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Synthesis of the glycosaminoglycan–protein linkage tetraosyl peptide moieties of betaglycan, which serve as a hexosamine acceptor for enzymatic glycosyl transfer

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ABSTRACT

Betaglycan, also known as TGF-β type III receptor, is a membrane-anchored proteoglycan, which has two glycosaminoglycan (GAG) attachment sites (López-Casillas, F.; Payne, H. M.; Andres, J. L.; Massagué, J. *J. Cell Biol.* **1994**, *124*, 557–568). Chondroitin sulfate (CS) or heparan sulfate (HS) can attach to the first site, Ser^{535}, whereas only CS attaches to the second, Ser^{546}. Although the mechanism behind the assembly of CS and HS is not fully understood, it has been reported that the assembly of HS requires not only a cluster of acidic residues but also hydrophobic residues located near the Ser-Gly attachment sites (Esko, J. D. Zhang, L. *Curr. Opin. Struct. Biol.* **1996**, *6*, 663–670). To further understand the effects of amino acids close to the Ser residues of the GAG-attachment sites on the glycosyltransferases, two tetraosyl peptides derived from the CS attachment sites of betaglycan, GlcA-Gal-Gal-Xyl-SerGlyAspAsnGly (**1**) and GlcA-Gal-Gal-Xyl-SerGlyAspAsnGlyPheProGly (**2**), were synthesized, and used as donor substrates for β1,4-*N*-acetylgalactosaminyltransferase-I (β4GalNAcT-I) and α1,4-*N*-acetylglucosaminyltransferase-I (α4GlcNAcT-I). Both the chemically synthesized linkage region tetrasaccharides were far better acceptors for β4GalNAcT-I than for α4GlcNAcT-I in vitro, although they also showed appreciable acceptor activity for α4GlcNAcT-I.

1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharide side chains of proteoglycans (PGs), and are composed of two parts, a linkage tetrasaccharide, glucuronic acid-galactose-galactose-xylose (GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-), which is covalently linked to the respective core-protein through the hydroxyl group of the serine residue(s), and a repeating disaccharide region formed by hexosamine and hexuronic acid. GAGs are classified into a chondroitin sulfate (CS) type and a heparan sulfate (HS) type based on the hexosamine residues of the repeating disaccharides (Fig. 1). In the endoplasmic reticulum and Golgi apparatus, the tetrasaccharide of the GAG–protein linkage region is elongated by stepwise additions of monosaccharides to the non-reducing terminal by the corresponding glycosyltransferases from uridine diphosphate (UDP)-monosaccharides.[1] Following completion of the synthesis of the linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser, the first GalNAc or GlcNAc residue is transferred to the non-reducing terminal GlcA in the linkage region by β1,4-*N*-acetylgalactosaminyltransferase-I (β4GalNAcT-I) or α1,4-*N*-acetylglucosaminyltransferase-I (α4GlcNAcT-I), which initiates the synthesis of a CS or HS chain, respectively. Then, the repeating disaccharide region of CS and HS is further elongated by the chondroitin synthase family and exostosin (EXT) family, respectively.[2–4] Thus, the first transfer of the hexosamine residue, α-GlcNAc or β-GalNAc, which is the fifth saccharide from the reducing terminal, is crucial in determining the type of GAG, HS, or CS.

Betaglycan, also known as transforming growth factor (TGF)-β type III receptor, contains two Ser residues bearing a CS and/or HS chain in most mammalian cells.[5] Ser^{535} can have either CS or HS, whereas Ser^{546} only bears CS.[5–7] Zhang and Esko demonstrated that the synthesis of a HS chain on betaglycan requires a cluster of acidic amino acids and tryptophan residues located near the Ser-Gly of the GAG-attachment site using betaglycan chimeras, created by fusing short peptides to Protein A and/or polyhistidine tags and expression in Chinese hamster ovary cells.[7] However, the molecular mechanism behind the assembly of HS and CS at GAG-attachment sites is still unclear. The use of synthetically available substrates is one of the best approaches to elucidating the regulation of the enzymatic elongation of glycan. To further understand the differential assembly of HS and CS chains, this paper reports

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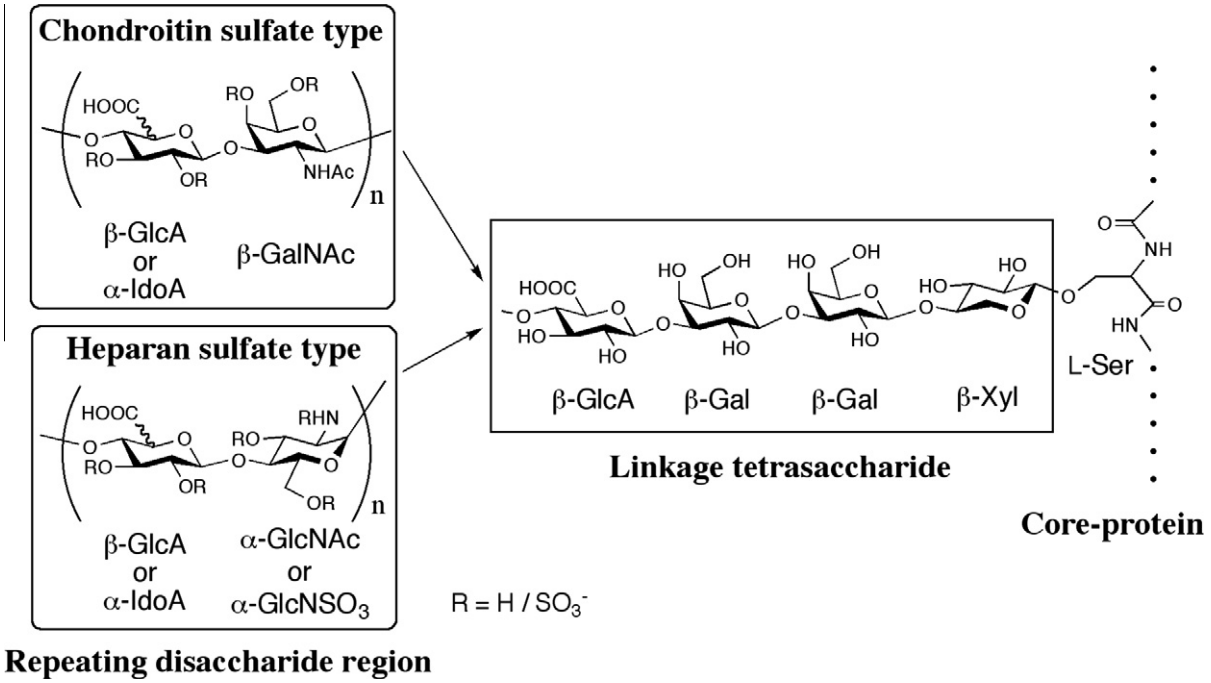


Figure 1. Structure of glycosaminoglycans.

the facile synthesis of partial structures of betaglycan having the GAG–protein linkage tetrasaccharide attached to Ser[546] with peptides of different lengths, GlcA-Gal-Gal-Xyl-SerGlyAspAsnGly (**1**) and GlcA-Gal-Gal-Xyl-SerGlyAspAsnGlyPheProGly (**2**). In addition, enzymatic transfers of β-GalNAc and α-GlcNAc to the non-reducing terminus of not only these linkage region tetrasaccharide-peptides but also GlcA-Gal-Gal-Xyl-Ser[535]GlyTrpProAspGly (**3**),[8] which was synthesized previously, were performed to produce acceptor substrates for β4GalNAcT-I and α4GlcNAcT-I using human CS *N*-acetylgalactosaminyltransferase 2 (CSGalNAcT2)[9] and exostosin-like 3 (EXTL3)[4,10] as enzyme sources, respectively.

