An Engineered Biocatalyst for the Synthesis of Glycoconjugates: Utilization of β1,3-*N*-Acetyl-D-glucosaminyltransferase from *Streptococcus agalactiae* Type Ia Expressed in *Escherichia coli* as a Fusion with Maltose-Binding Protein

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Abstract: A fusion protein composed of β1,3-*N*-acetyl-D-glucosaminyltransferase (β1,3-GlcNAcT) from *Streptococcus agalactiae* type Ia and maltose-binding protein (MBP) was produced in *Escherichia coli* as a soluble and highly active form. Although this fusion protein (MBP-β1,3-GlcNAcT) did not show any sugar-elongation activity to some simple low-molecular weight acceptor substrates such as galactose, Galβ(1→4)Glc (lactose), Galβ(1→4)GlcNAc (*N*-acetyllactosamine), Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc (lacto-*N*-tetraose), and Galβ(1→4)GlcβCer (lactosylceramide, LacCer), the multivalent glycopolymer having LacCer-mimic branches (LacCer mimic polymer, LacCer primer) was found to be an excellent acceptor substrate for the introduction of a β-GlcNAc residue at the O-3 position of the non-reducing galactose moiety by this engineered enzyme. Subsequently, the polymer having GlcNAcβ(1→3)Galβ(1→4)Glc was subjected to further enzymatic modifications by using recombinant β1,4-D-galactosyltransferase (β1,4-GalT), α2,3-sialyltransferase (α2,3-SiaT), α1,3-L-fucosyltransferase (α1,3-FucT), and ceramide glycanase (CGase) to afford a biologically important ganglioside; Neu5Aα(2→3)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→3)Galβ(1→4)GlcCerα(IV3Neu5Acα,III3Fucα-nLc4Cer) in 40% yield (4 steps). Interestingly, it was suggested that MBP-β1,3-GlcNAcT could also catalyze a glycosylation reaction of the LacCer mimic polymer with *N*-acetyl-D-galactosamine served from UDP-GalNAc to afford a polymer carrying trisaccharide branches, GalNAcβ(1→3)Galβ(1→4)Glc. The versatility of the MBP-β1,3-GlcNAcT in the practical synthesis was preliminarily demonstrated by applying this fusion protein as an immobilized biocatalyst displayed on the amylose resin which is known as a solid support showing potent binding-affinity with MBP.

Keywords: β1,3-*N*-acetyl-D-glucosaminyltransferase; carbohydrate synthesis; cluster effect; immobilized glycosyltransferase; maltose-binding protein; multivalency.

Introduction

The emerging understanding of the critical structural and functional roles of glycoconjugates in cellular biology and the promise of therapeutics based on carbohydrate-related compounds create an urgent need for an efficient synthetic methodology for these biomolecules.[1–3] Although the progress made in the carbohydrate chemistry in recent years has been remarkable, chemical synthesis of oligosaccharides still involves difficult and time-consuming processes. These problems are mainly due to the requirement of multistep transformations of sugar intermediates involving iterative "protection → glycosylation → deprotection" reactions and tedious purification procedures of the products. Enzyme-assisted synthesis of carbohydrates is a potentially promising alternative method to conventional chemical synthesis because biocatalysts have materialized highly stereo- and regioselective glycosylation reactions without any protective groups under aqueous and mild conditions.[4–6] Recently, a large number of eukaryotic glycosyltransferases has been cloned and some of their recombinant forms have been produced.[7] However, the number of recombinant glycosyltransferases is still limited and they are too expensive and unstable to be used as versatile reagents for practical oligosaccharide synthesis.

In the course of our studies on "multivalency" in carbohydrate-protein interactions,[8–10] it has been generally accepted that the drastically enhanced affinity of multiple sugar derivatives supported on the water-soluble polymers with recombinant glycosyltransferases can be applied for the efficient and practical synthesis of glycoconjugates.[11–17] Although some of the eukaryotic glycosyltransferases such as β1,4-GalT, α2,3-SiaT, α2,6-SiaT, and α1,3-FucT have been produced and employed for synthetic studies on some sphingoglycolipids[14,16] or glycopeptides,[18,19] a family of *N*-acetyl-D-glucosaminyltransferases (GlcNAcTs) has also been regarded as one of the most important classes of glycosyltransferases in the biosynthetic processes of a variety of glycoconjugates.[20] In the present paper, our attention is directed toward expression and utilization of a β1,3-GlcNAcT that catalyzes glycoside bond formation with D-galactose residues found abundantly in glycolipids and glycoproteins. Since GlcNAcβ(1→3)Gal disaccharide is an essential structure for constructing functional

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oligosaccharides involving poly-*N*-acetyllactosamine as well as sphingoglycolipids containing lacto and lactoneo series sequences,[21–23] the β1,3-GlcNAcT might become a key synthetic tool to construct glycoconjugates involving this abundant disaccharide sequence. Although some mammalian β1,3-GlcNAcT have been characterized to show highly limited substrate specificity,[24,25] we considered that use of bacterial glycosyltransferases having a broad acceptor specificity might be suited for the construction of carbohydrate libraries in a combinatorial manner. Actually, it has been reported that β1,3-GlcNAcT of *Neisseria meningitidis*,[26] α2,3-siaT of *N. meningitidis*[27] and α2,6-siaT of *Photobacterium damsela* JT0160[28] exhibit much broader substrate specificity towards glycosyl acceptors than those of mammalian glycosyltransferases. Since it was suggested that capsular polysaccharides synthesized by *Streptococcus agalactiae* type Ia also contain an interesting GlcNAcβ(1 → 3)Galβ(1 → 4)Glc motif and the genetic structure that encodes a specific β1,3-GlcNAcT has been reported,[29] we aimed to produce this novel glycosyltransferase in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP)[30] that would greatly contribute to the development of a highly practical "enzyme-based" synthesis of glycoconjugates.[1]

Results and Discussion

A Fusion Protein as a Designed Biocatalyst: MBP-β1,3-GlcNAcT

The basic concept for the use of glycosyltransferases (GTs) as fusion proteins with maltose-binding protein (MBP) is summarized in Figure 1. Although MBP has been used basically for the purpose of the production of fusion proteins as soluble forms,[24] it should be emphasized in the present study that the merits of this strategy lie in the functional roles of the MBP domain as "a specific tag" for both purification and immobilization of this engineered biocatalyst as well as solubilization. Here, we considered that the specific affinity of carbohydrate binding proteins with carbohydrates could be utilized for the development of a novel and practical method to achieve immobilized enzymes without any tedious procedures based on the chemical cross-linkings with cyanogen bromide or some activating reagents.

