Enzymatic Synthesis of 4-Methylumbelliferyl Glycosides of Trisaccharide and Core Tetrasaccharide, Gal(β1-3)Gal(β1-4)Xyl and GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl, Corresponding to the Linkage Region of Proteoglycans

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Fluorescence labeled trisaccharide, Gal(β1-3)Gal(β1-4)Xyl(β)–MU [MU = 4-methyl-2-oxo-2*H*-chromen-7-yl (4-methylumbelliferyl)], and tetrasaccharide, GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl(β)–MU, corresponding to the linkage region between glycosaminoglycans and core proteins in proteoglycans were synthesized from Xyl–MU by stepwise enzymatic transglycosidation using β-galactosidase (*Escherichia coli*) and β-glucuronidase (bovine liver). Introduction of the second galactosyl residue at the 3′-position of Gal–Xyl–MU was achieved by minimal protection of the disaccharide intermediate whose reactive primary hydroxy function was selectively protected with an acetyl group by using lipase catalyzed transacetylation. Regioselective β-glucuronylation was effected by the high substrate specificity of the β-glucuronidase without any protection of the glycosyl acceptor.

The connective tissue proteoglycans are complex glycoconjugates that are composed of core proteins and glycosaminoglycan (GAG) chains. The GAG chains are covalently bound to the core protein via a common core tetrasaccharide, GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl(β) as the linkage region (Fig. 1). Though the small details are not fully elucidated yet, the fundamental route of GAG biosynthesis has already been elucidated: The sequence is initiated by the transfer of a xylose moiety to a serine residue of a core protein, followed by completion of the tetrasaccharide structure and subsequent synthesis of the chain of the repeating uronic acid and hexosamine by the stepwise incorporation of the monosaccharide units.[1] Biosynthetic formation of GAG chains in cultured cells was studied recently[1a—d] by the use of fluorescent 4-methyl-2-oxo-2*H*-chromen-7-yl (4-methylumbelliferyl) β-D-xyloside (Xyl–MU, **4**) as a primer. The primer-induced oligosaccharides, such as Gal(β1-4)-Xyl(β)–MU (**1**) and Gal(β1-3)Gal(β1-4)Xyl(β)–MU (**2**), were produced in these cells in addition to GAG–MU. Other oligosaccharides, such as GlcA(β1-4)Xyl(β)–MU, GlcA-(β1-3)Xyl4SO_3(β)–MU, Xyl(β1-4)Xyl(β)–MU, and Sia-(α2-3)Gal(β1-3)Gal(β1-4)Xyl(β)–MU (Sia = sialyl), which are not related to GAGs, were also produced by certain cells. However, the key tetrasaccharide, GlcA(β1-3)Gal(β1-3)-Gal(β1-4)Xyl(β)–MU (**3**), has not been found in any cells. We therefore planned to synthesize MU-labeled key oligosaccharides, Gal(β1-4)Xyl(β)–MU (**1**), Gal(β1-3)Gal(β1-4)-Xyl(β)–MU (**2**), and GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl-(β)–MU (**3**) to be used as standards and primers in the biosynthetic study of GAG chains (Fig. 2). Recently, Neumann et al. reported chemical synthesis of a serine-linked pentasaccharide, GlcNAc(α1-4)GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl(β1-*O*)–L-Ser, which possesses an additional *N*-acetylglucosamine residue, for a purpose similar to our present work.[2]

Enzymatic synthesis of oligosaccharides has been of much interest because stereoselective glycosidation can be achieved without multistep reactions of selective protection and deprotection procedures.[3,4] Among enzymatic procedures, glycosidation reactions based on transglycosidation activity of glycosidases possess obvious advantages in the ready availability of enzymes[4] and their rather low specificity for glycosyl acceptors, allowing the application of this method to a variety of compounds. One of the major issues in transglycosidation, however, has been how to improve the regioselectivity which is not always high enough. In the present study, we achieved a regioselective transgalactosidation using β-D-galactosidase (*Escherichia coli*) by employing minimal protection of a reactive primary hydroxy group in an acceptor. Regio- and stereoselective β-glucuronidation was effected, by contrast, by the use of bovine β-glucuronidase without protection of reactive primary hydroxy groups. This was the first successful β-glucuronidation by means of transglycosidation using a β-glucuronidase.