2. Results and discussion

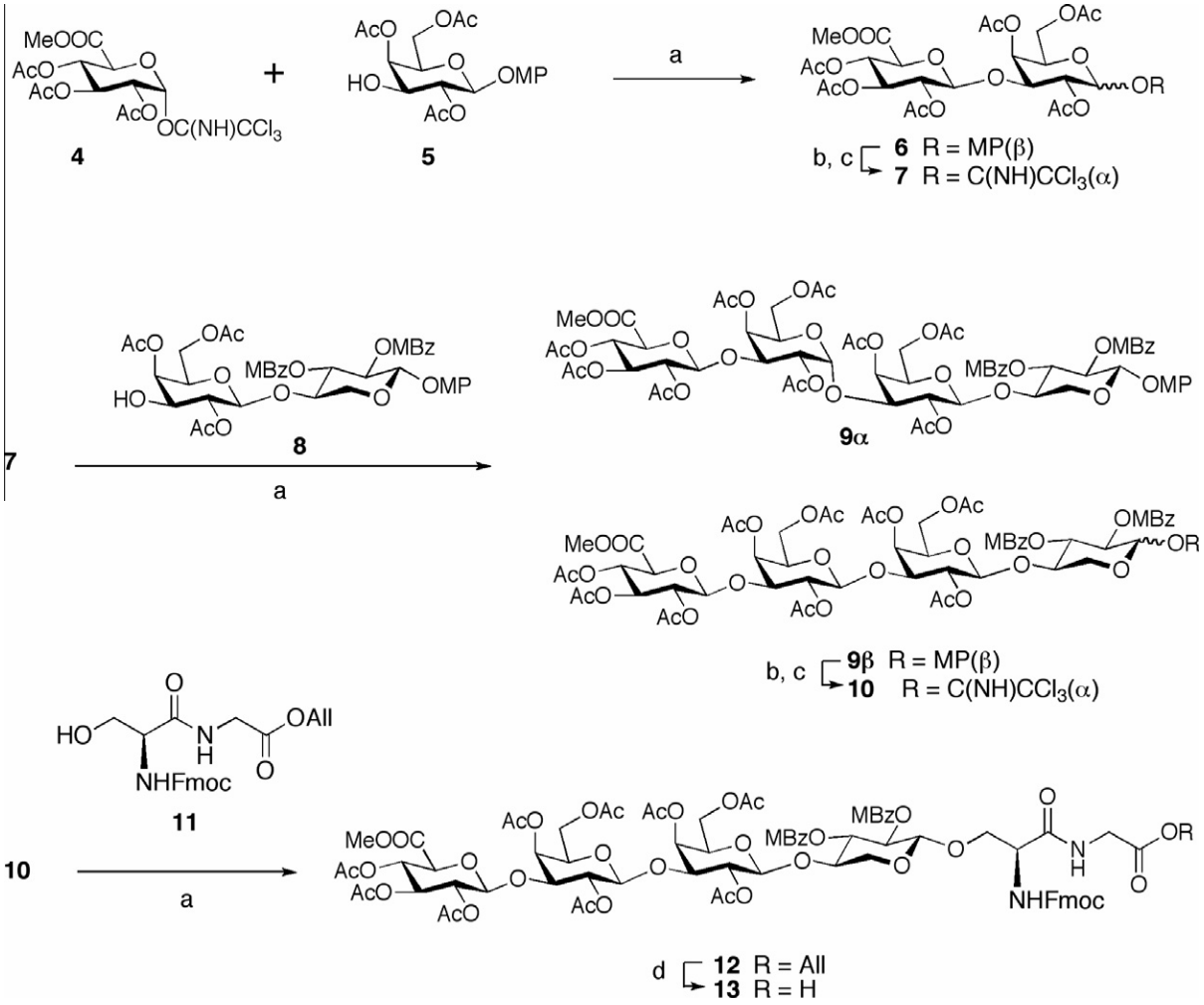
To obtain the linkage tetrasaccharide in fewer steps than that previously reported,[8] GlcA-Gal + Gal-Xyl glycosylation was adopted. As depicted in Scheme 1, the GlcA-Gal moiety was prepared by coupling glucuronosyl trichloroacetimidate (**4**)[11] with the galactosyl acceptor (**5**)[12] in the presence of TMSOTf in CH_2Cl_2 to give the β-linked disaccharide (**6**) in 24% yield together with the recovery of **5** in 60% yield. The coupling yield to obtain GlcA-Gal depends on the substrates.[13,14] Some efforts have been made to increase the yield by protecting the acceptors with benzyl ether[15] or silylene acetal.[14] Although these modifications are effective, here, importance was attached to shortening steps for immediate synthesis. The disaccharide (**6**) was converted to the corresponding hemiacetal by the removal of the 4-methoxyphenyl group with CAN, and subsequent trichloroacetimidation with CCl_3CN and DBU afforded **7** in 69% and 87% yields, respectively. Compound **7** was coupled with the disaccharide acceptor (**8**)[8] in the presence of TMSOTf to give the desired tetrasaccharide (**9β**) in 47% yield. Even with the acetyl group at the 2-O position of the glycosyl donor, the α-isomer (**9α**) was obtained in 24% yield. Starting from methyl tetra-*O*-acetyl-β-D-glucopyranosyl uronate, 4-methoxyphenyl 2,4,6-tri-*O*-acetyl-3-*O*-allyl-β-D-galactopyranoside, and **8**, tetrasaccharide (**9β**) was obtained in seven steps, while 11 steps were needed previously.[8] The tetrasaccharide (**9β**) was converted to the corresponding imidate (**10**) in 88% yield (two steps) in the same manner as mentioned above.

The serylglycine allyl ester protected with Fmoc at the N-terminal (**11**)[16] was glycosylated with **10** in a TMSOTf-mediated manner to give **12** in 92% yield. The allyl group of **12** was removed with $Pd(PPh_3)_4$ and *N*-methylmorpholine to obtain the corresponding carboxylic acids (**13**) in 93% yield.

As shown in Scheme 2, a commercially available Boc-asparagine was coupled with glycine methyl ester in the presence of HOBt, WSCD·HCl, and Et_3N to give the dipeptide (**14**) in 39% yield. The Boc group of **14** was quantitatively removed with TFA to give the corresponding amine (**15**) which was elongated with Fmoc-(O-*t*Bu)-aspartic acid in the same manner as for the synthesis of **14** to afford the tripeptide (**16**) in 63% yield. The Fmoc group of **16** was quantitatively removed with morpholine to give **17**, which was used for the coupling with **13** in the presence of HOBt and HBTU in DMF. Subsequent TFA treatment afforded the tetraosyl pentapeptide (**18**) in 98% yield (two steps). Mild saponification was required for the deprotection of **18** to avoid the elimination of the glycan from the peptide: (1) aq LiOH in THF for 1 h, (2) 0.25 M NaOH in MeOH for 6 h, then (3) 0.1 M NaOMe in aq MeOH for 5 days. Purification was performed by gel-permeation (LH-20) to give **1** in 80% yield.

Another target, **2**, was synthesized on the Sieber amide resin (Scheme 3). The elongation was performed manually by the Fmoc procedure employing HOBt, HBTU, and DIPEA in NMP. Fmoc was removed with piperidine in NMP. The N-terminal of the hexapeptide on the resin was coupled with **13** in the same manner as for the peptide elongation. Then the glycosyl peptide on resin was treated with a TFA cocktail to give the corresponding tetraosyl octapeptide without the *tert*-butyl and trityl groups. Careful saponification as described for the synthesis of **1** furnished the desired tetraosyl octapeptide **2**, which was purified by gel-permeation (LH-20) and HPLC (C8). Tetraosyl penta- and octapeptides (**1** and **2**) were completely assigned by ^1H NMR spectroscopy and by MALDI-TOFMS.

To study the mechanism behind the assembly of CS and HS, the glycosyltransferase activities were assayed using the tetraosyl peptides (**1**, **2**, and **3**) as the sugar acceptors and either UDP-GalNAc



Scheme 1. Reagents and conditions: (a) TMSOTf, MSAW300, CH_2Cl_2, −20 to −10 °C; (b) CAN, CH_3CN, H_2O, 0 °C; (c) CCl_3CN, DBU, CH_2Cl_2, 0 °C; (d) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 2 h. Abbreviations: MP, 4-$MeOC_6H_4$; MBz, 4-MeC_6H_4CO; All, $CH_2{=}CHCH_2$.

or UDP-GlcNAc as a sugar donor. The enzymes used were soluble forms of CSGalNAcT2[9] and EXTL3[4] secreted into the culture medium by COS-7 cells transfected with the respective expression vectors. CSGalNAcT2 and EXTL3 transfer β-GalNAc and α-GlcNAc at the GlcA terminal to elongate the linkage tetrasaccharide toward CS and HS via the glycosyltransferase activities of β4GalNAcT-I and α4GlcNAcT-I, respectively. It is worth noting that β-GalNAc and α-GlcNAc were transferred to both the tetraosyl oligopeptides, GlcA-Gal-Gal-Xyl-S$^{(546)}$GDNG and GlcA-Gal-Gal-Xyl-S$^{(546)}$GDNGFPG (**1** and **2**), though with remarkably higher activity for β-GalNAc than for α-GlcNAc (Table 1 and Fig. 2). These results were compatible with the fact that the naturally occurring betaglycan has only CS at Ser^{546}. The next issue addressed was whether the tetraosyl oligopeptide, GlcA-Gal-Gal-Xyl-S$^{(535)}$GWPDG (**3**), in which the Ser residue corresponds to a potential GAG-attachment site for both HS and CS,[5,7] serves as an acceptor substrate for α4GlcNAcT-I and β4GalNAcT-I. Similar β4GalNAc transferase activity of CSGalNAcT2 was observed using **1**, **2**, or **3** as an acceptor, suggesting β4GalNAcT-I to be little influenced by the amino acid sequences around the GAG-attachment site, consistent with previous reports.[7,9,17] It has been proposed that α4GlcNAcT-I requires a hydrophobic aglycon moiety of the core-protein in the acceptor substrate, which enhanced HS biosynthesis and α4GlcNAcT-I activity in a cell system and in vitro, respectively.[6,7,17] Unexpectedly, the amount of α4GlcNAc transferred by EXTL3 to GlcA-Gal-Gal-Xyl-SGWPDG (**3**) was comparable to that transferred to GlcA-Gal-Gal-Xyl-SGDNG (**1**) and GlcA-Gal-Gal-Xyl-SGDNGFPG (**2**) (Table 1). Therefore, α4GlcNAcT-I of EXTL3 may not always require tryptophan and/or a hydrophobic residue near the GAG-attachment site although they appear to be enhancer elements.[6] Alternatively or in addition, other EXT family members such as EXT1 and EXT2 may be involved in the initiation/elongation of a HS chain at this particular site. In fact, the EXT1/EXT2 complex, HS-polymerase, could synthesize heparan polymers in vitro on GlcA-Gal-Gal-Xyl-SGWPDG (**3**) but not on GlcA-Gal-Gal-Xyl-(G)SGE, which corresponds to the CS attachment site of α-thrombomodulin.[3]

