peaks represent interresidue NOEs (Figure 5 and Figure S-22 in Supporting Information). It is noteworthy that NOESY spectra of this glycopeptide involved significant NOEs between the peptide moiety and the reducing GalNAc residue as indicated in Figure S-23 (Supporting Information). It was suggested that the amide proton (N*H*COAc at C-2 position) of the GalNAc residue at 7.40 ppm exhibits significant correlations with side chains of Val4 (γH, 0.82 and 0.87 ppm) and Thr5 (γH, 1.13 ppm). In addition, the cross-peaks of the acetyl protons (C*H*$_3$CONH at C-2 position) of the GalNAc residue at 1.89 ppm with Ala7 residue (NH, 8.35 ppm, and βH, 1.28 ppm) may indicate the specific structural feature of the MUC1 glycopeptides bearing *O*-glycan chains.[11b,14d] It seems likely that these NOEs will become valuable for revealing the important conformation required for displaying crucial epitope structures of MUC1 glycopeptides as tumor-associated antigens.

In our previous paper,[16] we communicated the total synthesis of naturally occurring sphingoglycolipid, Neu5Acα(2 → 3)Galβ-(1 → 4)[Fucα(1 → 3)]GlcNAcβ(1 → 3)Galβ(1 → 4)Glcβ1 → Cer (IV^{3}Neu5Acα,III^{3}Fucα-nLc^{4}Cer), by means of enzyme-assisted processes on the lactosyl ceramide mimic polymer support (LacCer mimic primer).[17] Herein, we have expressed a bacterial glycosyltransferase, *Streptococcus agalactiae* type Ia β1,3-*N*-acetylglucosaminyltransferase (β1,3GlcNAcT), in *E. coli* as a fusion protein with maltose-binding protein. It was suggested that this enzyme catalyzes the GlcNAc transfer reaction toward LacCer primer and afforded multiple trisaccharide GlcNAcβ(1 → 3)Galβ(1 → 4)Glc branches. Although this key intermediate could be subjected to further modification reactions by three different glycosyltransferases and ceramide glycanase to complete rapid and efficient synthesis of the target glycosphingolipid, it seemed that *Streptococcus agalactiae* type Ia β1,3GlcNAcT did not show any GlcNAc extension activity

(16) Toda, A.; Yamada, K.; Nishimura, S.-I. *Adv. Synth. Catal.* **2002**, *344*, 61−69.

(17) (a) Nishimura, S.-I.; Yamada, K. *J. Am. Chem. Soc.* **1997**, *119*, 10555−10556. As for the feasibility of LgtA in the synthesis of glycolipid mimics and polymer-supported oligosaccharides, see: (b) Blixt, O.; Norberg, T. *Carbohydr. Res.* **1999**, *319*, 80−91.

SCHEME 5. Enzymatic Synthesis of Sphingoglycolipid Mimic Polymers Containing *N*-Acetyllactosamine Unit



to the polymer carrying the tetrasaccharide-type intermediate [Gal $\beta(1 \rightarrow 4)$GlcNAc$\beta(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glc].

As indicated in Scheme 5, we preliminarily examined the acceptability of LacCer primer **20** for the GlcNAc extension reaction by using LgtA. As expected, combined use of LgtA with β1,4GalT allowed for quantitative sugar elongation reactions on this water-soluble polymer support, suggesting that LgtA is a nice tool for the synthesis of various glycosphingolipids having lactosaminoglycans (LacNAc repeating unit) as well as for the synthesis of glycopeptides. High-resolution HSQC spectra (Supporting Information) of compounds **21**−**24** clearly suggest the feasibility of the present protocol for the synthesis of glycosphingolipid libraries, even though the procedures were conducted on polymer support. Practical

synthesis and application to biochemical study of sphingoglycolipids bearing different LacNAc repeats are underway, and the results will be reported as soon as possible.

Conclusion

In conclusion, we have established an efficient procedure for the construction and precise structural characterization of a lactosaminoglycan-related compound library based on the unique substrate specificity and properties of a bacterial β1,3GlcNAcT (*Neisseria meningitidis* LgtA) as a key extension enzyme. Our observations demonstrate that LgtA exhibits unique β1,3GlcNAcT activity in the presence of GlcNAcβ(1 → 6)[Galβ(1 → 3)]GalNAcα1 → Fmoc-Ser-OH (**1a**), while GlcNAcβ(1 → 6)[Galβ(1 → 3)]GalNAcα1 → Fmoc-Thr-OH (**1b**) cannot be modified by LgtA at all. On the other hand, LgtA shows potential β1,3GlcNAcT activity toward Gal residues linking with the GlcNAc residue of the core-2 structure (upper branching of core-2-type structures). Combined use of LgtA and human β1,4GalT allows for the synthesis of various building blocks having different size and structures of poly-*N*-acetyllactosamine chains from some Fmoc-Ser/Thr derivatives. Our results demonstrate the versatility of these building blocks by applying them to the construction of novel MUC1-related glycopeptides on the basis of solid-phase peptide synthesis under microwave irradiation. In addition, it should be emphasized that the present strategy can be employed for the synthesis of glycosphingolipids, even though the procedures are carried out on the ceramide mimic polymer supports. It is our belief that the versatile compound library constructed in this study will greatly accelerate the synthesis of highly complicated mammalian glycoconjugates for achieving insight into the essential roles of lactosaminoglycans and their specific partner molecules.