Results and Discussion

Disaccharide, Gal(β1-4)Xyl(β)–MU (**1**) was synthesized by glycosylation at the hydroxy group of the 4-position

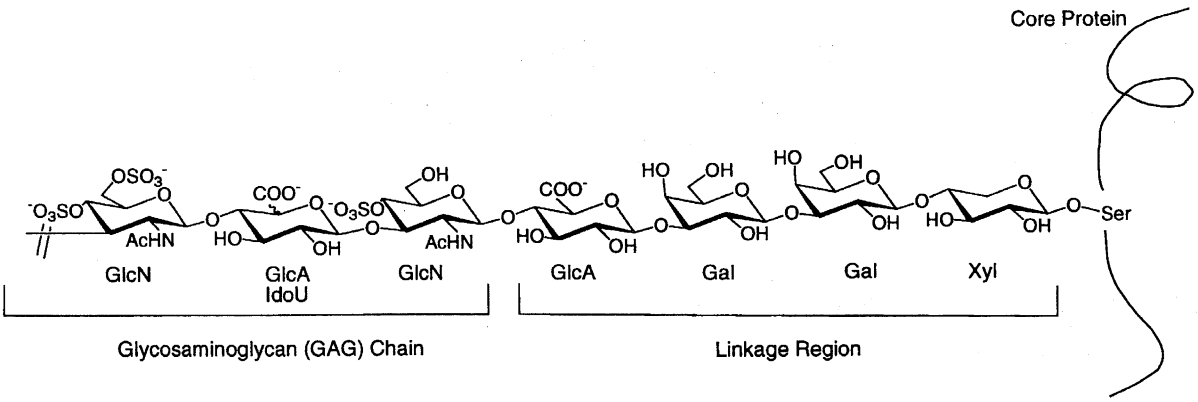


Fig. 1. A typical structure of proteoglycan.

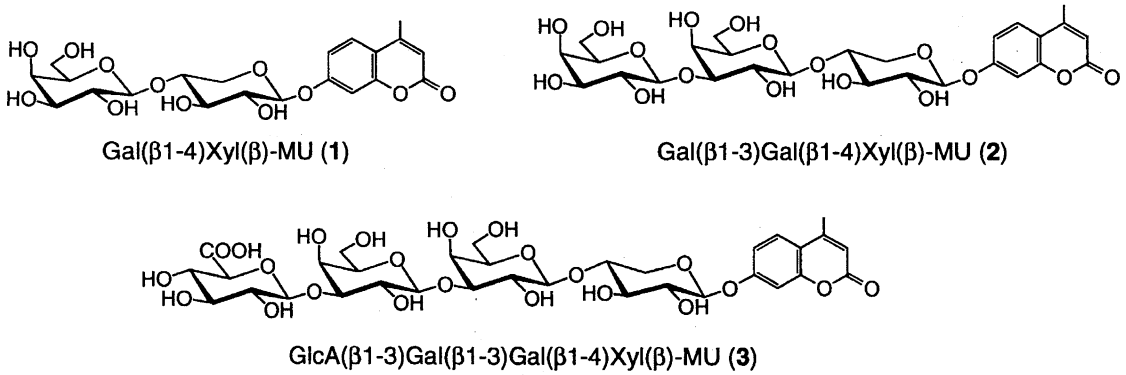
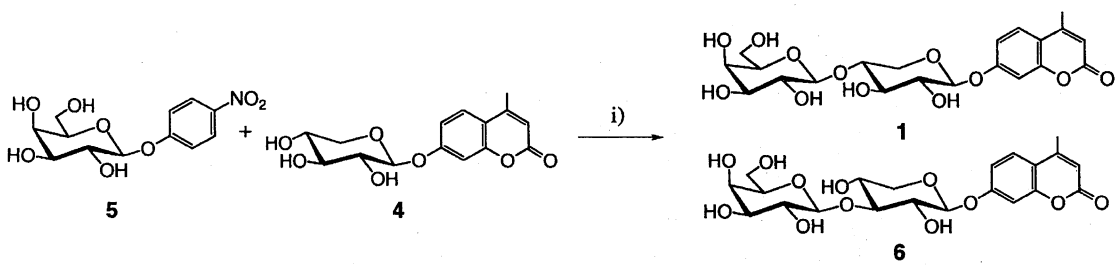