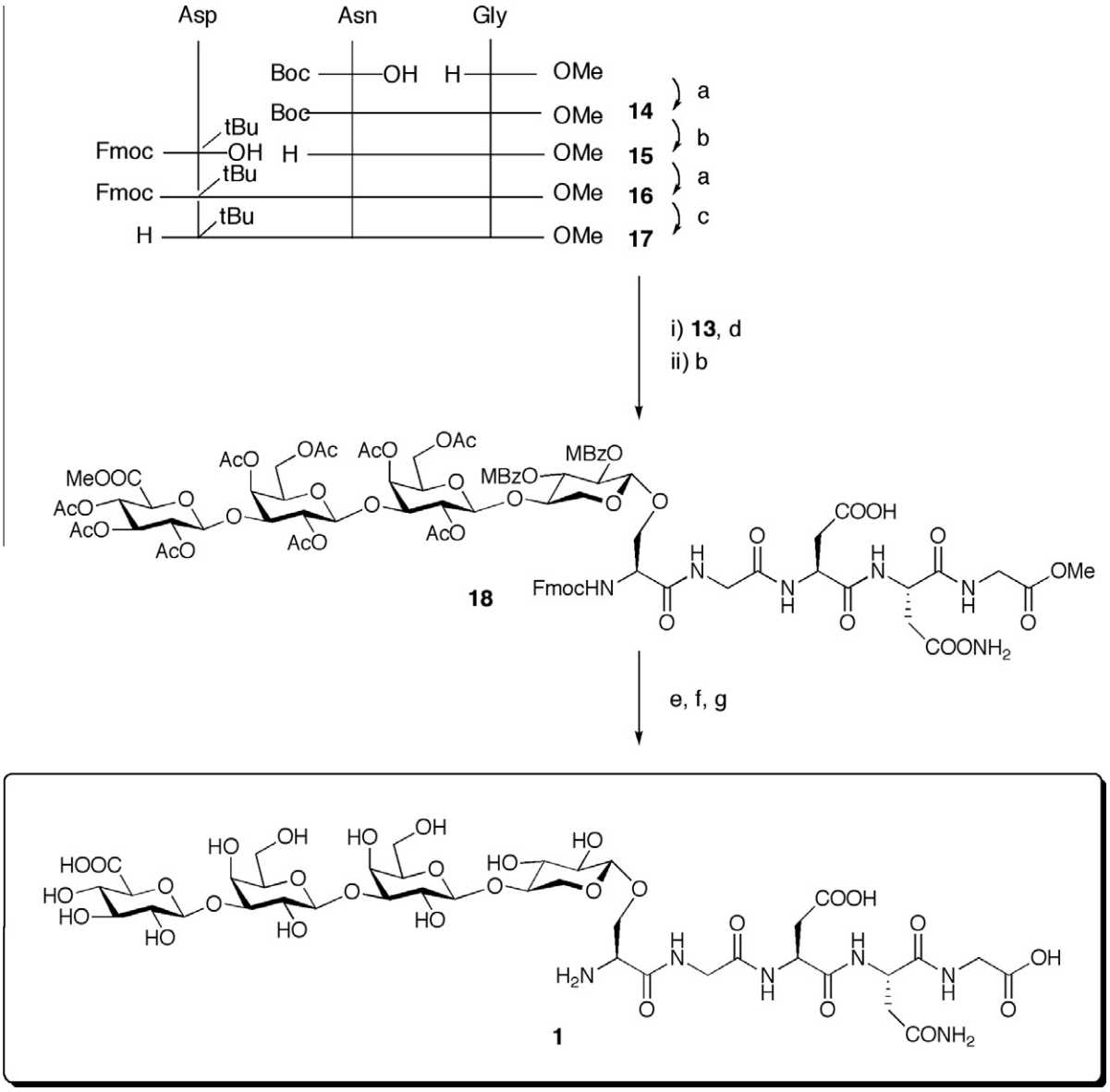
3. Conclusions

Partial sequences of betaglycan having a linkage tetrasaccharide at Ser^{546}, the site of attachment of CS but not HS in vivo, were synthesized with peptides of different lengths. The newly synthesized PG primers **1** and **2** proved to be superior acceptors for β-GalNAc than for α-GlcNAc transfers. However, they also showed appreciable activity as an acceptor for α4GlcNAc.

4. Experimental

4.1. General methods

Optical rotations were measured at 22 ± 3 °C with a JASCO polarimeter DIP-18-1. ^{1}H NMR assignments were confirmed by

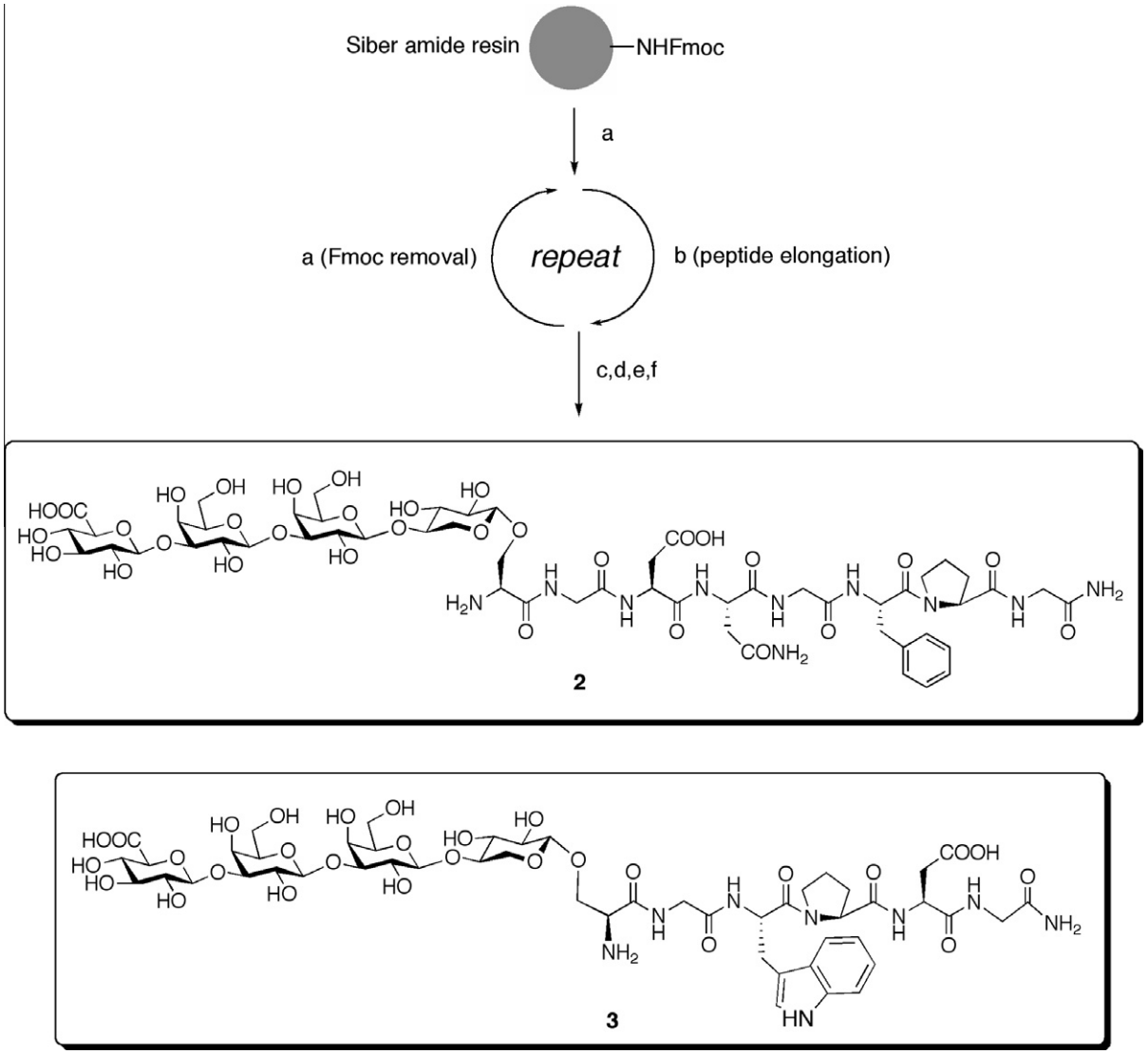


Scheme 2. Reagents and conditions: (a) WSCD·HCl, HOBt, Et_3N, CH_2Cl_2, −20 °C to rt, overnight; (b) TFA, CH_2Cl_2; (c) morpholine, CH_2Cl_2; (d) HBTU, HOBt, *i*-Pr_2EtN, DMF, −20 °C to rt, 2 h; (e) 1.25 M LiOH, THF, H_2O, 0 °C, 1 h, then 1 M AcOH; (f) 0.25 M NaOH, MeOH, H_2O, overnight, then 1 M AcOH; (g) 0.1 M NaOMe, MeOH, H_2O, 5 days, then 1 M AcOH.