Experimental Section

Production and Purification of *Neisseria meningitidis* β1,3-*N*-Acetylglucosaminyltransferase (LgtA). *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase (β1,3GlcNAcT) was prepared according to the method reported in previous papers[9,10] with some modifications. The *lgt A* gene encoding β1,3GlcNAcT was amplified by polymerase chain reaction (PCR) of *N. meningitides* MC 58 chromosomal DNA. Then, the appropriately digested PCR fragment was inserted in-frame pTrc99A vector. Recombinant enzyme was produced in *E. coli* JM109 with pTrc/LgtA. Transformed cells were inoculated in 2 × YT medium (1 L) containing 100 μg/mL of ampicillin at 37 °C until absorbance at 600 nm reached 0.7−0.9 (about 2 h), then IPTG (final concentration 1 mM) was added and cultivated at 20 °C for 20 h. The cells were harvested and frozen. After thawing, the cells were suspended in 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM 2-ME and then sonicated at 0 °C for 5 × 2 min. The cell debris was removed by centrifugation at 10 000*g* for 10 min. The supernatant was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM 2-ME and 1% (w/v) glycerol. The dialyzed solution was applied to a HiTrap Q FF column and pre-equilibrated with buffer A (25 mM Tris-HCl, 1 mM 2-ME, 1% (w/v) glycerol, pH 8.0). The enzyme was eluted with a linear gradient of 0.5 M NaCl in buffer A. Fractions (each 5.0 mL) showing LgtA (β1,3GlcNAcT) activity were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM 2-ME, 0.05% (w/v) CHAPS, and 1% (w/v) glycerol. The dialyzed solution was subjected to chromatography on a HiTrap Q FF column pre-equilibrated with buffer B [25 mM Tris-HCl, 1 mM 2-ME, 1% (w/v) glycerol, 0.05% (w/v) CHAPS, pH 8.0]. The enzyme was eluted with a linear gradient of 0.5 M NaCl in buffer B. Fractions having β1,3GlcNAcT activity were collected and pooled and concentrated up to 2.0 mL by Centriprep YM-10. The concentrated enzyme solution obtained from the above steps was loaded onto a gel-filtration column, Superdex 200 pg, and pre-equilibrated with buffer C (25 mM Tris-HCl, 1 mM 2-ME, 1% (w/v) glycerol, 0.2 M NaCl, pH 8.0). The column was then washed with the same buffer, and the eluted fractions containing β1,3GlcNAcT were concentrated by Centriprep YM-10, ULTRAFREE-MC until β1,3GlcNAcT activity condensed to 500 mU/mL. The purified enzyme can be stored as stable and active form for more 18 months.

Optimization of LgtA Activity. Mixtures of $MgCl_2$ (10 mM), $MnCl_2$ (10 mM), lactose (20 mM), and UDP-GlcNAc (10 mM) with various 100 mM buffered solutions (acetate/HCl: pH 4.5, 5.0, 5.5, 6.0; MES/NaOH: pH 5.0, 5.5, 6.0, 6.5, 7.0; Tris-HCl: pH 7.0, 7.5, 8.0, 8.5, 9.0; glycine/NaOH pH 8.5, 9.0, 9.5, 10.0, 10.5) were prepared. To the solution was added β1,3GlcNAcT (final concentration = 30 mU/mL), and the reaction mixture was incubated at 30 °C for 1 h. The reaction was quenched by addition of 1 M NaOH. The reaction mixture was centrifuged, and the filtrate was subjected to quantitative analysis by a Dionex HPAEC-PAD system.

To determine the optimal temperature, β1,3GlcNAcT (final concentration = 30 mU/mL) was added to a mixture of glycine/NaOH buffer (100 mM, pH 10.0), $MgCl_2$ (10 mM), $MnCl_2$ (10 mM), lactose (20 mM), and UDP-GlcNAc (10 mM). The reaction mixture was incubated for 1 h at various temperatures from 5 to 45 °C. The reaction was quenched by addition of 1 M NaOH and analyzed by the HPAEC-PAD system in a similar procedure described above.