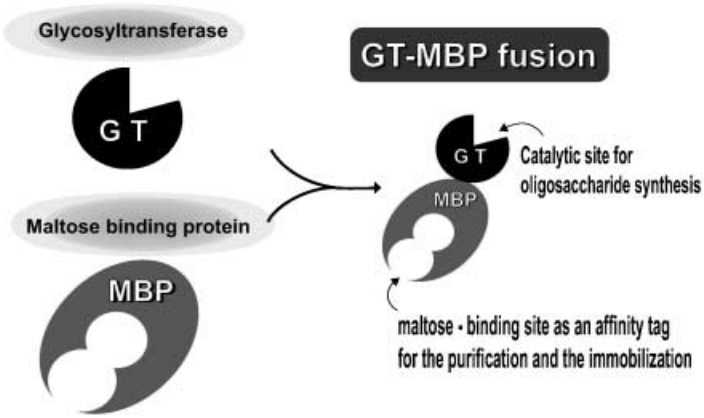


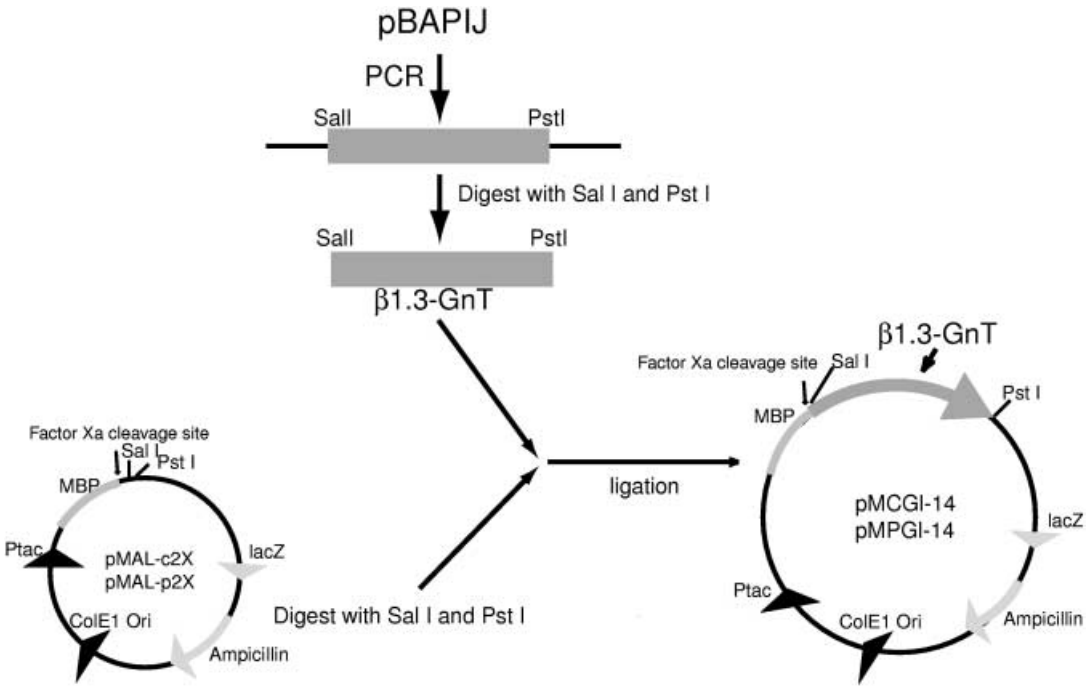
Figure 1. A concept of the engineered glycosyltransferases: A fusion protein of glycosyltransferase with maltose-binding protein is designed.

The nucleotide sequence of the *S. agalactiae cpsIaI* gene contains an open reading frame encoding a protein of 333 amino acids with a calculated molecular mass (*Mw* = 38,534). Since the DNA sequence of this protein involves two nucleotide sequences corresponding to hydrophobic segments near the *N* terminus of this enzyme, we have subcloned the *cpsIaI* gene of *S. agalactiae* type Ia encoding a β1,3-GlcNAcT and expressed as an active form of β1,3-GlcNAcT without hydrophobic and putative transmembrane regions.[32] The fragment amplified from pBAPIJ by PCR was placed into pMAL-p2X or pMAL-c2X vectors encoding MBP (42 kDa). Scheme 1 indicates a procedure for the production of the expression plasmids, pMCGl-14 and pMPGl-14. Here, pMAL-p2X vectors are designated to secrete MBP fusion protein to the *E. coli* periplasm, whereas pMAL-c2X vectors lack an MBP signal peptide and express proteins in the cytoplasm. Since the efficiency of the production of the fusion protein in the cytoplasm was much higher than that in the periplasm, the expression plasmid pMCGl-14 derived from pMAL-c2X was introduced to *E. coli* BL21(DE3) cells. Although analyses by SDS-PAGE revealed that high levels of MBP-β1,3GlcNAcT were expressed by BL21 (DE3) cells harboring the *cpsIaI* gene, a large part of the products was found in the insoluble fraction of the cells when the induction was conducted at 37 °C. Fortunately, it was demonstrated by optimization of the procedure that this fusion protein can be obtained as a soluble form by lowering the incubation temperature during the induction. Figure 2 shows SDS-PAGE and Western immunoblot analysis of the soluble fractions of cells induced with or without IPTG. Consequently, the production of the MBP-β1,3-GlcNAcT was performed in the presence of IPTG after 18 h of induction at 20 °C.

The soluble fraction of the lysate of a 10-liter batch of *E. coli* BL21 (DE3) was subjected to the several procedures for the purification including affinity chromatography by using amylase resin and 11.6 mg of the pure MBP-β1,3-GlcNAcT were finally obtained. As shown in Figure 3, the subunit molecular weight of this fusion protein was found to be 80.1 kDa. The molecular weight measured by means of gel permeation chromatography was also estimated to be 80.0 kDa, indicating that MBP-β1,3-GlcNAcT exists as a monomer of the identical subunit.