two-dimensional HH COSY experiments with a JEOL ECP 500 MHz spectrometer. As an example of signal assignments, 1^{III} stands for a proton at C-1 of sugar residue III. Silica gel chromatography, analytical TLC, and preparative TLC (PTLC) were performed on a column of Silica Gel 60 (E. Merck), Silica Gel 60N (spherical neutral) (Kanto Kagaku), and glass plates coated with Silica Gel F_{254} (E. Merck), respectively. The gel for size-exclusion chromatography (Sephadex LH-20) was from GE Healthcare. Molecular sieves (MS) were from GL Science, Inc. and activated at 200 °C under diminished pressure prior to use. All reactions in organic solvents were performed under a dry Ar-containing atmosphere. As a usual work-up, the organic phase of the reaction mixture was washed with aq $NaHCO_3$ and brine, and dried over anhyd $MgSO_4$. ^{3}H-Labeled uridine diphosphate *N*-acetyl-D-galactosamine (UDP-GalNAc) (20 Ci/mmol) and ^{3}H-labeled uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc) (34.6 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO) and PerkinElmer Life Sciences (Boston, MA), respectively. Unlabeled UDP-GalNAc, UDP-GlcNAc, p3XFLAG-CMV™-8, and anti-FLAG® M2 affinity resin were from Sigma (St. Louis, MO). COS-7 cells were from Japanese Collection of Research Bioresources-Cell Bank (Osaka, Japan).

4.2. 4-Methoxyphenyl *O*-[methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate]-(1→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranoside (6)

To a solution of methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronosyl trichloroacetimidate (**4**,[11] 9.82 g, 20.5 mmol) and 4-methoxyphenyl 2,4,6-tri-*O*-acetyl-β-D-galactopyranoside (**5**,[16] 3.30 g, 8.00 mmol) in CH_2Cl_2 (220 mL) was added MS AW300 (25 g). This mixture was stirred for 50 min at room temperature and cooled to −20 °C. To this solution was added TMSOTf (1.12 mL, 6.19 mmol) with stirring and the reaction temperature was gradually raised to −10 °C for 2 h. Then, an excess amount of satd $NaHCO_3$ was added. Insoluble materials were filtered on Celite. The organic phase was treated as described in Section 4.1. The volatiles were



Scheme 3. Reagents and conditions: (a) piperidine, NMP; Fmoc-AA, HBTU, HOBt, *i*-Pr_2EtN, CH_2Cl_2; (c) TFA, CH_2Cl_2; (d) 1.25 M LiOH, THF, H_2O, 0 °C, 1 h, then 1 M AcOH; (e) 0.25 M NaOH, MeOH, H_2O, 8.5 h, then 1 M AcOH; (g) 0.1 M NaOMe, MeOH, H_2O, overnight, then 1% AcOH. Fmoc-AA: Fmoc-Gly, Fmoc-Pro, Fmoc-Phe, Fmoc-Gly, Fmoc-Asn(Trt), Fmoc-Asp(O-*t*Bu), and **13**.

Table 1
Comparison of the acceptor specificity of the recombinant CSGalNAcT2 and EXTL3

Acceptor	Activity[a] (pmol/mL medium/h)	
	GalNAcT-I (CSGalNAcT2[b])	GlcNAcT-I (EXTL3[b])
1	110	3
2	57	2
3	99	4

The recombinant CSGalNAcT2 and EXTL3 were assayed using UDP-[^{3}H]GalNAc and UDP-[^{3}H]GlcNAc as the sugar donors, respectively, and tetraosyl peptides as the sugar acceptors (5 nmol) as described in Section 4. The reaction products were separated from UDP-[^{3}H]GalNAc or UDP-[^{3}H]GlcNAc by gel-permeation chromatography on a Superdex Peptide column (Fig. 2B and C). The radioactivity of each fraction was measured by liquid scintillation counting.

[a] No detectable glycosyltransferase activities were detected toward tetraosyl peptides tested when the empty vector (mock) was used for expression as an enzyme source.

[b] GalNAcT-I and GlcNAcT-I activities were measured using CSGalNAcT2 and EXTL3 as the enzyme sources, and UDP-[^{3}H]GalNAc and UDP-[^{3}H]GlcNAc as the sugar donors, respectively.

removed under diminished pressure and the residue was subjected to gel-permeation (LH-20, 1:1 $CHCl_3$–MeOH) and applied to a column of silica gel (5:1–4:1–3:1–2:1 toluene–EtOAc) to give **6** (1.40 g) in 24% yield as a syrup. Acceptor (**5**, 1.97 g) was recovered in 60% yield. $[\alpha]_D$ +2.9 (*c* 1.77, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 6.94–6.92 (m, 2H, Ar H), 6.82–6.79 (m, 2H, Ar H), 5.46 (d, 1H, $J_{3,4}$ = 3.4 Hz, H-4^{III}), 5.43 (dd, 1H, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.1 Hz, H-2^{III}), 5.20 (dd, 1H, $J_{3,4}$ = 8.7 Hz, $J_{4,5}$ = 9.6 Hz, H-4^{IV}), 5.19 (dd, 1H, $J_{2,3}$ = 8.9 Hz, H-3^{IV}), 4.94 (dd, 1H, H-2^{IV}), 4.80 (d, 1H, H-1^{III}), 4.70 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1^{IV}), 4.19 (dd, 1H, $J_{5,6b}$ = 5.4 Hz, J_{gem} = 11.6 Hz, H-6b^{III}), 4.13 (dd, 1H, $J_{5,6a}$ = 7.4 Hz, H-6a^{III}), 4.02 (d, 1H, $J_{4,5}$ = 9.6 Hz, H-5^{IV}), 3.96 (dd, 1H, H-3^{III}), 3.94 (m, 1H, H-5^{III}), 3.78, 3.77 (2s, 3H × 2, MeOPh, COOMe), 2.15, 2.13, 2.07, 2.04, 2.01, 2.01 (6s, 3H × 6, 6MeCO). Anal. Calcd for $C_{32}H_{40}O_{19}\cdot 0.5H_2O$: C, 52.09; H, 5.61. Found: C, 52.02; H, 5.43.

4.3. 4-Methoxyphenyl *O*-[methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate]-(1→3)-*O*-2,4,6-tri-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (7)

To a solution of **6** (257.3 mg, 0.353 mmol) in CH_3CN (10 mL) and H_2O (2.5 mL) was added cerium(IV) ammonium nitrate (CAN) (773.8 mg, 1.411 mmol) at 0 °C with stirring. After 1 h, additional CAN (207.0 mg, 0.378 mmol) was supplied and the reaction mix-

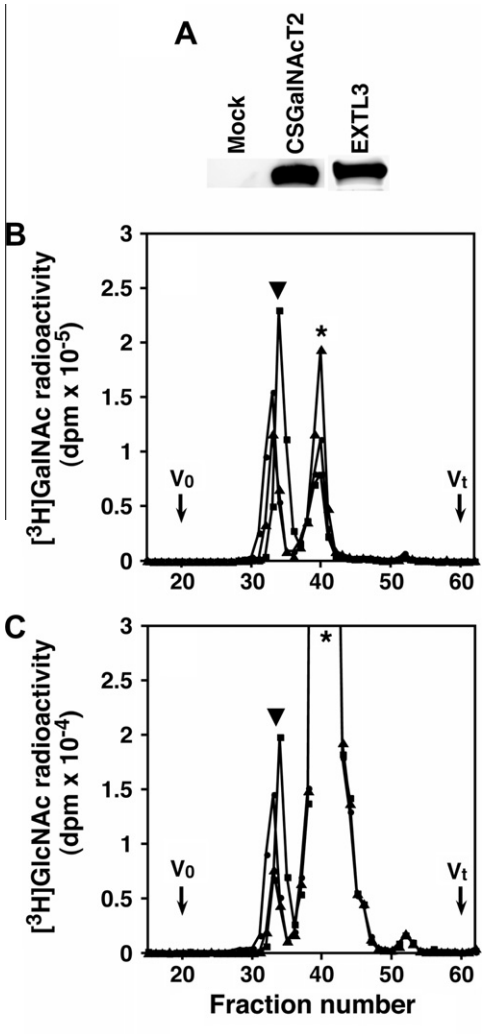


Figure 2. Western blotting of the recombinant CSGalNAcT2 and EXTL3 (A) and gel-filtration chromatographic analysis of the reaction products individually labeled with [^{3}H]GalNAc (B) and [^{3}H]GlcNAc (C). (A) The recombinant CSGalNAcT2 and EXTL3, which were purified and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, were detected with the anti-FLAG antibody in the Western blotting. Mock indicates the conditioned medium transfected with an empty vector as a negative control. (B) ^{3}H-Labeled GalNAcT-I reaction products obtained using the recombinant CSGalNAcT2 as the enzyme source, UDP-[^{3}H]GalNAc as the donor substrate, and **1** (solid circles), **2** (solid triangles), and **3** (solid squares) as the acceptor substrates were analyzed by gel-permeation on a Superdex Peptide column as described in Section 4, and the radioactivity in the effluent fractions (0.4 mL each) was analyzed. (C) ^{3}H-Labeled GlcNAcT-I reaction products obtained using the recombinant EXTL3 as the enzyme source, UDP-[^{3}H]GlcNAc as the donor substrate, and the tetraosyl peptides described above as the acceptor substrates were analyzed by gel-permeation on the same column. Arrowheads and asterisks indicate the elution positions of [^{3}H]GalNAc- or [^{3}H]GlcNAc-labeled reaction products, and UDP-[^{3}H]GalNAc or UDP-[^{3}H]GlcNAc, respectively. The void volume and the total volume of the column are shown by V_o and V_t, respectively.