Synthesis of Glycosylated Amino Acids Containing Poly-*N*-acetyllactosamine. *N*-(9-Fluorenylmethoxycarbonyl)-*O*-{*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (4) and *N*-(9-Fluorenylmethoxycarbonyl)-*O*-{*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (7). Compound **1a** (8.0 mg, 8.9 μmol) was modified by using 200 mU/mL of β1,4GalT and UDP-Gal (27.3 mg, 44.5 μmol) in a total volume of 8.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM $MnCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was centrifuged and lyophilized to afford crude **3**. HPLC and MALDI-TOF MS analyses of this reaction mixture suggested that galactosylation of **1a** proceeded almost quantitatively (Figure S-6 in Supporting Information). Then, crude intermediate **3** was used without further purification for the glucosaminylation by using 240 mU of LgtA and UDP-GlcNAc (31.6 mg, 48.6 μmol) in a total volume of 8.0 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mg/mL of BSA, and 100 mU/mL of calf intestine alkaline phosphatase. After incubation for 24 h at 20 °C, the reaction mixture was centrifuged, and the filtrate was lyophilized. Subsequently, the lyophilized crude material was directly subjected to galactosylation by using 1.6 U of β1,4GalT and UDP-Gal (27.3 mg, 44.5 μmol) in a total volume of 8.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM $MnCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was purified by analytical reversed-phase HPLC to give compound **4** (9.8 mg, 6.9 μmol, 78%) and compound **7** (2.54 mg, 1.4 μmol, 16%). **4:** RP-HPLC retention time = 20.1 min; ^{1}H NMR (600 MHz, D_2O; data for the ring and the exocyclic protons are summarized in Table S-1 in Supporting Information) δ 7.83−7.34 (m, 8 H, Ar*H*), 4.57 (m, 1 H, Ser-β*H*), 4.46 (m, 1 H, Ser-β*H*), 4.27 (m, 1 H, Ser-α*H*), 4.11 (m, 1 H, Fmoc-C*H*), 3.69 (m, 2 H, Fmoc-CH_2), 1.94 (s, 6 H, GlcNAc-NHCH_3), 1.85 (s, 3 H, GalNAc- NHCH_3); ^{13}C NMR (150 MHz, D_2O; data for the skeletal carbon atoms are summarized in Table S-2) δ 128.0, 127.3, 125.2, 120.2 (Ar), 68.7 (Fmoc-CH_2),