Fig. 2. Structures of fluorescence labeled oligosaccharides synthesized in this work.

of Xyl–MU (**4**) with *p*-nitrophenyl β-D-galactopyranoside (Gal–PNP, **5**) as a donor and a β-D-galactosidase (EC 3.2.1.23; *E. coli*) (Scheme 1). Although **4** was poorly soluble in water owing to the presence of the hydrophobic MU group, its solubility was found to increase in the presence of Gal–PNP (**5**), probably by a detergent-like effect of the latter.[5] The transglycosidation reaction was thus carried out in a phosphate buffer (0.05 M, pH 7.3, 1 M = 1 mol dm^{-3}) at 37 °C using an excess amount of Gal–PNP (**5**) against Xyl–MU (**4**) to give the desired Gal(β1-4)Xyl(β)–MU (**1**) together with a minor regioisomer, Gal(β1-3)Xyl(β)–MU (**6**). Since *p*-nitrophenol, which was liberated as the reaction proceeded, inhibited the activity of the enzyme at a concentration higher than 0.17 M, the transglycosidation was carried out at 37 °C by using a 0.17 M Gal–PNP (**5**) solution. With this constant concentration of the donor **5**, the yield of **1** increased depending on the molar ratio of **5** to **4** but seems to reach a maximum of ca. 21% (Table 1). Regioselectivity of the reaction remained almost constant under these conditions (**1**/**6** = 3.1—3.3). For the preparative purpose, 4 molar amounts of Gal–PNP (**5**) was used against the acceptor **4** to give the disaccharide **1** in 17% yield after HPLC purification. The structures of both **1** and **6** were confirmed by ^{1}H-^{1}H COSY of their hexaacetates.

Enzymatic galactosylation of the hydroxy group of the 3′-position of the disaccharide **1** was next investigated to form a trisaccharide, Gal(β1-3)Gal(β1-4)Xyl(β)–MU (**2**) (Scheme 2). Since the acceptor **1** itself in this reaction is a good substrate of galactosidase, facile enzymatic hydrolysis was expected when direct glycosylation to **1** would be attempted. Furthermore, glycosylation at the more reactive 6′-position rather than at the 3′-position would occur preferentially.[4t] These problems were overcome by modification of the substrate **1** through selective protection of the