Synthesis by using MBP-β1,3-GlcNAcT

Since the naturally occurring capsular polysaccharide in *S. agalactiae* type Ia contains a linear backbone of the repeating unit [→ 4)-β-D-Glc-(1 → 4)-β-D-Gal-(1 →] with trisaccharide side chains [α-NeuNAc-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 →] linked to the C-3 position of each β-D-galactose residue of the backbone structure (Figure 4),[29,33] the desirable acceptor substrates for MBP-β1,3-GlcNAcT were supposed to involve a non-reducing terminal D-galactose residue. Thus, some simple compounds and glycopolymer (LacCer mimic primer) listed in Figure 5 were tested as tentative candidates



Scheme 1. Construction of the plasmid for the expression of β1,3-GlcNAcT in *E. coli* as a fusion with MBP. The capsular polysaccharide synthesis genes of *Streptococcus agalactiae* type Ia was cloned in pBAPIJ. The pMAL-c2X lacks an MBP signal peptide and expresses MBP fusion protein in the cytoplasm. The pMAL-p2X was designed to secrete MBP fusion protein to the *E. coli* periplasm.

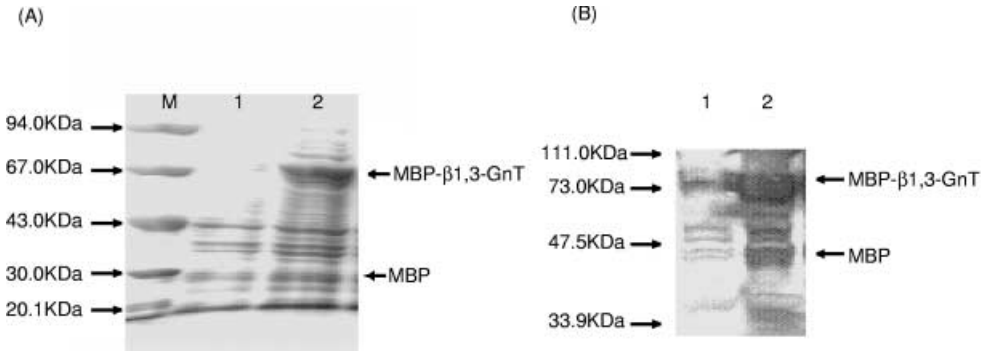


Figure 2. SDS-PAGE and Western immunoblots analysis of MBP-β1,3-GlcNAcT. (**A**) Coomassie brilliant blue detection; (**B**) immunological detection. Lane M, molecular weight markers; lane 1, soluble fraction of cells induced without IPTG; lane 2, soluble fraction of cells induced with IPTG. The arrows indicate the positions of MBP-β-1,3-GlcNAcT and MBP.

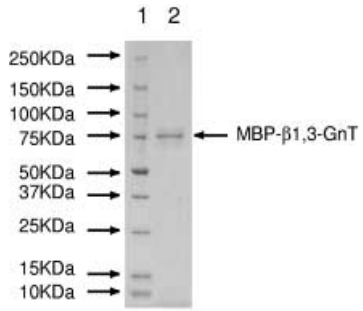


Figure 3. SDS-PAGE analysis of the purified MBP-β1,3-GlcNAcT. Lane 1, molecular weight markers; lane 2, MBP-β-1,3-GlcNAcT purified by HiPrep Sephacryl S-200 gel filtration chromatography.

for glycosyl acceptor substrates. Unexpectedly, it was suggested that MBP-β1,3-GlcNAcT did not catalyze any GlcNAc-transfer reaction to the lactose derivatives **3**, **5**, LacNAc **4**, and lacto-*N*-tetraose derivative **8** as well as D-galactose **6** which were supposed to be appropriate substrates for this enzyme. Exceptionally, a coupling reaction of the LacCer mimic primer **1** with the GlcNAc residue from UDP-GlcNAc proceeded smoothly in the presence of MBP-β1,3-GlcNAcT and the ^{1}H NMR spectrum of the product exhibited a quantitative sugar elongation reaction (Scheme 2 and Figure 6). In addition to the generation of the typical signals at $\delta = 4.63$ (H-1, GlcNAc), 3.84 (H-4, GlcNAc), and 3.72 ppm (H-3, Gal) due to the introduction of GlcNAc residue at C-3 position of the galactose residue,[26] the integration data of the CH_3 protons at $\delta = 1.96$ ppm, corresponding to the *N*-acetyl group at the C-2

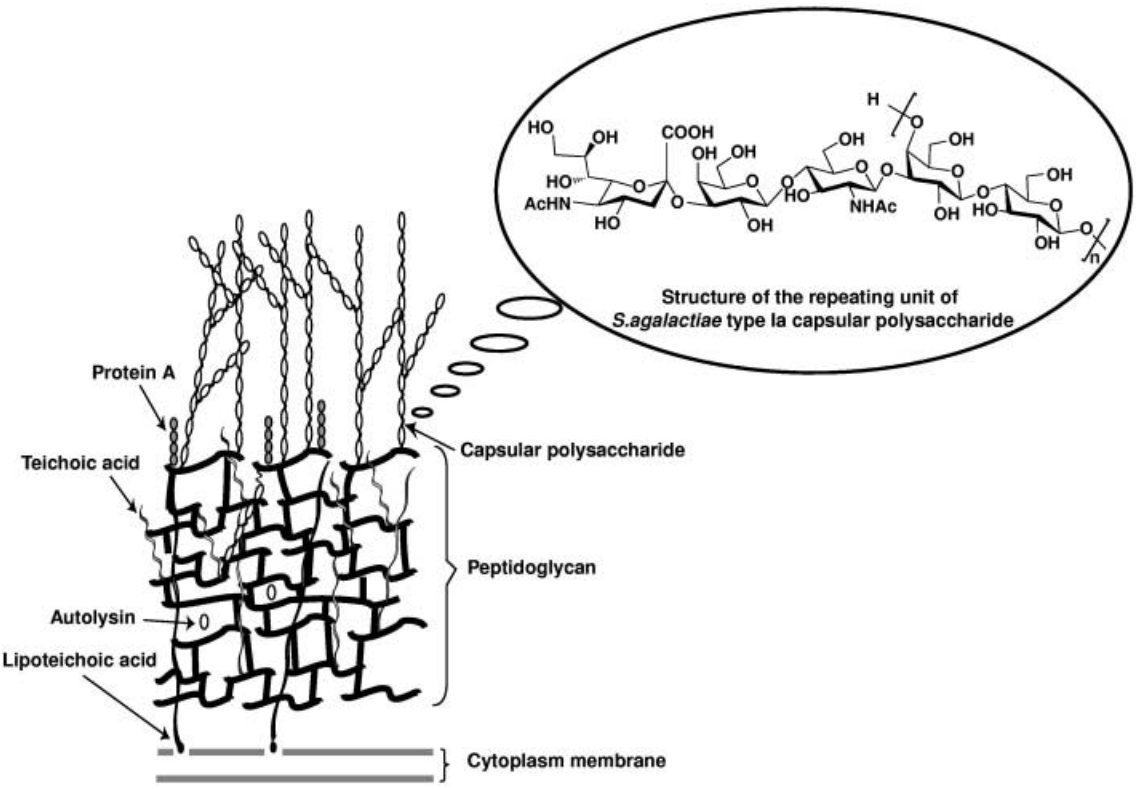


Figure 4. Proposed chemical structure of bacterial capsular polysaccharide of *Streptococcus agalactiae* type Ia.