66.5 (Ser-β*C*), 55.8 (Fmoc-*C*H), 47.1 (Ser-α*C*), 22.3 (GalNAc-NH*C*H$_3$, GlcNAc-NH*C*H$_3$); MALDI-TOFMS (*m/z*) calcd for $C_{60}H_{86}N_4O_{35}Na$ [M + Na]$^+$ 1445.50, found 1445.45; HR-ESIMS (*m/z*) calcd for $C_{60}H_{85}N_4O_{35}$ [M − H]$^-$ 1421.4994, found 1421.4995. **7:** RP-HPLC retention time = 18.9 min; ^{1}H NMR (600 MHz, D$_2$O; data for the ring and the exocyclic protons are summarized in Table S-1) δ 7.83−7.35 (m, 8 H, Ar*H*), 4.55 (m, 1 H, Ser-β*H*), 4.42 (m, 1 H, Ser-β*H*), 4.29 (m, 1 H, Ser-α*H*), 4.04 (m, 1 H, Fmoc-C*H*), 3.68 (m, 2 H, Fmoc-C*H*$_2$), 1.93 (s, 12 H, NHC*H*$_3$), 1.85 (s, 3 H, GalNAc-NHC*H*$_3$); ^{13}C NMR (150 MHz, D$_2$O; data for the skeletal carbon atoms are summarized in Table S-2) δ 128.2, 127.6, 125.0, 120.1 (Ar), 68.9 (Fmoc-*C*H$_2$), 66.3 (Ser-β*C*), 56.2 (Fmoc-*C*H), 46.9 (Ser-α*C*), 22.1 (GalNAc-NH*C*H$_3$, GlcNAc-NH*C*H$_3$); MALDI-TOFMS (*m/z*) calcd for $C_{74}H_{109}N_5O_{45}Na$ [M + Na]$^+$ 1810.63, found 1810.82; HR-ESIMS (*m/z*) calcd for $C_{74}H_{108}N_5O_{45}$ [M − H]$^-$ 1786.6316, found 1786.6343.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-{*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (5).** Compound **4** (9.0 mg, 6.3 μmol) was modified by means of 240 mU of LgtA and UDP-GlcNAc (20.5 mg, 31.6 μmol) in a total volume of 8.0 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM MnCl$_2$, 10 mM MgCl$_2$, 0.1 mg/mL of BSA, and 100 mU/mL of calf intestine alkaline phosphatase. After incubation for 24 h at 20 °C, the reaction mixture was centrifuged, and the supernatant was lyophilized. The crude material was subsequently treated with a mixture of 1.6 U of β1,4GalT and UDP-Gal (19.3 mg, 31.6 μmol) in a total volume of 8.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM MnCl$_2$. After incubation for 24 h at 20 °C, the reaction mixture was subjected to the purification by means of analytical reversed-phase HPLC to give compound **5** (9.3 mg, 5.2 μmol, 82%): RP-HPLC retention time = 19.5 min; ^{1}H NMR (600 MHz, D$_2$O; data for the ring and the exocyclic protons are summarized in Table S-1) δ 7.84−7.35 (m, 8 H, Ar*H*), 4.56 (m, 1 H, Ser-β*H*), 4.42 (m, 1 H, Ser-β*H*), 4.29 (m, 1 H, Ser-α*H*), 4.04 (m, 1 H, Fmoc-C*H*), 3.69 (m, 2 H, Fmoc-C*H*$_2$), 1.93 (s, 9 H, GlcNAc-NHC*H*$_3$), 1.85 (s, 3 H, GalNAc-NHC*H*$_3$); ^{13}C NMR (150 MHz, D$_2$O; data for the skeletal carbon atoms are summarized in Table S-2) δ 128.0, 127.6, 125.0, 120.1 (Ar), 69.0 (Fmoc-*C*H$_2$), 66.4 (Ser-β*C*), 56.2 (Fmoc-*C*H), 47.0 (Ser-α*C*), 22.2 (GalNAc-NH*C*H$_3$, GlcNAc-NH*C*H$_3$); MALDI-TOFMS (*m/z*) calcd for $C_{74}H_{109}N_5O_{45}Na$ [M + Na]$^+$ 1810.63, found 1810.62; HR-ESIMS (*m/z*) calcd for $C_{74}H_{108}N_5O_{45}$ [M − H]$^-$ 1786.6316, found 1786.6304.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-{*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (6).** Compound **5** (757 μg, 423 nmol) was treated with a mixture of 15 mU of β1,3GlcNAcT and UDP-GlcNAc (1.38 mg, 2.12 μmol) in a total volume of 500 μL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM MnCl$_2$, 10 mM MgCl$_2$, 0.1 mg/mL of BSA, and 100 mU/mL of calf intestine alkaline phosphatase. After incubation for 24 h at 20 °C, the reaction mixture was centrifuged, and the filtrate was lyophilized. The crude material was directly dissolved in a solution of 100 mU of β1,4GalT and UDP-Gal (1.29 mg, 2.12 μmol) in a total volume of 500 μL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM MnCl$_2$. After incubation for 24 h at 20 °C, the reaction mixture was subjected to the purification with analytical reversed-phase HPLC to give compound **6** (328 μg, 152 nmol, 36%): RP-HPLC retention time = 19.5 min; MALDI-TOFMS (*m/z*) calcd for $C_{74}H_{109}N_5O_{45}Na$ [M + Na]$^+$ 2175.76, found 2175.70.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-{2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (8).** Compound **7** (1.9 mg, 1.1 μmol) was modified by using 120 mU of β1,3GlcNAcT and UDP-GlcNAc (7.2 mg, 11.1 μmol) in a total volume of 2.0 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM MnCl$_2$, 10 mM MgCl$_2$, 0.1 mg/mL of BSA, and 100 mU/mL of calf intestine alkaline phosphatase. After incubation for 24 h at 20 °C, the reaction mixture was purified by analytical reversed-phase HPLC to give compound **8** (2.0 mg, 0.95 μmol, 86%): RP-HPLC retention time = 18.4 min; ^{1}H NMR (600 MHz, D$_2$O; data for the ring and the exocyclic protons are shown in Table S-1) δ 7.83−7.35 (m, 8 H, Ar*H*), 4.54 (m, 1 H, Ser-β*H*), 4.41 (m, 1 H, Ser-β*H*), 4.29 (m, 1 H, Ser-α*H*), 4.04 (m, 1 H, Fmoc-C*H*), 3.68 (m, 2 H, Fmoc-C*H*$_2$), 1.93 (s, 15 H, GlcNAc-NHC*H*$_3$), 1.85 (s, 3 H, GalNAc-NHC*H*$_3$); ^{13}C NMR (150 MHz, D$_2$O; data for the skeletal carbon atoms are shown in Table S-2) δ 128.1, 127.6, 125.1, 120.2 (Ar), 69.0 (Fmoc-*C*H$_2$), 66.4 (Ser-β*C*), 56.2 (Fmoc-*C*H), 47.0 (Ser-α*C*), 22.1 (GalNAc-NH*C*H$_3$, GlcNAc-NH*C*H$_3$); MALDI-TOFMS (*m/z*) calcd for $C_{90}H_{135}N_7O_{55}Na$ [M + Na]$^+$ 2216.79, found 2216.75; HR-ESIMS (*m/z*) calcd for $C_{90}H_{134}N_7O_{55}$ [M − H]$^-$ 2192.7904, found 2192.7930.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-{*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-serine (9).** Compound **8** (1.3 mg, 0.59 μmol) was treated with a mixture of 200 mU of β1,4GalT and UDP-Gal (3.6 mg, 5.9 μmol) in a total volume of 1.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM MnCl$_2$. After incubation for 24 h at 20 °C, the reaction mixture was purified by analytical reversed-phase HPLC to give compound **9** (1.3 mg, 0.53 μmol, 90%): RP-HPLC retention time = 17.9 min; ^{1}H NMR (600 MHz, D$_2$O; data for the ring and the exocyclic protons are shown in Table S-1) δ 7.83−7.34 (m, 8 H, Ar*H*), 4.59 (m, 1 H, Ser-β*H*), 4.54 (m, 1 H, Ser-β*H*), 4.29 (m, 1 H, Ser-α*H*), 4.27 (m, 1 H, Fmoc-C*H*), 3.72 (m, 2 H, Fmoc-C*H*$_2$), 1.93 (s, 15 H, GlcNAc-NHC*H*$_3$), 1.84 (s, 3 H, GalNAc-NHC*H*$_3$); ^{13}C NMR (150 MHz, D$_2$O; data for the skeletal carbon atoms are shown in Table S-2) δ 128.0, 127.6, 125.0, 120.2 (Ar), 67.7 (Fmoc-*C*H$_2$), 66.3 (Ser-β*C*), 54.4 (Fmoc-*C*H), 47.1 (Ser-α*C*), and 22.2 (GalNAc-NH*C*H$_3$, GlcNAc-NH*C*H$_3$); MALDI-TOF MS (*m/z*) calcd for $C_{102}H_{155}N_7O_{65}Na$ [M + Na]$^+$ 2540.89, found 2540.90; HR-ESI MS (*m/z*) calcd for $C_{102}H_{154}N_7O_{65}$ [M − H]$^-$ 2516.8960, found 2516.8992.