ture was stirred for another 1 h, then diluted with $CHCl_3$ and brine, and extracted with $CHCl_3$. The organic phase was washed with brine and the residue was eluted from a column of silica gel (2:1–1:1–1:2 toluene–EtOAc) to give the corresponding hemiacetal (151.6 mg, 69%) as a syrup. The hemiacetal was diluted with CH_2Cl_2 (4 mL). To the solution were added CCl_3CN (1 mL) and a small amount of DBU at 0 °C. The reaction mixture was stirred for 1.5 h and directly applied to a column of silica gel (3:1–1:1–1:3 *n*-hexane–EtOAc) to give an α-isomer of **7** (161.7 mg) in 87% yield as a syrup, which was used for the next reaction without further purification. ^{1}H NMR ($CDCl_3$): δ 8.67 (s, 1H, NH), 6.53 (s, 1H, $J_{1,2}$ = 3.67 Hz, H-1), 5.58 (d, 1H, $J_{3,4}$ = 2.75 Hz, H-4^{III}), 5.33 (dd, 1H, $J_{2,3}$ = 10.31 Hz, H-2^{III}), 5.21 (m, 2H, H-3^{IV},4^{IV}), 4.96 (m, 1H, H-2^{IV}), 4.76 (d, 1H, $J_{1,2}$ = 7.33 Hz, H-1^{III}), 4.37 (br t, 1H, J = 5.19 Hz, H-5^{III}), 4.27 (dd, 1H, H-3^{III}), 4.21 (dd, 1H, $J_{5,6b}$ = 5.04 Hz, J_{gem} = 11.69 Hz, H-6b^{III}), 4.05 (m, 1H, H-5^{IV}), 3.98 (dd, 1H, $J_{5,6a}$ = 7.33 Hz, H-6a^{III}), 3.78 (s, 3H, COOMe), 2.14, 2.07, 2.03, 2.02, 2.01 (5s, 3H × 6, 6MeCO).

4.4. 4-Methoxyphenyl *O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-αβ-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-β-D-xylopyranoside (9α and 9β)

To a solution of **6** (1.48 g, 1.93 mmol) and 4-methoxyphenyl *O*-(2,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-β-D-xylopyranoside (**8**,[8] 2.46 g, 3.15 mmol) in CH_2Cl_2 (137 mL) was added MS AW300 (2.53 g). This mixture was stirred for 20 min at room temperature and cooled to −20 °C. To this solution was added TMSOTf (138 μL, 762 μmol) with stirring and the reaction temperature was gradually raised to −10 °C for 1 h. An excess amount of satd $NaHCO_3$ was added. Insoluble materials were filtered on Celite. The organic phase was treated as described in Section 4.1. The volatiles were removed under diminished pressure and the residue was subjected to gel-permeation (LH-20, 1:1 $CHCl_3$–MeOH) and applied to a column of silica gel (1:2–1:3–1:10 *n*-hexane–EtOAc) to give **9β** (1.28 g) and **9α** (650 mg) in 47% and 24% yields, respectively, as syrups. Compound **9β**: $[\alpha]_D$ +16.5 (*c* 1.20, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.95 (m, 4H, Ar H), 7.24 (m, 4H, Ar H), 6.96 (m, 2H, Ar H), 6.81 (m, 2H, Ar H), 5.61 (t, 1H, J = 5.96 Hz, H-3^{I}), 5.42 (dd, 1H, $J_{1,2}$ = 5.04 Hz, $J_{2,3}$ = 5.93 Hz, H-2^{I}), 5.37 (d, 1H, $J_{3,4}$ = 3.67 Hz, H-4^{III}), 5.30 (d, 1H, H-1^{I}), 5.25 (d, 1H, $J_{3,4}$ = 3.67 Hz, H-4^{II}), 5.20 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.62 Hz, H-4^{IV}), 5.16 (t, 1H, $J_{2,3}$ = 9.62 Hz, H-3^{IV}), 5.14 (dd, 1H, $J_{1,2}$ = 7.79 Hz, $J_{2,3}$ = 10.54 Hz, H-2^{III}), 5.02 (dd, 1H, $J_{1,2}$ = 7.79 Hz, $J_{2,3}$ = 10.08 Hz, H-2^{II}), 4.88 (br t, 1H, J = 8.25 Hz, H-2^{IV}), 4.62 (d, 1H, $J_{1,2}$ = 7.33 Hz, H-1^{IV}), 4.54 (d, 1H, H-1^{III}), 4.39 (d, 1H, H-1^{II}), 4.23 (dd, 1H, $J_{4,5eq}$ = 3.67 Hz, J_{gem} = 12.37 Hz, H-5eq^{I}), 4.13 (dd, 1H, $J_{5,6a}$ = 6.41 Hz, J_{gem} = 11.46 Hz, H-6a^{III}), 4.04 (dd, 1H, $J_{5,6a}$ = 6.42 Hz, H-6b^{III}), 3.99 (d, 1H, H-5^{IV}), 3.76 (m, 1H, H-4^{I}), 3.86 (dd, 1H, $J_{5,6a}$ = 5.50 Hz, J_{gem} = 11.46 Hz, H-6a^{II}), 3.77 (m, 2H, H-3^{II}, 3^{III}), 3.76 (s, 6H, MeOPh, COOMe), 3.75 (m, 2H, H-5^{II}, 5^{III}), 3.64 (dd, 1H, $J_{4,5ax}$ = 5.96 Hz, H-5ax^{I}), 3.54 (dd, 1H, $J_{5,6a}$ = 6.87 Hz, H-6b^{II}), 2.40, 2.38 (2s, 3H × 2, 2MePh), 2.12, 2.07, 2.05, 2.03, 2.00, 1.93 (6s, 3H × 9, 9MeCO). Anal. Calcd for $C_{65}H_{76}O_{33}$: C, 56.36; H, 5.53. Found: C, 56.13; H, 5.56.

Compound 9α: $[\alpha]_D$ +46.5 (*c* 3.21, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.93 (m, 4H, Ar H), 7.22 (m, 4H, Ar H), 6.96 (m, 2H, Ar H), 6.81 (m, 2H, Ar H), 5.66 (br t, 1H, J = 7.0 Hz, H-3^{I}), 5.44 (dd, 1H, $J_{1,2}$ = 5.5 Hz, $J_{2,3}$ = 7.1 Hz, H-2^{I}), 5.43 (m, 1H, H-4^{III}), 5.26 (d, 1H, H-1^{I}), 5.23 (d, 1H, $J_{3,4}$ = 2.7 Hz, H-4^{II}), 5.21–5.17 (m, 3H, H-2^{III}, 3^{IV}, 4^{IV}), 5.13 (dd, 1H, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.4 Hz, H-2^{II}), 5.10 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1^{III}), 4.89 (m, 1H, H-2^{IV}), 4.68 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1^{IV}), 4.52 (d, 1H, H-1^{II}), 4.21 (dd, 1H, $J_{4,5eq}$ = 4.4 Hz, J_{gem} = 6.2 Hz, H-5eq^{I}), 4.19 (br t, 1H, J = 5.6 Hz, H-5^{III}), 4.09 (dd, 1H, $J_{5,6a}$ = 6.1 Hz, J_{gem} = 11.4 Hz, H-6a^{III}), 4.09 (d, 1H, $J_{4,5}$ = 8.7 Hz, H-5^{IV}), 4.05 (dd, 1H, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 3.5 Hz, H-3^{III}), 4.01 (m, 1H, H-4^{I}), 3.94 (dd, 1H, $J_{5,6a}$ = 6.4 Hz, H-6b^{III}), 3.76 (m, 1H, H-6a^{II}), 3.76, 3.75 (2s, 3H × 2, *Me*OPh, COOMe), 3.74 (m, 1H, H-3^{II}), 3.68 (br t, 1H, J = 6.8 Hz, H-5^{II}), 3.63 (dd, 1H, $J_{5,6b}$ = 7.1 Hz, J_{gem} = 12.3 Hz, H-6b^{II}), 3.60 (dd, 1H, $J_{4,5ax}$ = 10.5 Hz, H-5ax^{I}), 2.40, 2.38 (2s, 3H × 2, 2MePh), 2.09, 2.08, 2.02, 2.01, 2.00, 1.98 (6s, 3H × 9, 9MeCO). Anal. Calcd for $C_{65}H_{76}O_{33}$: C, 56.36; H, 5.53. Found: C, 56.12; H, 5.66.