position of the GlcNAc residue, clearly supports a successful glycosylation reaction. The signals observed in the ^{13}C NMR spectrum at $\delta = 103.5$ (C-1, Gal), 102.5 (C-1, GlcNAc), and 101.5 ppm (C-1, Glc) also support the formation of this trisaccharide sequence. This result suggests that MBP-β1,3-GlcNAcT produced by means of genetic information of a bacterial GlcNAcT, *S. agalactiae* type Ia, can catalyze glycosylation reactions of the non-reducing galactose residues of polymeric glycoconjugates as glycosyl acceptor substrates. As indicated in our recent report,[17] an enhanced affinity of multivalent LacCer branches bound to a water-soluble polymer with MBP-β1,3-GlcNAcT might be a crucial step for the efficient glycosylation reaction. Since it was revealed that β1,3-GlcNAcT found in *Neisseria meningitidis* has potent activity for transferring the GlcNAc residue to simple compounds such as galactose **6**, lactose **3**, and *N*-acetyllactosamine **4**,[26] the acceptability of MBP-β1,3-GlcNAcT toward glycosyl acceptors seems to be different from that of β1,3-GlcNAcT of *Neisseria meningitidis* in terms of the molecular size of the acceptor substrates. This result may indicate a key mechanism for the construction of the bacterial cell membranes containing a GlcNAcβ(1 → 3)Galβ(1 → 4)Glc motif. Further biochemical evaluation of this enzyme will be reported in the nearest future.

When UDP-GalNAc instead of UDP-GlcNAc was employed as a donor substrate for the reaction with LacCer primer **2** (Scheme 3), a polymer carrying GalNAcβ(1 → 3)Galβ(1 → 4)Glc branches **10** was obtained in 20% efficiency which was estimated from the integration data of the signal at $\delta = 2.04$ ppm due to the methyl protons of the *N*-acetyl group [^1H NMR (D_2O): $\delta = 4.62$ (H-1, GalNAc), 4.12 (H-4, Gal), 3.90 (H-4, Glc), 3.82 (H-3, Glc), 3.72 (H-2, Glc), 2.04 ppm ($COCH_3$, GalNAc)]. This result also indicates the versatility of MBP-β1,3-GlcNAcT in the synthesis of a variety of glycoconjugates containing the GalNAcβ(1 → 3)Gal structure as well as GlcNAcβ(1 → 3)Gal. For instance, MBP-β1,3-GlcNAcT can be applied for the synthesis of globo series sphingoglycolipids containing a GalNAcβ(1 → 3)Gal element as an indispensable core structure of these bioactive sphingoglycolipids.

The importance and versatility of the intermediate polymer carrying GlcNAcβ(1 → 3)Galβ(1 → 4)Glc branches **9** in the efficient synthesis of neolacto series of sphingoglicolipids was demonstrated by the construction of a naturally occurring sphingoglycolipid (**11**), Neu5Aα(2 → 3)Galβ(1 → 4)[Fucα (1 → 3)]GlcNAcβ(1 → 3)Galβ(1 → 4)GlcCerα(IV3Neu5Acα, III3Fucα-nLc4Cer). This sphingoglycolipid has been known as one of the most important cell adhesive gangliosides which involve the sialyl Lewis X tetrasaccharide.[18] The first total chemical synthesis was also reported by Kameyama et al.[34] As shown in Figure 7, a series of enzymatic manipulations of the intermediate **9** was carried out according to the conditions reported previously [14,19] and gave a target compound **11** in 40% overall yield [^1H NMR (D_2O): $\delta = 5.58$ (m, 1H, $J =$ 6.4 Hz, 7.4 Hz, and 15.6 Hz, H-4, Cer), 5.33 (dd, 1H, $J =$ 7.4 Hz and 15.6 Hz, H-5, Cer), 4.89 (d, $J = 3.3$ Hz, H-1, Fuc), 4.04 and 4.01 (each d, $J = 3.6$ Hz, H-4, two galactose residues) 2.74 (dd, $J = 3.4$ Hz and 12.5 Hz, H-3eq, Neu5Ac), 3.95 – 3.72 (m, H-2, H-3, and H-4, Glc), 2.08 (t, 2H, $J = 8.5$ Hz, $COCH_2$), 1.90 and 1.88 (each s, 6H, two $COCH_3$), 1.06 and 1.05 ppm (each t, 6H, $J = 3.4$ Hz and 3.6 Hz, H-6 of fucose residue and H-3ax of sialic acid residue), and 0.77 ppm (t, 6H, $J = 7.0$ Hz, $2 \times CH_3$)].

Finally, we examined the feasibility of the immobilized MBP-β1,3-GlcNAcT adsorbed on amylase resin prepared on