***O*-{2-Acetamido-*O*-[2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)]-2-deoxy-*O*-[*β*-D-galactopyranosyl-(1 → 3)]-α-D-galactopyranosyl}- N^α-(fluoren-9-ylmethoxycarbonyl)-L-threonine *tert*-butyl ester (13).** Compound **12** (67.6 mg, 70 μmol) was dissolved in a solution of 10 mM sodium hydroxide in methanol (14 mL), and the mixture was stirred for 1.5 h at room temperature. After neutralization with acetic acid, the solution was concentrated in vacuo. To a solution of the residue and NaHCO$_3$ (11.8 mg, 140 μmol) in 60% aqueous acetone (2.8 mL) was added a solution of 9-fluorenylmethyl-*N*-succimidylcarbonate (35.4 mg, 105 μmol) in acetone (4.2 mL). The mixture was stirred at room temperature for 2 h and evaporated in vacuo. The residual syrup was subjected to the chromatography with a preparative-scale reversed-phase HPLC to give **13** (45.1 mg, 47 μmol, 67%): ^{1}H NMR (600 MHz, DMSO-*d*$_6$; data for the ring and the exocyclic protons are shown in Table S-3) δ 7.91−7.33 (m, 8 H, Ar*H*), 4.48, 4.45 (m, 2 H, Fmoc-C*H*$_2$), 4.31 (m, 1 H, Fmoc-C*H*), 4.20 (m, 1 H, Thr-β*H*), 4.06 (m, 1 H,

Thr-α*H*), 1.83 (s, 6 H, NH*Ac*), 1.36 (s, 3 H, O*^t^Bu*), and 1.12 (d, 3 H, Thr-δ*H*); HR-ESIMS (*m/z*) calcd for $C_{45}H_{63}N_3O_{20}Na$ [M + Na]$^+$ 988.3903, found 988.3890.

***O*-{2-Acetamido-*O*-[*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)]-2-deoxy-*O*-[*β*-D-galactopyranosyl-(1 → 3)]-α-D-galactopyranosyl}- *N*^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine *tert*-butyl ester (14).** Compound **13** (45.1 mg, 46.7 μmol) was subjected to the treatment with 4.7 U of β1,4GalT and UDP-Gal (57 mg, 93.4 μmol) in a total volume of 23.4 mL of 50 mM HEPES buffer, pH 7.0, 10 mM $MnCl_2$, 0.1% NaN_3, 0.1 mg/mL of BSA, and 10 mM 2,6-di-*O*-methyl-β-cyclodextrin. After incubation for 15 h at 25 °C, the reaction mixture was purified by a preparative reversed-phase HPLC to give compound **14** (45.6 mg, 40.4 μmol, 87%): ^{1}H NMR (600 MHz, DMSO-d_6; data for the ring and the exocyclic protons are shown in Table S-3) δ 7.91−7.44 (m, 8 H, Ar*H*), 4.47, 4.45 (m, 2 H, Fmoc-C*H*$_2$), 4.31 (m, 1 H, Fmoc-C*H*), 4.20 (m, 1 H, Thr-β*H*), 4.05 (m, 1 H, Thr-α*H*), 1.82 (s, 6 H, NH*Ac*), 1.35 (s, 3 H, O*^t^Bu*), 1.12 (d, 3 H, Thr-δ*H*); HR-ESIMS (*m/z*) calcd for $C_{51}H_{73}N_3O_{25}Na$ [M + Na]$^+$ 1150.4431, found 1150.4440.