4.5. 4-Methoxyphenyl *O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-D-xylopyranosyl trichloroacetimidate (10)

To a solution of **9β** (1.28 g, 924 mmol) in CH_3CN (178 mL) and H_2O (30 mL) was added CAN (2.03 g, 3.70 mmol) at 0 °C with stirring. After 1.5 h, additional CAN (0.51 g, 0.93 mmol) was supplied and the reaction mixture was stirred for another 1 h, then diluted with $CHCl_3$ and brine, and extracted with $CHCl_3$. The organic phase was washed with brine and the residue was eluted from a column of silica gel (2:3–3:7–1:10 toluene–EtOAc) to give the corresponding hemiacetal (1.04 g, 88%) as a syrup. The hemiacetal was diluted with CH_2Cl_2 (19 mL). To the solution were added CCl_3CN (816 μL) and DBU (48 μL) at 0 °C. The reaction mixture was stirred for 1.5 h and applied directly to a column of silica gel (1:2–1:4 toluene–EtOAc) to give **10** quantitatively as a syrup. The characteristic anomeric proton and NH signals of **10** were detected with ^{1}H NMR measurements. The imidate **10** was used for the next reaction without further purification.

4.6. *N*-(9-Fluorenylmethoxycarbonyl)-*O*-[(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-β-D-xylopyranosyl]-L-serylglycine allyl ester (12)

To a solution of *N*-(9-fluorenylmethoxycarbonyl)-L-serylglycine allyl ester (**11**,[16] 486.0 mg, 1.14 mmol) and **10** (814.4 mg, 572 μmol) in CH_2Cl_2 (83 mL) was added MS AW300 (2.3 g). This mixture was stirred for 30 min at room temperature, and then cooled to −20 °C. To this solution was added TMSOTf (72 μL, 397 μmol) with stirring for 2 h. The reaction was quenched and treated as described for the synthesis of **9β**. The residue was subjected to gel-permeation (LH-20, 1:1 $CHCl_3$–MeOH) to give **12** (884.4 mg) in 92% yield: $[\alpha]_D$ +27.6 (*c* 1.34, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.85 (m, 4H, Ar H), 7.77 (m, 2H, J = 7.56 Hz, Ar H), 7.57 (br t, 1H, J = 7.43 Hz, Ar H), 7.40 (m, 1H, Ar H), 7.29 (m, 2H, Ar H), 7.17 (m, 4H, Ar H), 6.90 [m, 1H, NH(Gly)], 5.86 (m, 1H, =CH), 5.80 [m, 1H, NH(Ser)], 5.60 (br t, 1H, J = 8.02 Hz, H-3^{I}), 5.37 (dd, 1H, $J_{3,4}$ = 3.21 Hz, $J_{4,5}$ = 0.92 Hz, H-4^{III}), 5.30 (m, 2H, =CH_2), 5.23 (m, 2H, H-2^{I}, 4^{II}), 5.20 (br t, 1H, J = 9.40 Hz, H-4^{IV}), 5.16 (br t, 1H, J = 9.16 Hz, H-3^{IV}), 5.08 (dd, 1H, $J_{1,2}$ = 8.25 Hz, $J_{2,3}$ = 9.85 Hz, H-2^{II}), 5.01 (dd, 1H, $J_{1,2}$ = 8.02 Hz, $J_{2,3}$ = 10.08 Hz, H-2^{III}), 4.87 (dd, 1H, $J_{1,2}$ = 7.56 Hz, $J_{2,3}$ = 8.94 Hz, H-2^{IV}), 4.74 (m, 1H, H-1^{I}), 4.61 (d, 1H, H-1^{IV}), 4.51 (m, 2H, OCH_2), 4.50–4.31 [m, 8H, OCH_2 (Fmoc), H-9(Fmoc), Serαβ, Gly], 4.46 (d, 1H, H-1^{II}), 4.38 (d, 1H, H-1^{III}), 4.19 (m, 1H, H-5eq^{I}), 4.13 (dd, 1H, $J_{5,6a}$ = 5.96 Hz, J_{gem} = 11.45 Hz, H-6a^{III}), 4.05 (dd, 1H, $J_{5,6b}$ = 7.27 Hz, H-6b^{III}), 4.00 (m, 1H, H-4^{I}), 3.99 (d, 1H, $J_{4,5}$ = 9.63 Hz, H-5^{IV}), 3.82 (dd, 1H, $J_{5,6a}$ = 5.73 Hz, J_{gem} = 11.23 Hz, H-6a^{II}), 3.76 (s, 3H, COOMe), 3.75 (m, 1H, H-3^{III}), 3.73 (m, 2H, H-3^{II}, 5^{III}), 3.66 (br t, 1H, J = 6.66 Hz, H-5^{II}), 3.60 (m, 1H, H-5a^{I}), 3.52 (m, 1H, H-6b^{II}), 2.36, 2.33 (2s, 6H, 2*Me*Ph), 2.12, 2.07, 2.05, 2.04, 2.03, 2.00, 2.00, 1.95 (8s, 3H × 9, 9MeCO). Anal. Calcd for $C_{81}H_{92}N_2O_{37}$: C, 57.72; H, 5.50; N, 1.66. Found: C, 57.37; H, 5.50; N, 1.63.

4.7. *N*-(9-Fluorenylmethoxycarbonyl)-*O*-[(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-β-D-xylopyranosyl]-L-serylglycine (13)

Tetrakis(triphenylphosphine)palladium(0) (27.8 mg, 24.1 μmol) and *N*-methylaniline (65 μL, 0.60 mmol) were added to a solution of **12** (101.4 mg, 60.2 μmol) in THF (3 mL) with stirring. Two hours later, volatiles were removed under diminished pressure and the residue was applied to columns of gel-permeation (LH-20, 10:10:1 $CHCl_3$–MeOH −AcOH) and silica gel (100:1–50:1 EtOAc–MeOH containing 1% AcOH) to give **13** (91.7 mg) in 93% yield. Complete removal of the allyl ester was confirmed with ^{1}H NMR measurements. The compound (**13**) was used for the next reaction without further purification.

4.8. *N*-(Butoxycarbonyl)-L-asparaginylglycine methyl ester (14)

Boc-Asn-OH (2.70 g, 11.6 mmol) and H-Gly-*O*-Me·HCl (1.46 g, 11.6 mmol) were dissolved in CH_2Cl_2 (150 mL). To this solution were added Et_3N (1.60 mL, 11.5 mmol) and HOBt (3.15 g, 23.3 mmol) with stirring. The mixture was cooled to −20 °C, and then a solution of WSCD·HCl (2.45 g, 12.8 mmol) in CH_2Cl_2 (21 mL) was added. The reaction mixture was stirred overnight at room temperature. The volatiles were removed under diminished pressure and the residue was applied to a column of silica gel (50:1–16:1 EtOAc–MeOH containing 1% AcOH) to give **14** (1.36 g) in 39% yield. This compound was used for the next reaction without further purification. ^{1}H NMR (CD_3OD): δ 4.39 (br t, 1H, J = 4.39 Hz, Asnα), 3.88, 3.82 (ABq, 2H, J = 17.53 Hz, Gly), 3.62 (s, 3H, OMe), 2.60 (dd, 1H, $J_{\alpha,\beta a}$ = 5.27 Hz, J_{gem} = 15.35 Hz, Asnβa), 2.50 (dd, 1H, $J_{\alpha,\beta b}$ = 7.79 Hz, Asnβb), 1.35 (s, 9H, *tert*-Bu).

4.9. L-Asparaginylglycine methyl ester (15)

Trifluoroacetic acid (13 mL) was added to a solution of **14** (602 mg, 1.98 mmol) in CH_2Cl_2 (13 mL) and the mixture was stirred for 1 h. The volatiles were removed under diminished pressure to give **15** quantitatively. All of this compound was used for the next reaction without further purification. ^{1}H NMR (CD_3OD): δ 4.17 (dd, 1H, $J_{\alpha,\beta a}$ = 4.58 Hz, $J_{\alpha,\beta b}$ = 8.70 Hz, Asnα), 3.96, 3.88 (ABq, 2H, J = 17.87 Hz, Gly), 3.64 (s, 3H, OMe), 2.84 (dd, 1H, J_{gem} = 16.95 Hz, Asnβa), 2.70 (dd, 1H, Asnβb).

4.10. *N*-(9-Fluorenylmethoxycarbonyl)-*O*-(*tert*-butyl)-L-aspartyl-L-asparaginylglycine methyl ester (16)

FmocAsp(*O*-*t*Bu)-OH (1.39 g, 3.39 mmol) and the dipeptide (**15**, 1.98 mmol) obtained above were dissolved in CH_2Cl_2 (13 mL). To this solution were added Et_3N (353 μL, 2.55 mmol) and HOBt (0.92 g, 6.8 mmol) with stirring. The mixture was cooled to −20 °C, and then a solution of WSCD·HCl (1.30 g, 6.78 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred overnight at room temperature, then diluted with $CHCl_3$, and washed with 1 M HCl and brine. The crude mixture obtained was applied to a column of silica gel (30:1–25:1 EtOAc–MeOH containing 1% AcOH) to give **16** (850.2 mg) in 63% yield. $[\alpha]_D$ −29.1 (*c* 0.44, $CHCl_3$); ^{1}H NMR (CD_3OD): δ 7.70 (d, 2H, J = 7.56 Hz, Ar H), 7.57 (m, 2H, Ar H), 7.29 (br t, 2H, J = 7.45 Hz, Ar H), 7.21 (br t, 2H, J = 7.45 Hz, Ar H), 4.67 (br t, 1H, J = 6.19 Hz, Aspα), 4.40 [m, 1H, H-9(Fmoc)], 4.27 [m, 1H, OCH_2 (Fmoc)], 4.14 (br t, 1H, J = 6.87 Hz, Asnα), 3.83 (s, 2H, Gly), 3.58 (s, 3H, OMe), 2.74 (dd, 1H, $J_{\alpha,\beta a}$ = 5.50 Hz, J_{gem} = 16.50 Hz, Asnβa), 2.66 (dd, 1H, $J_{\alpha,\beta a}$ = 5.73 Hz, J_{gem} = 15.58 Hz, Aspβa), 2.62 (dd, 1H, $J_{\alpha,\beta b}$ = 6.88 Hz, Aspβb), 2.51 (dd, 1H, $J_{\alpha,\beta b}$ = 8.25 Hz, Asnβb), 1.33 (s, 9H, *tert*-Bu). Anal. Calcd. for $C_{30}H_{36}N_4O_9$: C, 60.40; H, 6.08; N, 9.39. Found: C, 59.90; H, 6.31; N, 9.39.