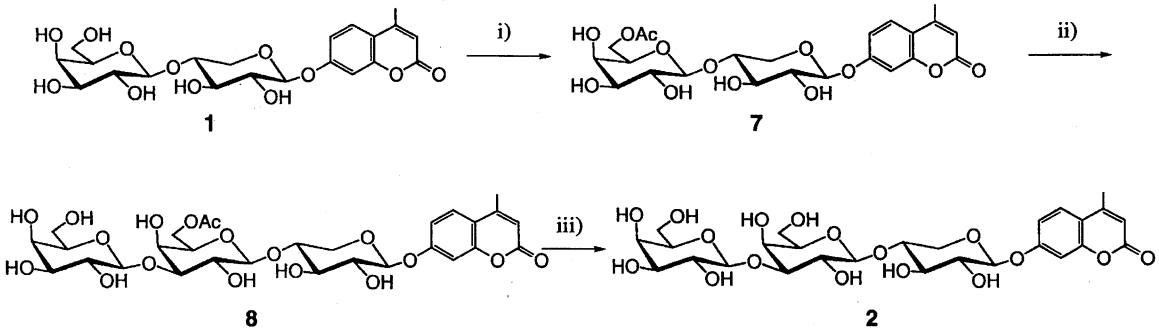


Scheme 1. Reagent, conditions, and yields. i) β-galactosidase (*E. coli*), pH 7.3, 37 °C; **1**, 16.7%; **6**, 4.63%.

Table 1. The Yields of Disaccharide **1** and Its Regioisomer **6** on Transgalactosidation of Xyl–MU (**4**) with Gal–PNP (**5**)[a)]

Entry	Molar ratio of **5** to **4**	Concentration of **4**/mg ml^{-1}	Disaccharide	
			Yield of β(1-4) (**1**)[b)]/%	Ratio of β(1-4) (**1**) to β(1-3) (**6**)
1	3	17.0	15.6	3.2
2	4	12.5	17.2	3.3
3	5	10.0	18.6	3.1
4	8	6.3	20.6	3.2
5	10	5.0	20.9	3.1

a) All reactions were carried out with the same initial concentration (0.17 M) of the donor **5**. b) Based on HPLC.



Scheme 2. Reagents, conditions, and yields. i) lipase (Amano PS), vinyl acetate, THF, r.t., 89.8%; ii) β-galactosidase (*E. coli*), pH 7.3, 37 °C, 15.8%; iii) K_2CO_3, MeOH, 85.0%.

6-hydroxy group of its galactosyl residue.[6)] In our previous preliminary synthesis of the trisaccharide **2**, a methoxymethyl (MOM) group was employed for that purpose.[7)] But the regioselective introduction of the MOM group to the 6′-hydroxy group in **1** was not satisfactory. In the present work, an acetyl group was therefore chosen for the protection of the particular hydroxy group. Selective introduction of an acetyl group to the 6′-position was readily carried our by the use of lipase[8)] in vinyl acetate and THF (1 : 1). Among several lipases tested, (Table 2), celite-immobilized Amano PS[9)] gave the best result in view of both the yield and regioselectivity. The 6′-*O*-monoacetylated disaccharide **7** was thus obtained in 89.8% isolated yield.

Regioselective glycosylation at the 3′-position was then effected by the use of Gal–PNP (**5**) (5 mol amt.) in a phosphate buffer (0.05 M, pH 7.3) at 37 °C for 5 h under supersaturated conditions of the 6′-*O*-monoacetylated disaccharide **7** as the acceptor.[10)] The desired trisaccharide **8** was successfully obtained in 15.8% yield with 72% recovery of **7**. Neither hydrolysis of the β-galactosyl bond in the substrate **7** nor formation of any other regioisomeric byproducts was observed by this transglycosidation. The acetyl group in compound **8** was removed by potassium carbonate in methanol to afford the desired MU-labeled trisaccharide **2** in 85.0% yield. The structure of **2** was confirmed by the NMR spectra (^{1}H-^{1}H COSY, HMQC, and HMBC) and the matrix-assisted laser desorption/ionization (MALDI) mass spectrum. Synthetic **2** was identified by direct HPLC comparison with a corresponding biosynthetic specimen isolated from cells fed with Xyl–MU.[1c)] The result unequivocally proved the proposed structure of the latter and confirmed the occurrence of this trisaccharide as an intermediate in the biosynthetic

Table 2. The Yields of the 6′-Monoacetylated Product and Its Ratio to Other Acetylation Products on Lipase-Catalyzed Acetylation of Gal(β1-4)Xyl(β)–MU (**1**)

Entry	Lipase	Yield of **7**[a)]/%	Ratio of **7** to all acetylated products
1	Porcine pancreas	59.8	0.945
2	Amano PS	81.1	0.845
3	Amano PS (immobilized)	88.7	0.957
4	*Pseudomonas aerugirosa*	22.7	0.227

a) Based on HPLC.

pathway starting from Xyl–MU.