***O*-{2-Acetamido-*O*-[2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)]-2-deoxy-*O*-[*β*-D-galactopyranosyl-(1 → 3)]-α-D-galactopyranosyl}- *N*^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine *tert*-butyl ester (15).** Compound **14** (45.6 mg, 40.4 μmol) was treated by a mixture of 606 mU of β1,3GlcNAcT and UDP-GlcNAc (65.8 mg, 101 μmol) in a total volume of 20.2 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mg/mL of BSA, 0.1% NaN_3, 0.2% Triton X-100, and 100 mU/mL of calf intestine alkaline phosphatase. After incubation for 48 h at 25 °C, the reaction mixture was subjected to the purification by a preparative reversed-phase HPLC to give **15** (38.7 mg, 29.1 μmol, 72%): ^{1}H NMR (600 MHz, DMSO-d_6; data for the ring and the exocyclic protons are shown in Table S-3) δ 7.90−7.32 (m, 8 H, Ar*H*), 4.47, 4.45 (m, 2 H, Fmoc-C*H*$_2$), 4.30 (m, 1 H, Fmoc-C*H*), 4.20 (m, 1 H, Thr-β*H*), 4.05 (m, 1 H, Thr-α*H*), 1.82 (s, 6 H, NH*Ac*), 1.36 (s, 3 H, O*^t^Bu*), 1.12 (d, 3 H, Thr-δ*H*); HR-ESIMS (*m/z*) calcd for $C_{59}H_{86}N_4O_{30}Na$ [M + Na]$^+$ 1353.5223, found 1353.5199.

***O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1 → 3)]- 4-*O*-acetyl-α-D-galactopyranosyl}-*N*^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine *tert*-butyl ester (16).** Compound **15** (38.7 mg, 29.1 μmol) was dissolved and stirred in a solution of Ac_2O/pyridine/AcOH (5:3:2, v/v/v, 29.1 mL) for 19 h at room temperature. The reaction mixture was evaporated, and the residual syrup was subjected to the purification by a preparative reversed-phase HPLC to give **16** (51.6 mg, 27.5 μmol, 94%): ^{1}H NMR (600 MHz, $CDCl_3$; data for the ring and the exocyclic protons are shown in Table S-3) δ 7.79−7.26 (m, 8 H, Ar*H*), 4.57, 4.45 (m, 2 H, Fmoc-C*H*$_2$), 4.26 (m, 1 H, Fmoc-C*H*), 4.19 (m, 1 H, Thr-α*H*), 3.96 (m, 1 H, Thr-β*H*), 2.15−1.93 (s, 48 H, *Ac*), 1.27 (s, 3 H, O*^t^Bu*), 1.12 (d, 3 H, Thr-δ*H*); HR-ESIMS (*m/z*) calcd for $C_{85}H_{112}N_4O_{43}Na$ [M + Na]$^+$ 1899.6598, found 1899.6631.

***O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1 → 3)]- 4-*O*-acetyl-α-D-galactopyranosyl}-*N*^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine (11).** Compound **16** (51.6 mg, 27.5 μmol) was treated with 95% aqueous trifluoroacetic acid (10 mL) for 1 h at room temperature. The reaction mixture was concentrated by streaming nitrogen gas, and the residue was subjected to the purification by a preparative reversed-phase HPLC to give **11** (39.2 mg, 21.5 μmol, 78%): ^{1}H NMR (600 MHz, CD_3OD; data for the ring and the exocyclic protons are shown in Table S-3) δ 7.84−7.33 (m, 8 H, Ar*H*), 4.59, 4.52 (m, 2 H, Fmoc-C*H*$_2$), 4.29 (m, 1 H, Fmoc-C*H*), 4.34 (m, 1 H, Thr-α*H*), 3.94 (m, 1 H, Thr-β*H*), 2.12−1.86 (s, 48 H, *Ac*), 1.22 (d, 3 H, Thr-δ*H*); HR-ESIMS (*m/z*) calcd for $C_{81}H_{104}N_4O_{43}Na$ [M + Na]$^+$ 1843.5972, found 1843.5978.