4.11. *O*-(*tert*-Butyl)-L-aspartyl-L-asparaginylglycine methyl ester (17)

Morpholine (7.1 mL) was added to a solution of **16** (716.2 mg, 1.200 mmol) in CH_2Cl_2 (21.4 mL) and stirred overnight. The crude

mixture was evaporated to dryness with toluene, and the residue was subjected to gel-permeation (LH-20, 1:1 $CHCl_3$–MeOH) to afford **17** quantitatively. This compound was used for the next reaction without further purification. ^{1}H NMR (D_2O): δ 4.86 (m, 1H, Aspα), 4.35 (br t, 1H, J = 5.95 Hz, Asnα), 4.05 (s, 2H, Gly), 3.77 (s, 3H, OMe), 3.07 (dd, 1H, $J_{\alpha,\beta a}$ = 5.73 Hz, J_{gem} = 17.64 Hz, Aspβa), 3.02 (dd, 1H, $J_{\alpha,\beta b}$ = 6.42 Hz, Aspβb), 2.90 (dd, 1H, $J_{\alpha,\beta a}$ = 5.50 Hz, J_{gem} = 15.81 Hz, Asnβa), 2.79 (dd, 1H, $J_{\alpha,\beta b}$ = 8.48 Hz, Asnβb), 1.47 (s, 9H, *tert*-Bu).

4.12. *N*-(9-Fluorenylmethoxycarbonyl)-*O*-[(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-(4-methylbenzoyl)-β-D-xylopyranosyl]-L-serylglycyl-L-aspartyl-L-asparaginylglycine methyl ester (18)

Tetraosyl dipeptide (**13**, 63.5 mg, 29.5 μmol) was dissolved in DMF (170 μL), and HOBt (11.9 mg, 38 μmol) was added with stirring. This solution was cooled to −20 °C and HBTU (10.2 mg, 26.9 μmol) was added. The cooling-bath was removed, the reaction mixture was stirred at room temperature for 30 min, and **17** (32.9 mg, 87.9 μmol) and Hünigs base (15.3 μL, 87.8 μmol) were added. The reaction mixture was stirred overnight. Same amounts of additional Hünigs base, HOBt, and HBTU were added and the reaction mixture was diluted with $CHCl_3$ after 2 h. The organic phase was washed with 1 N HCl, aq $NaHCO_3$, and brine, and dried over $MgSO_4$. The volatiles were removed under diminished pressure. The crude materials were subjected to gel-permeation (1:1 $CHCl_3$–MeOH) to give a coupling product (60.5 mg) which was diluted with CH_2Cl_2 (5.0 mL), and TFA (5.0 mL) was added to the solution with stirring. After 1 h all the volatiles were removed under diminished pressure to give **18** (62.5 mg) in 98% yield (two steps). $[\alpha]_D$ +49.2 (*c* 0.13, $CHCl_3$); ^{1}H NMR (CD_3OD) (selected): δ 7.92 [br, 1H, NH(Asp)], 8.82 (d, 4H, J = 7.79 Hz, Ar H), 7.73 [m, 3H, Ar H, NH(Gly)], 7.57 [br, 1H, NH(Asn)], 7.53–7.47 (m, 2H, Ar H), 7.37 (t, 2H, J = 7.33 Hz, Ar H), 7.28 (m, 2H, Ar H), 7.16–7.09 [m, 5H, Ar H, NH(Gly)], 6.48 [br, 1H, NH(Ser)], 5.57 (br t, 1H, J = 7.56 Hz, H-3^I), 5.36 (d, 1H, $J_{3,4}$ = 3.43 Hz, H-4^{III}), 5.22 (br s, 1H, H-4^{II}), 5.21–5.14 (m, 3H, H-2^I, 3^{IV}, 4^{IV}), 5.04 (dd, 1H, $J_{1,2}$ = 8.02 Hz, $J_{2,3}$ = 10.08 Hz, H-2^{II}), 5.00 (dd, 1H, $J_{1,2}$ = 8.02 Hz, $J_{2,3}$ = 10.08 Hz, H-2^{III}), 4.88 (br t, 1H, J = 8.25 Hz, H-2^{IV}), 4.83 (m, 1H, Aspα), 4.74 (d, 1H, H-1^I), 4.63 (m, 1H, Serα), 4.62 (d, 1H, $J_{1,2}$ = 7.57 Hz, H-1^{IV}), 4.51 (m, 1H, Asnα), 4.46 (d, 1H, H-1^{II}), 4.39 (d, 1H, H-1^{III}), 3.60 (s, 3H, COOMe), 2.95–2.57 (m, 4H, Aspβ, Asnβ),2.33, 2.25 (2s, 3H × 2, 2*Me*Ph), 2.11 (s, 3H, MeCO), 2.05, 2.05, 2.05 (3s, 9H, 3MeCO), 2.00, 2.00, 1.99 (3s, 12H, 4MeCO), 1.92 (s, 3H, MeCO). Anal. Calcd for $C_{89}H_{104}N_6O_{43}$: C, 54.94; H, 5.39; N, 4.32. Found: C, 54.72; H, 5.63; N, 4.18.

4.13. *O*-[β-D-Glucopyranuronosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-xylopyranosyl]-L-serylglycyl-L-aspartyl-L-asparaginylglycine (1)

To a solution of **18** (27.2 mg, 11.7 μmol) in THF (0.78 mL) and water (0.15 mL) was added 1.25 M LiOH (50 μL) at 0 °C and stirred for 1 h. The reaction was quenched with 1 M AcOH and the volatiles were removed with toluene under diminished pressure. The residue was diluted with 50% MeOH. Next, 0.25 M NaOH (122 μL) was added in portions over 6 h and the solution was stirred overnight. The reaction was quenched with 1 M AcOH and the reaction mixture was subjected to gel-permeation (LH-20, 1% AcOH) after evaporation. The fractions containing the glycopeptides were collected and diluted with 50% MeOH (0.6 mL) again. To this solution was added 0.1 M NaOMe (0.17 mL) portionwise during 5 days, and after quenching the reaction with 1 M AcOH the volatiles were removed with toluene under diminished pressure. The residue was subjected to gel-permeation (LH-20, 1% AcOH) to give **1** (10 mg) in 80% yield. ^{1}H NMR (D_2O): δ 4.73 (dd, 1H, $J_{\alpha,\beta a}$ = 5.27 Hz, $J_{\alpha,\beta b}$ = 8.02 Hz, Aspα or Asnα), 4.69 (dd, 1H, $J_{\alpha,\beta a}$ = 6.19 Hz, $J_{\alpha,\beta b}$ = 7.79 Hz, Asnα or Aspα), 4.67 (d, 1H, $J_{1,2}$ = 8.01 Hz, H-1^{IV}), 4.65 (d, 1H, $J_{1,2}$ = 7.79 Hz, H-1^{III}), 4.51 (d, 1H, $J_{1,2}$ = 8.02 Hz, H-1^{II}), 4.46 (d, 1H, $J_{1,2}$ = 7.56 Hz, H-1^I), 4.40 (br t, 1H, J = 4.35 Hz, Serα), 4.27 (dd, 1H, J_{gem} = 11.45 Hz, $J_{\alpha,\beta b}$ = 5.27 Hz, Serβa), 4.17 (2s, 2H, H-4^{II}, 4^{III}), 4.11 (dd, 1H, J_{gem} = 10.31 Hz, $J_{4,5e}$ = 6.19 Hz, H-5eq^I), 4.10 (dd, 1H, $J_{\alpha,\beta b}$ = 4.59 Hz, Serβb), 4.09, 3.95 (ABq, 2H, J = 16.72 Hz, Gly), 3.88 (m, 1H, H-4^I), 3.86, 3.83 (ABq, 2H, J = 17.18 Hz, Gly), 3.83 (m, 1H, H-3^{II}), 3.82 (m, 1H, H-3^{III}), 3.79–3.69 (m, 6H, H-5^{II}, 6ab^{II}, 5^{III}, 6ab^{III}), 3.76 (m, 1H, H-5^{IV}), 3.76 (m, 1H, H-2^{III}), 3.66 (br t, 1H, J = 8.76 Hz, H-2^{II}), 3.60 (br t, 1H, J = 9.28 Hz, H-3^I), 3.52 (m, 2H, H-3^{IV}, 4^{IV}), 3.42 (m, 2H, H-5ax^I, 2^{IV}), 3.36 (br t, 1H, J = 8.36 Hz, H-2^I), 2.88, 2,.6 (2dd, 2H, J_{gem} = 8.93 Hz, Aspβ or Asnβ), 2.85, 2.77 (2dd, 2H, J_{gem} = 10.31 Hz, Asnβ or Aspβ); FAB-MS (positive) *m*/*z*: 1103.25 (calcd for $C_{38}H_{60}N_6NaO_{30}$ 1103.33, $[M+Na]^+$).