Since enzymatic β-glucuronylation using glucuronidase has not been reported yet, transglycosidation activities of two commercially available β-glucuronidases were tested by the use of the disaccharide **1** as a model acceptor prior to the attempt to introduce the glucuronic acide residue to the 3″-position of the trisaccharide **2**. The enzymatic reaction of *p*-nitrophenyl β-D-glucuronide (GlcA–PNP, **9**) (5 mol amt.) with **1** by the use of *E. coli* glucuronidase in citrate buffer (pH 5) only resulted in the hydrolysis of **9**, no transglycosidation products being obtained. On the other hand, the desired trisaccharide **10** possessing a GlcA(β1-3)Gal linkage was obtained in 12.1% yield by using β-glucuronidase from bovine liver (Scheme 3). The structure of **10** was confirmed by HMBC measurement. Interestingly, no other regioisomers were formed in this reaction. This high regioselectivity is obviously due to the high specificity of this enzyme to the substrate.

The regioselective transglycosylation of the trisaccharide **2** was also achieved by using the bovine liver β-glucuronidase and 8 molar amounts of GlcA–PNP (**9**) as a donor (Scheme 3). The desired tetrasaccharide **3** was successfully obtained in 19.6% yield with 71% recovery of **2**. No other transglycosidation products were formed in this case either. The structure of **3** was confirmed by the NMR spectra (^{1}H-^{1}H COSY, HMQC, and HMBC) and the MALDI mass spectrum.

Conclusions

The combination of transglycosidation and minimal protection using lipase described in this paper provides a facile short step procedure for synthesis of Gal(β1-3)Gal(β1-4)-Xyl(β)–MU (**2**) and GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl-(β)–Mu (**3**) which have been desired for the biosynthetic study of GAG chains. The presence of MU moiety, which has characteristic adsorption at 320 nm and renders the oligosaccharides sufficient lipophilicity, enabled facile and complete purification of the product at each reaction step by reversed phase HPLC. As demonstrated in this and our previous works,[7] transglycosidation reactions with an enzyme of low specificity can be controlled by the use of an appropriate "protecting" group. The effect of such protection resides not only in the control of the position of glycosylation but also in the suppression of undesirable hydrolysis of substrates. Obviously the size of the protecting group is an important factor, since it may not interfere the interaction between the enzyme and the substrate. Acetyl and MOM groups have been so far shown to be satisfactory, but we are trying to find other groups for this purpose in order to extend the scope of this approach.

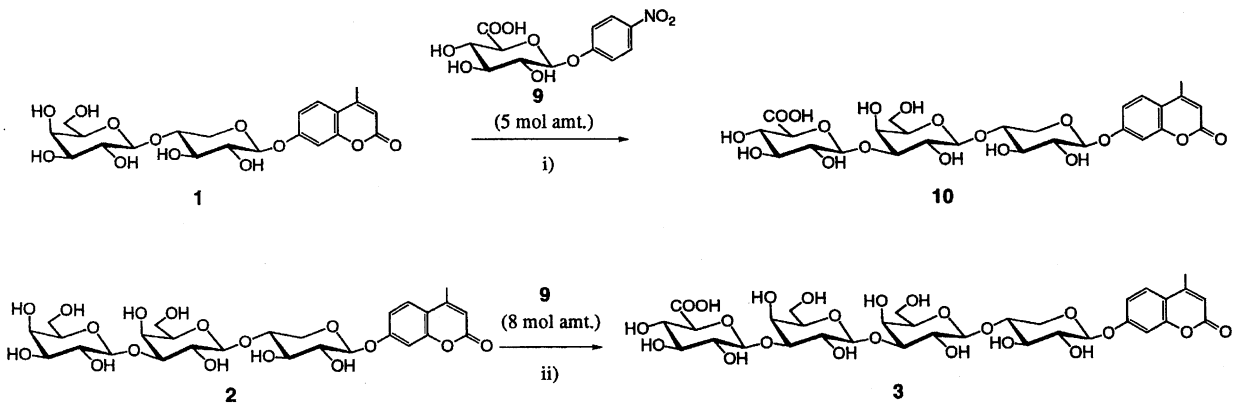
By employing a highly specific enzyme like the β-glucuronidase from bovine liver, on the contrary, selective formation of the glycosidic linkage at the desired position can be readily effected without any protection. Ajisaka et al. recently described isolation of enzymes which form certain specific linkages.[4m] Intensive search in this line which would provide a large set of specific enzymes will be also very important to expand the versatility of the enzymatic transglycosidation.