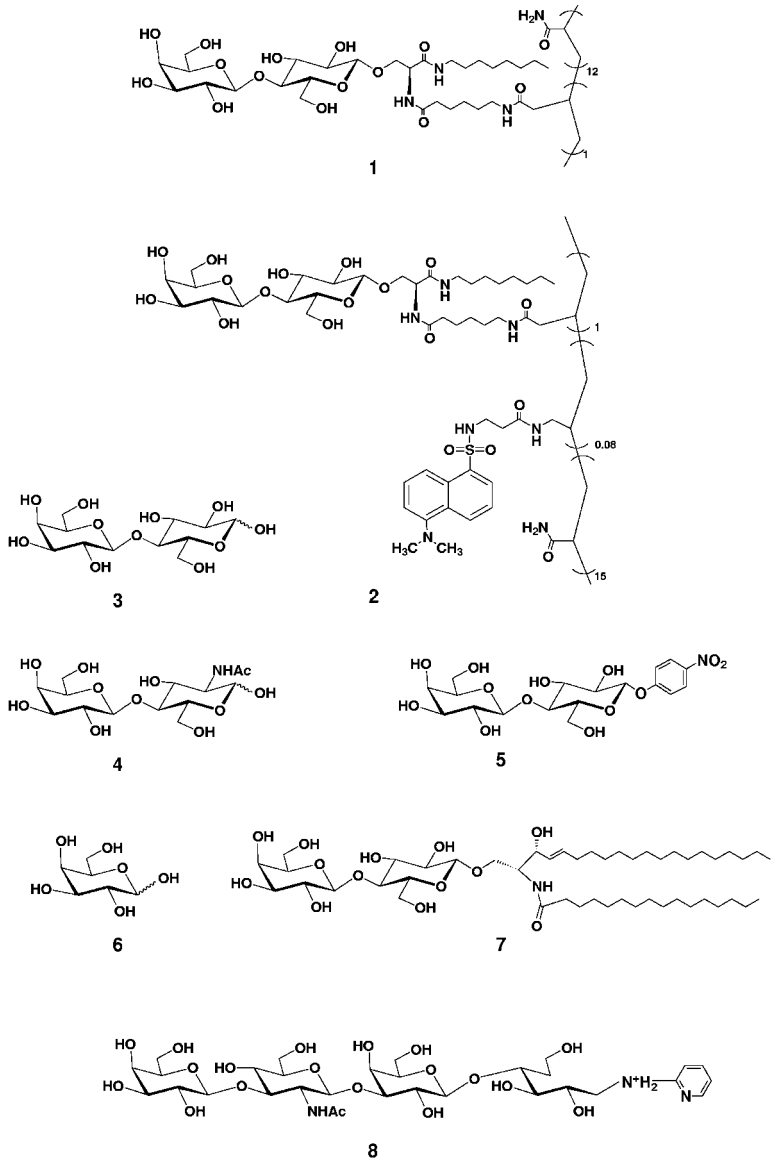
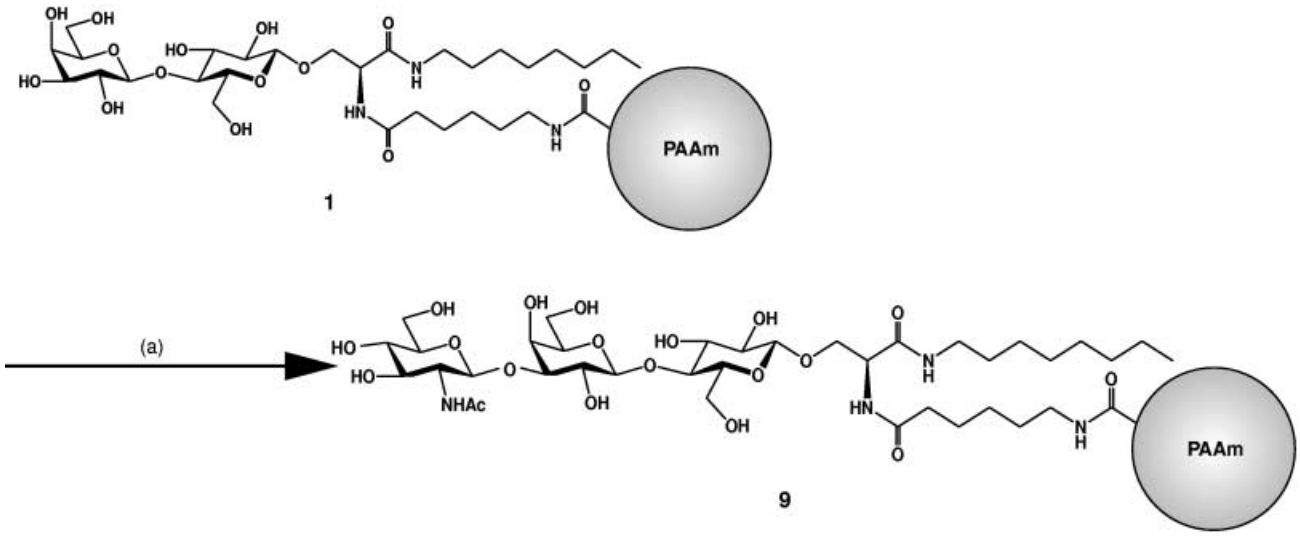


Figure 5. Compounds used in this study as glycosyl acceptor substrates.



Scheme 2. Synthetic scheme of a polymer carrying GlcNAcβ(1 → 3)Galβ(1 → 4)Glc structure by using MBP-β1,3-GlcNAcT in the presence of UDP-GlcNAc.