L-Alanyl-L-histidyl-L-glycyl-L-valyl-*O*-{2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-*O*-{2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-galactopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-α-D-galactopyranosyl}-L-threonyl-L-arginylamide (19). Solid-phase (glyco)peptide synthesis was manually performed on commercial Rink amide PEGA resin (0.05 mmol/g of wet resin, 276 mg, 13.8 μmol) using the microwave-assisted protocol as described previously.[11] Microwave-assisted glycopeptide synthesis was performed on a Green Motif I microwave synthesis reactor (IDX Corp., Tochigi, Japan). Sealed reaction vessel was placed inside the cavity, and the single mode microwave was irradiated at 2450 MHz using temperature control at 50 °C. The temperature of the reaction mixture was directly monitored by means of a metal-sheathed thermocouple wire. It usually took about 1 min to raise the temperature from room temperature to 50 °C. The resin was agitated with 20% piperidine/DMF (2 mL) for 3 min at 50 °C under microwave irradiation (0−40 W). After deprotection of the Fmoc group, the resin was filtered and was washed five times with DMF (3 mL). The resin was then stirred with an appropriate Fmoc amino acid (3 equiv, 138 μmol) except for building blocks **10** and **11**, 2-2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3 equiv, 138 μmol), *N*-hydroxybenzotriazole (HOBt) (3 equiv, 138 μmol), and diisopropylethylamine (DIEA) (6 equiv, 276 μmol) in DMF (700 μL). The coupling reaction was performed at 50 °C under microwave irradiation (0−40 W) for 10 min. The unreacted amino groups on the resin were acetylated by treating with a solution of 13 mM HOBt in 4.75% Ac_2O and 2.25% DIEA in DMF for 5 min at room temperature. After completion of the coupling reaction, the resin was filtered and washed in the same manner as described above. In the case of coupling reaction by means of **10** and **11** instead of common Fmoc amino acids, the coupling reaction was performed by using the solution of **10** (1.5 equiv, 83.7 μmol) or **11** (1.5 equiv, 83.7 μmol) in HBTU (1.5 equiv, 20.6 μmol), HOBt (1.5 equiv, 20.6 μmol), and DIEA (3.0 equiv, 41.3 μmol) in DMF (350 μL) at 50 °C under microwave irradiation (0−40 W) for 20 min. After completion of the synthesis, the resin was washed five times with DMF (3 mL) and CH_2Cl_2 (3 mL) and then treated with a solution of trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5, v/v/v, 3.5 mL) for 2 h at room temperature to release the intermediate **18** from the resin. The cleavage solution was filtered, and the resin was washed twice with the same cleavage cocktail (3 mL). These filtrates were combined and concentrated by streaming nitrogen gas, and then the resulting solution was precipitated from *tert*-butylmethyl ether to give crude product. The crude product was roughly purified by a semipreparative reversed-phase HPLC. Then, crude material was dissolved and stirred with 10 mM NaOH in MeOH (10 mL) for 1.5 h at room temperature. After neutralization with acetic acid, the reaction mixture was concentrated by streaming nitrogen gas. Final purification was carried out by a semipreparative reversed-phase HPLC to give **19** (1.90 mg, 0.73 μmol, overall yield 5.3%): ^{1}H NMR [(600 MHz, D_2O) and ^{13}C NMR (150 MHz, D_2O) data are summarized in Table S-4 and Figures S-21, S-22, and S-23 in Supporting Information; amino acid analysis (numbers in parentheses are theoretical values] Ala 1.9 (2), Arg 0.9 (1), Asp 1.0 (1), Gly 1.0 (1), His 0.9 (1), Pro 1.0 (1), Ser 0.9 (1), Thr 1.8 (2), Val 0.9 (1); HR-ESIMS (*m/z*) calcd for $C_{103}H_{169}N_{22}O_{56}$ [M − H]$^-$ 2610.1053, found 2610.1057.

Glycosphingolipid Mimic Polymer 21. Compound **20** (10.5 mg, 4.2 μmol of lactose moiety) was treated by a mixture of 30 mU of β1,3GlcNAcT and UDP-GlcNAc (13.8 mg, 21.1 μmol) in a total

volume of 1.0 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM $MnCl_2$, and 10 mM $MgCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was concentrated by a centrifugal UF unit (30 K Millipore, 500 μL). The retentate was washed with H_2O and lyophilized to give compound **21** (9.2 mg, 100%; conversion yield estimated from the integration data of the ^{1}H NMR spectrum): ^{1}H NMR (600 MHz, D_2O; data for the ring and the exocyclic protons are shown in Table S-5) δ 4.42 (m, 1 H, Ser-α*H*), 4.10 (m, 1 H, Ser-βH), 3.78 (m, 1 H, Ser-β*H*), 3.15−3.02 (m, 4 H, NHCH_2 × 2), 2.26 (m, 2 H, NHCOCH_2), 2.25−2.09 (m, 26 H, C*H*), 1.94 (s, 3 H, NHCH_3), 1.67−1.18 (m, 70 H, CH_2), 0.77 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, D_2O; data for the skeletal carbon atoms are shown in Table S-6) δ 68.5 (Ser-βC), 53.8 (Ser-αC), 41.8, 41.5, 41.3 (*C*H), 39.1 (NH*C*H_2), 35.8, 35.1 (*C*H_2), 35.0 (NHCO*C*H_2), 34.9, 34.6, 34.5, 33.9, 30.9, 28.1, 27.7, 25.6, 24.6, 21.9 (*C*H_2), 21.8 (NH*C*H_3), and 13.1 (*C*H_3).

Sphingoglycolipid Mimic Polymer 22. Compound **21** (8.2 mg, 3.1 μmol of GlcNAc-Lac residue) was treated by a mixture of 200 mU of β1,4GalT and UDP-Gal (9.3 mg, 15.2 μmol) in a total volume of 1.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM $MnCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was concentrated by a centrifugal UF unit (30 K, 500 μL). The retentate was washed with H_2O and lyophilized to give compound **22** (7.9 mg, 100%; conversion yield estimated from the integration data of the ^{1}H NMR spectrum): ^{1}H NMR (600 MHz, D_2O; data for the ring and the exocyclic protons are shown in Table S-5) δ 4.41 (m, 1 H, Ser-α*H*), 4.09 (m, 1 H, Ser-β*H*), 3.78 (m, 1 H, Ser-β*H*), 3.16−3.02 (m, 4 H, NHCH_2 × 2), 2.25 (m, 2 H, NHCOCH_2), 2.24−2.08 (m, 26 H, C*H*), 1.94 (s, 3 H, NHCH_3), 1.67−1.18 (m, 70 H, CH_2), 0.77 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, D_2O; data for the skeletal carbon atoms are shown in Table S-6) δ 68.5 (Ser-β*C*), 53.8 (Ser-α*C*), 41.9, 41.5, 41.3 (*C*H), 39.1 (NH*C*H_2), 35.9, 35.6 (*C*H_2), 35.0 (NHCO*C*H_2), 34.7, 34.6, 34.5, 33.6, 30.9, 28.2, 27.9, 25.7, 25.6, 24.7, 21.9 (*C*H_2), 21.9 (NH*C*H_3), and 13.2 (*C*H_3).