Although the yields of enzymatic transglycosidation are still generally moderate or low, this approach would give new alternative routes to preparation of complex glycoconjugates.

Experimental

β-Galactosidase (*E. coli*, EC 3.2.1.23, catalog No. G3153) and β-glucuronidases (bovine liver and *E. coli*, EC 3.2.1.31, catalog No. G0251 and G8271) were purchased from Sigma Chemical Co. Lipase (EC 3.1.1.3), Amano PS was a generous gift from Amano Pharmaceutical Co., Ltd. (Aichi, Japan). Other lipases were purchased from commercial sources and used without further purification. *p*-Nitrophenyl β-D-galactoside (Gal–PNP), 4-methyl-2-oxo-2*H*-chromen-7-yl (4-methylumbelliferyl) β-D-xyloside (Xyl–MU), and *p*-nitrophenyl β-D-glucuronide (GlcA–PNP) were purchased from Sigma Chemical Co.

HPLC was performed using a Shimadzu LC-6AD liquid chromatograph fitted with a YMC-GEL ODS-AM 120 S-5 column, 20×250 mm (designated column A), a Cosmosil 5C18AR column, 20×250 mm (column B), or a YMC-pack ODS SH-343-5 column, 20×250 mm (column C). The yields and regioselectivities of the products were determined from the peak areas in the analytical HPLC chromatograms. FAB-MS spectra were performed using a JEOL SX-102 mass spectrometer. Positive-ion MALDI-MS were



Scheme 3. Reagent, conditions, and yields. i) β-glucuronidase (bovine liver), pH 5, 37 °C, 12.1%; ii) β-glucuronidase (bovine liver), pH 5, 37 °C, 19.6%.

performed using a Voyager Elite XL time-of-flight mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystem, Framingham, MA).[11] α-Cyano-4-hydroxycinnamic acid was used as a matrix. NMR spectra were recorded on a JEOL JNM-GSX 270 (270 MHz), VARIAN-UNITY 600 NMR (600 MHz), or JEOL LA 500 (500 MHz) spectrometer, using D_2O or $CDCl_3$ as a solvent. The chemical shifts of the protons are given in δ values as determined either with TMS in $CDCl_3$ or HDO ($\delta = 4.65$) in D_2O as the internal standard. The galactose residue connected to the xylose in the compound **2**, **3**, or **8** is designated Gal_A and the other galactose is designated Gal_B, respectively, in the NMR assignment.