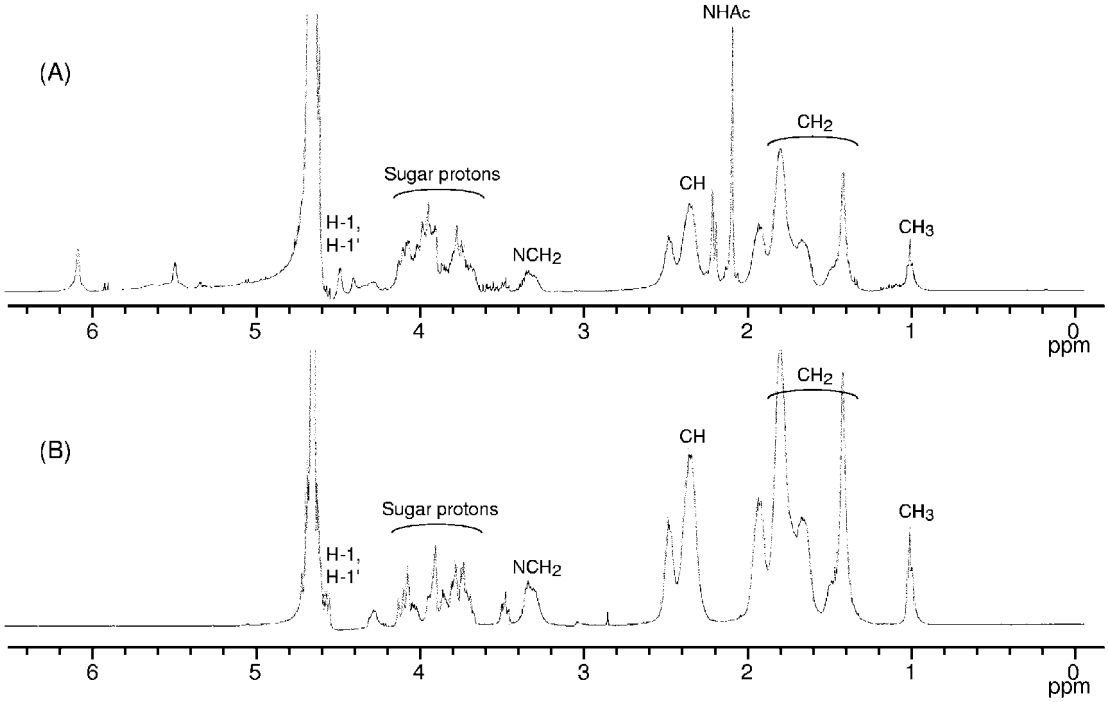
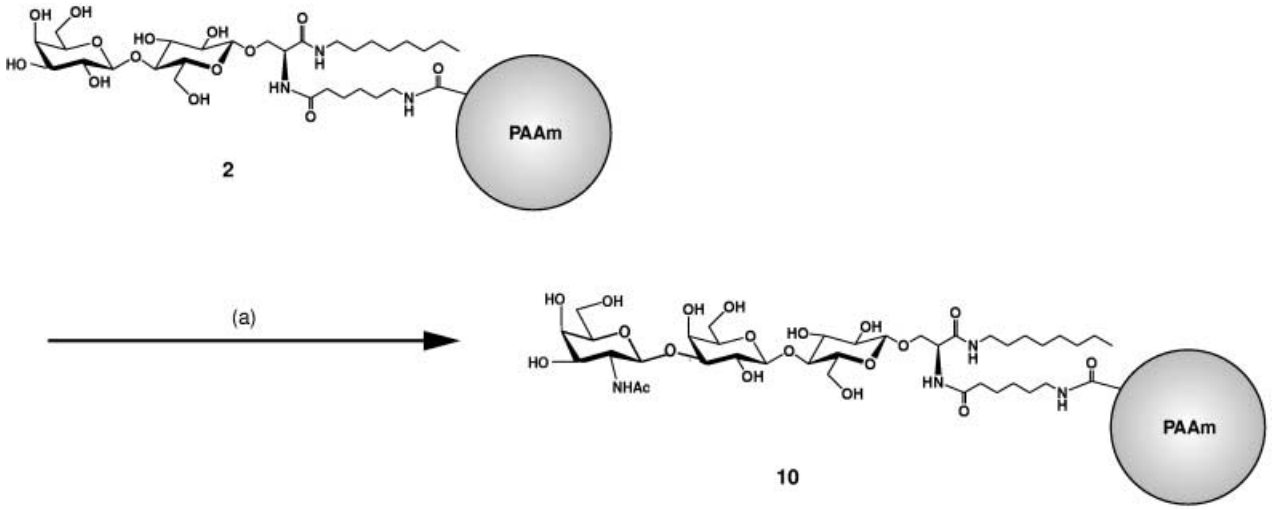


Figure 6. 400 MHz ^{1}H NMR spectra of the product after sugar elongation reaction using MBP-β1,3-GlcNAcT in the presence of UDP-GlcNAc as donor substrate at room temperature. (**A**) product; (**B**) LacCer polymer (starting material).



Scheme 3. Synthetic scheme of a polymer carrying the GalNAcβ(1 → 3)Galβ(1 → 4)Glc structure by using MBP-β1,3-GlcNAcT in the presence of UDP-GalNAc.

the basis of the specific sugar-protein interaction. As anticipated, the MBP domain of the fusion protein proved to act as an affinity tag for the immobilization of this engineered biocatalyst without any chemical treatment of cross-linking. 80% of transfer of GlcNAc residue from UDP-GlcNAc toward LacCer polymer was preliminarily achieved by using this immobilized enzyme, suggesting that an engineered biocatalyst developed here is suited for the construction of "polymer-based automated synthesis of glycoconjugates".[1]

Conclusion

In conclusion, a bacterial glycosyltransferase, *S. agalactiae* type Ia β1,3-GlcNAcT, was expressed in *E. coli* as a fusion protein with MBP. It was demonstrated that MBP-β1,3GlcNAcT exhibited specific GlcNAc (GalNAc)-transferring activity toward a polymeric glycosyl acceptor, LacCer mimic primer to give a novel sphingoglycolipid-type glycopolymer having GlcNAcβ(1 → 3)Galβ(1 → 4)Glc branches **9** that can be used for the efficient synthesis of further complicated bioactive sphingoglicolipids. The engineered biocatalyst described herein will become a key reagent for the efficient