4.14. General procedure for solid-phase synthesis

Glycopeptides were synthesized manually with 192 mg of Sieber amide resin (100 μmol) as follows. The Fmoc group was removed with 20% of piperidine/NMP (2 mL) (2 × 3 min and 1 × 20 min), as monitored with the Kaiser ninhydrin test. After washing with NMP (2.2 mL) (6 × 1 min) and CH_2Cl_2 (2.2 mL) (3 × 1 min), it was dried in vacuo. The N-terminal free resin or peptide-on-resin was swollen in NMP (2 mL) and shaken overnight with the corresponding Fmoc amino acid (250 μmol), HOBt (250 μmol), HBTU (250 μmol), and DIPEA (500 μmol). Coupling was monitored by the Kaiser test. The resin was washed with NMP (2.2 mL) (3 × 1 min) and CH_2Cl_2 (2.2 mL) (3 × 1 min), and dried in vacuo.

4.15. *O*-[β-D-Glucopyranuronosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-xylopyranosyl]-serylglycyl-L-aspartyl-L-asparaginylglycyl-L-phenylalanyl-L-prolylglycineamide (2)

Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Asp(O-*t*-Bu)-OH were coupled in turn on the Sieber amide resin (192.0 mg, 100 μmol). The resin was coupled with **13** (245.0 mg, 149.0 μmol) as described in the general procedure, in which case different amounts of HOBt (20.1 mg), HBTU (56.5 mg), and DIPEA (52 μL) were used. The resultant resin was exposed to 20% TFA in CH_2Cl_2 (4.5 mL) with shaking for 5.5 h and evaporated. The residue was subjected to gel-permeation (LH-20, 1% AcOH) to give a crude glycopeptide (89.9 mg) still having acyl groups and **13** (133.5 mg, 54%) was recovered. Some portion of the crude glycopeptide (31.7 mg) was diluted with 83% aq THF. To the solution was added 1.25 M LiOH (56 μL) with stirring at 0 °C for 1 h and the reaction was quenched with 1 M AcOH. The volatiles were removed under diminished pressure. The residue was diluted with 50% aq MeOH (3.4 mL). To this solution was added 0.25 M NaOH (220 μL) dropwise for 8.5 h with the pH kept at 8.5. The reaction mixture was neutralized with 1 M AcOH (90 μL) again and subjected to gel-permeation (LH-20, 1% AcOH). The fractions containing glycopeptides were collected (3.1 mg) and diluted with 50% aq MeOH (0.6 mL). Methanolic sodium methoxide (0.1 M, 119 μL) was added to the solution with stirring overnight. Then, the reaction mixture was neutralized with 1% AcOH (100 μL) and evaporated. The residue was purified by gel-permeation (LH-20, 1% AcOH) and HPLC (C18, 10%CH_3CN + 0.1%CF_3COOH ~ 90%CH_3CN + 0.1%CF_3COOH) to give **2** (1.7 mg). ^{1}H NMR (D_2O): δ 7.37–7.28 (m, 5H, Phe), 4.91 (dd, 1H, $J_{\alpha,\beta a}$ = 6.19 Hz, $J_{\alpha,\beta b}$ = 8.25 Hz, Pheα), 4.66 (d, 1H, $J_{1,2}$ = 7.79 Hz,

H-1^{IV}), 4.65 (m, 2H, Asnα, Aspα), 4.64 (d, 1H, $J_{1,2}$ = 7.79 Hz, H-1^{III}), 4.50 (d, 1H, $J_{1,2}$ = 8.02 Hz, H-1^{II}), 4.46 (d, 1H, $J_{1,2}$ = 7.56 Hz, H-1^{I}), 4.39 (m, 2H, Serα, Proα), 4.26 (dd, 1H, J_{gem} = 11.22 Hz, $J_{\alpha,\beta b}$ = 5.73 Hz, Serβa), 4.17 (2s, 2H, H-4^{II}, 4^{III}), 4.10 (m, 2H, H-5eq^{I}, Serβb), 4.06, 3.96 (ABq, 2H, J = 16.96 Hz, Gly), 3.90, 3.83 (ABq, 2H, J = 17.64 Hz, Gly), 3.88–3.63 (m, 9H, H-5^{II}, 6ab^{II}, 5^{III}, 6ab^{III}, Gly, Proδa), 3.88 (m, 1H, H-4^{I}), 3.82 (m, 2H, H-3^{II}, 3^{III}), 3.77 (m, 1H, H-2^{III}), 3.76 (m, 1H, H-5^{IV}), 3.68 (m, 1H, H-2^{II}), 3.60 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.16 Hz, H-3^{I}), 3.51 (m, 3H, H-3^{IV}, 4^{IV}, Proδb), 3.43 (m, 2H, H-5ax^{I}, 2^{IV}), 3.36 (dd, 1H, H-2^{I}), 3.13 (dd, 1H, J_{gem} = 14.43 Hz, Pheβa), 2.98 (dd, 1H, J_{gem} = 14.43 Hz, Pheβb), 2.86–2.67 (m, 4H, Asnβab, Aspβab), 2.25 (m, 1H, Proβa), 2.20–1.89 (m, 3H, Proβb, γab); FAB-MS (positive) *m*/*z*: 1381.48 (calcd for $C_{54}H_{81}N_{10}O_{32}$ 1381.50, $[M+H]^+$), 1425.62 (calcd for $C_{54}H_{79}N_{10}Na_2O_{32}$ 1425.47, $[M+2Na-H]^+$), 1447.63 (calcd for $C_{54}H_{78}N_{10}Na_3O_{32}$ 1447.45, $[M+3Na-2H]^+$).

4.16. Construction of an expression vector containing a cDNA fragment encoding the soluble form of CSGalNAcT2 or EXTL3

cDNA fragments encoding human chondroitin sulfate *N*-acetylgalactosaminyltransferase 2 (CSGalNAcT2[9]) and exostosin-like 3 (EXTL3[4]) proteins lacking the first N-terminal 36 and 90 amino acids, respectively, were individually amplified by PCR with IMAGE clones (ID numbers, 5223190 and 4131101) containing the full-length forms of CSGalNAcT2 and EXTL3 as templates using *KOD*-Plus DNA polymerase (Toyobo, Tokyo, Japan). The PCR fragments were subcloned into the *Hind*III and *Bam*HI (or *Eco*RV) sites of p3XFLAG-CMV™-8. The resultant vector contained cDNA encoding a fusion protein that had an NH_2-terminal cleavable preprotrypsin leader peptide and a 3xFLAG tag peptide followed by a truncated form of either CSGalNAcT2 or EXTL3.

4.17. Expression of a soluble form of CSGalNAcT2 and EXTL3, and enzyme assays

The expression plasmids (6.7 μg) were individually introduced into COS-7 cells (2.2×10^6), which were cultured on 100-mm plates in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (Gibco, Carlsbad, CA), using FuGENE™6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After three days of culture at 37 °C, 0.1 mL of the culture medium was collected and incubated with 10 μL of anti-FLAG® M2 affinity resin at 4 °C for 4 h. The resin was washed with 25 mM Tris-buffered saline containing 0.05% Tween-20 and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was carried out using anti-FLAG M2 monoclonal antibody (Sigma) and the ECL Advance™ western blotting detection kit (GE Healthcare, Buckinghamshire, UK).

GalNAcT-I and GlcNAcT-I activities of the recombinant CSGalNAcT2 and EXTL3, respectively, toward tetrasaccharide-peptides derived from the GAG–protein linkage region were assayed by a method described previously[4,9] with slight modifications. Briefly, the standard reaction mixture (30 μL) contained 10 μL of the enzyme-bound anti-FLAG resin, 50 mM 2-morpholinoethanesulfonic acid-NaOH, pH 6.5, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 167 μM ATP, 8.3 μM UDP-[^{3}H]GalNAc, or UDP-[^{3}H]GlcNAc ($\sim 5 \times 10^5$ dpm) as a donor substrate, and 5 nmol of each linkage region-peptide as an acceptor substrate. The reaction mixtures were incubated at 37 °C for 1 or 4 h, and the radiolabeled products were separated from UDP-[^{3}H]GalNAc or UDP-[^{3}H]GlcNAc by gel-filtration chromatography on a Superdex™ Peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden) equilibrated and eluted with 0.2 M NH_4HCO_3.[4] Fractions (0.4 mL each) were collected at a flow rate of 0.4 mL/min, and radioactivity was measured by liquid scintillation counting in an LS6500 (Beckman coulter Inc., Brea, CA).

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