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-β-D-Galactopyranosyl-(1→4)-β-D-xylopyranoside (1) and 4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-β-D-Galactopyranosyl-(1→3)-β-D-xylopyranoside (6). Gal–PNP (**5**) (390 mg, 1.29 mmol) and Xyl–MU (**4**) (100 mg, 0.324 mmol) were dissolved in a phosphate buffer (0.05 M, pH 7.3, 7.8 ml) at 80 °C. After the solution was cooled to 37 °C, the β-galactosidase (390 U[12]) was added. After the mixture was allowed to stand at 37 °C for 4 h, the reaction was stopped by heating the mixture at 100 °C for 1 min. The mixture was filtered and the filtrate concentrated in vacuo. The residue was purified by HPLC [column A, solvent: 0.1% aqueous AcOH–MeCN (16%), flow rate: 10 ml min^{-1}, detection: UV at 320 nm, retention time: 22.0 min (**1**) and 29.0 min (**6**)] and the appropriate eluates lyophilized to give Gal(β1-4)Xyl(β)–MU (**1**) (25.4 mg, 16.7%), and Gal(β1-3)Xyl-(β)–MU (**6**) (7.05 mg, 4.6%) as colorless powders, respectively. The structures of **1** and **6** were confirmed by ^1H NMR spectrometry after hexaacetylation (acetic anhydride and pyridine).

1: FAB-MS [positive, matrix: nitrobenzyl alcohol (NBA)] *m/z* 471.1 [$(M+H)^+$]; $[\alpha]_D^{25}$ −58.0 (*c* 2.24, H_2O); ^1H NMR (600 MHz, D_2O) $\delta = 7.71$ (1H, d, $J = 8.4$ Hz, H-5: MU), 7.04—7.02 (2H, m, H-6 and H-8: MU), 6.25 (1H, d, $J = 1.2$ Hz, H-3: MU), 5.16 (1H, d, $J = 7.8$ Hz, H-1: Xyl), 4.25 (1H, $J = 7.2$ Hz, H-1: Gal), 3.94 (1H, dd, $J = 11$, 5.4 Hz, H-5ex: Xyl), 3.64—3.40 (10H, m), 2.41 (3H, s, CH_3: MU). Found: C, 53.60; H, 5.61%. Calcd for $C_{21}H_{26}O_{12}$: C, 53.12; H, 5.38%.

6: FAB-MS (positive) *m/z* 471.1 [$(M+H)^+$]; ^1H NMR (600 MHz, D_2O) $\delta = 7.71$ (1H, d, $J = 8.8$ Hz, H-5: MU), 7.06—7.02 (2H, m, H-6 and H-8: MU), 6.25 (1H, s, H-3: MU), 5.21 (1H, d, $J = 7.4$ Hz, H-1: Xyl), 4.33 (1H, d, $J = 7.7$ Hz, H-1: Gal), 3.86 (1H, dd, $J = 4.9$, 10.9 Hz, H-5eq: Xyl), 3.62 (1H, d, $J = 2.9$ Hz), 3.55—3.85 (9H, m), 2.50 (3H, s, CH_3: MU).

Hexaacetylated 1: ^1H NMR (600 MHz, $CDCl_3$) $\delta = 7.52$ (1H, d, $J = 8.8$ Hz, H-5: MU), 6.96 (1H, s, H-8: MU), 6.93 (1H, d, $J = 8.5$ Hz, H-6: MU), 6.19 (1H, s, H-3: MU), 5.38 (1H, d, $J = 3.6$ Hz, H-4: Gal), 5.26—5.17 (2H, m, H-1 and H-3: Xyl), 5.16 (1H, dd, $J = 8.0$, 10.4 Hz, H-2: Gal), 5.10 (1H, dd, $J = 7.1$, 5.5 Hz, H-2: Xyl), 5.01 (1H, dd, $J = 10.4$, 3.3 Hz, H-3: Gal), 4.56 (1H, d, $J = 7.8$ Hz, H-1: Gal), 4.14—4.07 (3H, m, H-5eq: Xyl; H-6: Gal), 3.93—3.87 (2H, m, H-4: Xyl; H-5: Gal), 3.55 (1H, dd, $J = 12.0$, 7.7 Hz, H-5ax: Xyl), 2.40 (3H, s, CH_3: MU), 2.15—1.98 (12H, M, Ac).