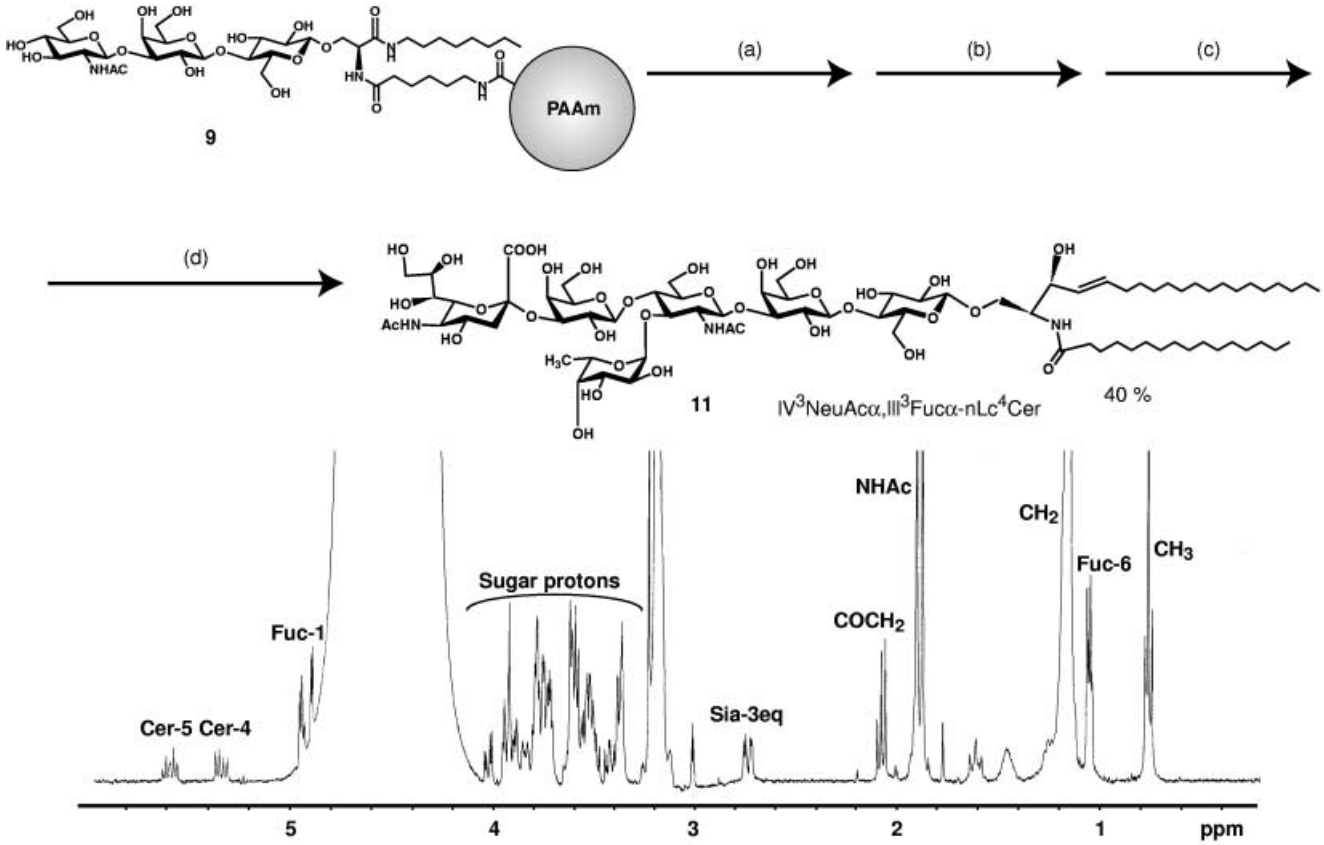


Figure 7. Synthesis of a sphingoglycolipid, Neu5Aα(2→3)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→3)Galβ(1→4)GlcCerα(IV3-Neu5Acα,III3Fucα-nLc4Cer). (**a**) Compound **9** (7 mg), β-1,4-GalT (1.0 unit), UDP-Gal disodium salt (1.2 eq, 2.60 mg), α-lactalbumin (0.26 mg), 50 mM HEPES buffer (pH 6.0), 37 °C, 24 h; (b) α-2,3-SiaT (0.03 unit), CMP-NANA disodium salt (1.4 eq, 3.27 mg), 50 mM sodium cacodylate buffer (pH 7.4), 10 mM $MnCl_2$, 1 mM NaN_3, CIAP (20 unit), Triton CF-54 (0.5% v/v), 37 °C, 72 h, (c) α-1,3-FucT (0.08 unit), GDP-Fuc disodium salt (1.4 eq, 3.14 mg), 50 mM sodium cacodylate buffer (pH 6.5), 10 mM $MnCl_2$, 1 mM NaN_3, 37 °C, 72 h; (d) ceramide glycanase (0.03 unit), ceramide (5.0 eq, 9.52 mg), 50 mM sodium citrate buffer (pH 6.0), Triton CF-54 (0.5% v/v), 37 °C, 17 h. Polymer product was isolated by gel filtration chromatography using Sephadex G-25 column in each step and crude material was employed for the next step without further purification.

synthesis of a variety of glycoconjugates based on the combined use of immobilized glycosyltransferses[17,35] and water-soluble primers.[1]

Experimental Section

General Methods and Materials

Proton and ^{13}C NMR spectra were recorded on a JEOL lambda 400 spectrometer or a Bruker ADVANCE 600 spectrometer. Analytical HPLC was performed on a Shimadzu HPLC system equipped with a LC-6A pump, SPV-6AV UV detector, reversed-phase C18 column, and Inertsil ODS-3 column at a flow rate of 0.5 mL min^{-1}. Matrix-associated laser-desorption ionization/time-of-flight/mass spectroscopy (MALDI-TOF-MS) was performed on a Bruker BIFLEX™ III. DNA sequencing was performed with an Applied Biosystems model 310 automated DNA sequencer using manufacturer's cycle sequencing kit. All *E. coli* strains used in this study were purchased from Toyobo Co. Ltd. (Tokyo, Japan). The pMAL-c2X vector was purchased from New England Biolabs Inc. (Massachusetts, USA). Plasmid pBAPIJ from *S. agalactiae* type Ia[29] was kindly provided by Dr. IIjima and Dr. Miyake of Nagoya University. Plasmids were propagated in the *E. coli* JM109 and DH5. The *E. coli* BL21 (DE3) was used as host for the expression of a fusion protein composed of MBP and β1,3-GlcNAcT (MBP-β1,3-GlcNAcT). Restriction endonucleases, ligation high and KOD-Plus-polymerase were purchased from Toyobo Co. Ltd. (Tokyo, Japan) and used according to the manufacturer's instructions. Routine DNA manipulations, such as purification of plasmid DNA and PCR amplified products were performed according to Mag Extractor (Toyobo, Tokyo Japan) protocol by using Mag Extractor-Plasmid and Mag Extractor-PCR & Gel Clean up. Synthetic oligonucleotide primers were obtained from Hokkaido System Science (Sapporo, Japan). Water-soluble polymers carrying LacCer branches **1** (LacCer mimic primer) were prepared according to the method reported previously.[15] Recombinant bovine β1,4-galactosyltransferase (β1,4-GalT), recombinant rat α2,3-sialyltransferase (α2,3-siaT), and recombinant human α1,3-fucosyltranseferase (α1,3-FucT) were purchased from Calbiochem Co Ltd. Calf intestine alkaline phosphatase was obtained from Sigma Co. Ltd. UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, and GDP-Fuc were purchased from Yamasa Co. Ltd. (Tokyo, Japan) and UDP-GalNAc from Sigma Chemical Co. Ltd. *p*-Nitrophenyl lactoside was purchased from Toronto Research Chemicals Ltd. Pyridylaminated derivative of lacto-*N*-tetraose was obtained from Takara Co. Ltd. Japan. The MW-MARKER PROTEINS (glutamate dehydrogenase, *Mw* 290,000; lactate dehydrogenase, *Mw* 142,000; enolase, *Mw* 67,000; myokinase, *Mw* 32,000; cytochrome-c, *Mw* 12,400, Oriental Yeast Co. Ltd., Tokyo, Japan) were used for the estimation of the molecular weight of the fusion protein. Amylose resin as a polymer support both for affinity chromatography and for enzyme-immobilization was purchased from New England Biolabs, Inc., Massachusetts, USA. All other chemicals were purchased from Wako Pure Chemical Industries Ltd. Tokyo, Japan.