Glycosphingolipid Mimic Polymer 23. Compound **21** (3.9 mg, 1.4 μmol of LacNAc-Lac) was treated by a mixture of 30 mU of β1,3GlcNAcT and UDP-GlcNAc (4.5 mg, 6.9 μmol) in a total volume of 1.0 mL of 100 mM glycine/NaOH buffer, pH 10.0, 10 mM $MnCl_2$, and 10 mM $MgCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was concentrated by a centrifugal UF unit (30 K, 500 μL). The retentate was washed with H_2O and lyophilized to give compound **23** (4.3 mg, 90%; conversion yield estimated from the integration data of the ^{1}H NMR spectrum): ^{1}H NMR (600 MHz, D_2O; data for the ring and the exocyclic protons are shown in Table S-5) δ 4.41 (m, 1 H, Ser-α*H*), 4.10 (m, 1 H, Ser-β*H*), 3.78 (m, 1 H, Ser-β*H*), 3.15−3.02 (m, 4 H, NHCH_2 × 2), 2.25 (m, 2 H, NHCOCH_2), 2.24−2.09 (m, 26 H, C*H*), 1.94 (s, 3 H, NHCH_3), 1.68−1.17 (m, 70 H, CH_2), 0.77 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, D_2O; data for the skeletal carbon atoms are shown in Table S-6) δ 68.5 (Ser-β*C*), 53.8 (Ser-α*C*), 41.9, 41.5, 41.3 (*C*H), 39.1 (NH*C*H_2), 36.0, 35.7 (*C*H_2), 35.1 (NHCO*C*H_2), 34.9, 34.7, 34.6, 33.7, 31.0, 28.3, 28.1, 25.8, 25.7, 24.8, 21.9 (*C*H_2), 22.0 (NH*C*H_3), and 13.4 (*C*H_3).

Glycosphingolipid Mimic Polymer 24. Compound **23** (1.3 mg, 0.43 μmol of GlcNAc-LacNAc-Lac moiety) was modified by using 200 mU of β1,4GalT and UDP-Gal (1.3 mg, 2.1 μmol) in a total volume of 1.0 mL of 50 mM HEPES/NaOH buffer, pH 7.0, and 10 mM $MnCl_2$. After incubation for 24 h at 20 °C, the reaction mixture was concentrated by a centrifugal UF unit (30 K, 500 μL). The retentate was washed with H_2O and lyophilized to give compound **24** (1.2 mg, 100%; conversion yield estimated from the integration data of the ^{1}H NMR spectrum): ^{1}H NMR (600 MHz, D_2O; data for the ring and the exocyclic protons are shown in Table S-5) δ 4.41 (m, 1 H, Ser-α*H*), 4.09 (m, 1 H, Ser-β*H*), 3.78 (m, 1 H, Ser-β*H*), 3.16−3.03 (m, 4 H, NHCH_2 × 2), 2.25 (m, 2 H, NHCOCH_2), 2.24−2.09 (m, 26 H, C*H*), 1.94 (s, 3H, NHCH_3), 1.68−1.17 (m, 70H, CH_2), 0.77 (s, 3H, CH_3); ^{13}C NMR (150 MHz, D_2O; data for the skeletal carbon atoms are shown in Table S-6) δ 68.5 (Ser-βC), 53.8 (Ser-αC), 41.9, 41.6, 41.3 (*C*H), 39.2 (NH*C*H_2), 35.9, 35.6 (*C*H_2), 35.0 (NHCO*C*H_2), 34.7, 34.5, 33.6, 30.9, 28.1, 27.8, 25.6, 24.7, 21.9 (*C*H_2), 21.9 (NH*C*H_3), and 13.3 (*C*H_3). HSQC spectrum of compound **24** is shown in Figure S-28.

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Supporting Information Available: HPLC and MALDI-TOF MS data for the characterization of LgtA. MALDI-TOF/TOFMS of compounds **1a**, **3**, **4**, **6**, and **8** and tables for the ^{1}H NMR spectra of compounds **4**, **5**, **7**−**9**, **11**, **13**−**16**, **19**, and **21**−**24**, and ^{13}C NMR spectra of compounds **4**, **5**, **7**−**9**, **19**, and **21**−**24**. HSQC spectra of compounds **5**, **9**, **19**, and **24**. NOESY spectra of compound **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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