Construction of the Plasmid Expression Vector

The β1,3-GlcNAcT gene in which the putative transmembrane region of *S. agalactiae* type Ia *cpsIaI* gene was deleted was amplified by PCR using plasmid pBAPIJ as a template, nucleotides 5′-TCTGTCGACTACAACTCAGAAGCAT (*Sal* I cleavage site underlined) and 3′-CCCTTTCTCTTAAATATTGAAGACGTCGGT (*Pst* I cleavage site underlined) as the sense and antisense primers, respectively. The PCR product was cleaved with *Sal* I and *Pst* I and they were subsequently ligated with *Pst* I/*Sal* I vector backbone of pMAL-c2X. The resulting construct evaluated by restriction analysis and nucleotide sequencing was designated as pMCGl-14.

Protein Expression and Purification of MBP-β1,3-GlcNAcT

The plasmid pMCGl-14 that produces the mature form of the MBP-fusion under *tac* promoter control was transformed into *E. coli* BL21 (DE3). *E. coli* cells carrying pMCGl-14 were cultivated overnight at 37 °C in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) containing 100 μg/mL ampicillin, and the seed culture was then diluted to 100-fold with fresh LB broth containing 200 μg/ml ampicillin, 0.02% glucose. The cells were grown at 37 °C to saturation, harvested by centrifugation, and resuspended in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol. Crude cell extract was prepared by sonicating the suspended cells using a Bioruptor sonifier (Cosmo Bio Co. Ltd., Tokyo, Japan). Polyethyleneimine at a final concentration of 0.7% was added to the crude cell extract prepared as above and it was then incubated for 30 min to precipitate the nucleic acids. To the supernatant obtained after centrifugation, ammonium sulfate was added to 70% saturation. The precipitate collected by centrifugation was dissolved in a buffer solution of 20 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol. The solution was dialyzed against the same buffer. After dialysis, the precipitate formed in this step was removed by centrifugation. The crude protein solution was applied to the affinity column (1.6 × 20 cm) of amylose resin. The fractions containing fusion proteins were obtained by eluting with 20 mM Tris-HCl solution (pH 7.4) containing 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol. Next, the combined fractions were applied on a HiTrapQ column (1.6 × 10 cm, Amersham Pharmacia Biotech Co. Ltd., UK) equilibrated with 10 mM HEPES-NaOH (pH 7.0) solution containing 1 mM EDTA. The adsorbed protein was eluted with a linear gradient of 0 – 0.5 M NaCl in the same buffer. Finally, fractions containing fusion proteins were subjected to the purification by using a HiPrep Sephacryl S-200 gel filtration column (1.6 × 60 cm, Amersham Pharmacia Biotech Co. Ltd., UK) equilibrated with 10 mM HEPES-NaOH (pH 7.0) solution containing 200 mM NaCl, and 1 mM EDTA. All these purification procedures were conducted at 4 °C. Protein concentration was estimated by employing protein assay kit (Bio-Rad Laboratories, Co. Ltd. CA, USA). To monitor the expression of MBP-fusion protein and to assess the purity, samples were subjected to SDS-PAGE on 5 – 20% polyacrylamide gels and proteins were visualized by staining with Coomassie Brilliant Blue. Routine immunoblotting was carried out according to the manufacture's protocol (Bio-Rad Laboratories) by means of anti-MBP-rabbit antiserum as the primary antibody, and peroxidase-conjugated goat anti-rabbit IgGs as the secondary antibody. Protein bands were visualized by Immunostain HRP-1000 (Konica, Tokyo, Japan).

General Conditions of Enzymatic Synthesis

Enzymatic syntheses by using a novel fusion protein were carried out basically on the water soluble primers **1**, **2** as glycosyl acceptor substrate under a similar condition reported previously.[15] For instance, the reaction mixture (normally 3.5 mL) consisted of 100 mM HEPES-NaOH buffer (pH 7.2) containing 40 mM $MnCl_2$, 0.1% Triton X-100, 50 mM UDP-GlcNAc (114.0 mg), 15 mg of LacCer polymer **1**, and 20 mg of MBP-β1,3-GlcNAcT. After incubation for 48 h at 37 °C, the reaction mixture was centrifuged. The supernatant was subjected to the purification by using chromatography on a column of Bio-Gel P-2 (extra fine < 45 μm, 1.5 × 50 cm) eluted with 10 mM ammonium acetate. The polymer-containing fractions were pooled and lyophilized to give powdery product **9** (10.2 mg). Progress of the sugar elongation reactions and structural determination of the products were monitored and discussed by using NMR spectroscopy according to the condition reported previously.[15] Reactions using simple glycosyl acceptor substrates such as D-galactose **6**, lactose derivatives **3**, **5**, *N*-acetyllactosamine **4**, lacto-*N*-tetraose derivative **8**, and LacCer **7** were monitored by means of TLC analysis as well as HPLC method according to the condition reported previously.[31]

Immobilization of MBP-β1,3-GlcNAcT on the Amylose Resin

Immobilization of MBP-β-1,3-GlcNAcT on the amylose resin was performed as follows; MBP-β-1,3-GlcNAcT (11.6 mg protein) dissolved in 100 mM HEPES buffer (pH 7.2) containing 20 mM $MnCl_2$ and 200 mM NaCl was subjected to a column of amylose resin (3.5 mL, 1.0 × 5.0 cm) and washed with the same buffer solution (three column volumes). The amount of the immobilized enzyme was estimated to be 5.7 mg. To the column of the immobilized enzyme was loaded the mixture of LacCer polymer **2** (11.0 mg) bearing fluorescent probe and 50 mM UDP-GlcNAc (162.8 mg) in 100 mM HEPES buffer (pH 7.2, 5.0 mL) containing 20 mM $MnCl_2$ and 200 mM NaCl at a flow rate of 0.3 mL/h. After incubating the solution for 48 h at 37 °C, the column was washed with 3 column volumes of buffer solution. Polymer-containing fractions were pooled and evaporated. The crude product was purified by chromatography on Bio-Gel P2 column (extra fine < 45 μm, 1.5 × 50 cm) eluted with 10 mM ammonium acetate. The polymer-containing solution was concentrated and lyophilized to afford powdery product **9** (5.1 mg). The efficiency of glycosylation reaction was estimated by integration of the methyl protons of *N*-acetyl group at C-2 position of the GlcNAc residue.

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