Hexaacetylated 6: ^1H NMR (600 MHz, $CDCl_3$) $\delta = 7.71$ (1H, d, $J = 8.5$ Hz, H-5: MU), 7.02—6.99 (2H, m, H-6 and H-8: MU), 6.18 (1H, s, H-3: MU), 5.40 (1H, d, $J = 3.3$ Hz, H-4: Xyl), 5.36 (1H, d, $J = 3.9$ Hz, H-4: Gal), 5.24—5.98 (3H, m, H-1 and H-2: Xyl; H-3: Gal), 4.67 (1H, d, $J = 8.0$ Hz, H-1: Gal), 4.22—4.19 (2H, m, H-5 and H-6: Gal), 4.10 (1H, dd, $J = 11.2$, 7.2 Hz, H-6: Gal), 3.99—3.93 (2H, m, H-3 and H-5eq: Xyl), 3.60 (1H, dd, $J = 12.3$, 4.4 Hz, H-5ax: Xyl), 2.40 (3H, s, CH_3: MU), 2.17—1.99 (12H, m, Ac).

Recovery of **4**: 64.4 mg (64.4%).

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-(6-*O*-Acetyl-β-D-galactopyranosyl)-(1→4)-β-D-xylopyranoside (7). To a suspension of disaccharide **1** (33.5 mg, 0.107 mmol) in THF (30 ml) and vinyl acetate (30 ml) was added immobilized lipase (Amano PS) (2.5 g).[9] After the mixture was stirred at room temperature for 12 h, the mixture was filtered and the filtrate concentrated in vacuo. The residue was purified by HPLC [column B, solvent: H_2O–MeCN, flow rate: 8 ml min^{-1}, gradient: 20—50% (0—30 min), detection: UV at 320 nm, retention time: 17.7 min] to give **7** as a colorless powder after lyophilization. Yield: 32.6 mg (89.8%); $[\alpha]_D^{25}$ −21.6 (*c* 2.78, DMF); ^1H NMR (500 MHz, D_2O) $\delta = 7.59$ (1H, d, $J = 7.0$ Hz, H-5: MU), 7.00 (1H, dd, $J = 2.5$, 7.0 Hz, H-6: MU), 6.94 (1H, d, $J = 3$ Hz, H-8: MU), 6.13 (1H, s, H-3: MU), 5.10 (1H, d, $J = 7.5$ Hz, H-1: Xyl), 4.44 (1H, d, $J = 8.0$ Hz, H-1: Gal), 4.23 (2H, d, $J = 6.5$ Hz, H-6: Gal), 4.12 (1H, dd, $J = 5.0$, 11.5 Hz, H-5eq: Xyl), 3.90 (1H, d, $J = 3.5$ Hz, H-4: Gal), 3.88—3.82 (1H, m, H-4: Xyl), 3.68 (1H, dd, $J = 9.5$, 9.5 Hz), 3.62—3.56 (3H, m), 3.48 (1H, dd, $J = 7.5$, 10.0 Hz, H-5ax: Xyl), 2.32 (3H, s, CH_3: MU), 2.07 (3H, s, Ac); MALDI-MS. Found: *m/z* 535.1244. Calcd for $C_{23}H_{28}O_{13}Na$ (M+Na), 535.1428.

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-β-D-Galactopyranosyl-(1→3)-*O*-(6-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-β-D-xylopyranoside (8). Compound **7** (76.6 mg, 0.149 mmol) and Gal–PNP (**5**) (224 mg, 0.745 mmol) were dissolved in water (1.13 ml) at 60 °C. The solution was cooled to room temperature and adjusted to pH 7.3 by addition of phosphate buffer (0.2 M, pH 7.3, 3.37 ml).[10] After addition of β-galactosidase (225 U), the solution was left standing at 37 °C for 5 h; then the reaction was stopped by addition of AcOH (0.5 ml). The mixture was filtered and the filtrate concentrated in vacuo. The residue was purified by HPLC [column B, solvent: 0.1% aqueous AcOH–MeCN, flow rate: 8 ml min^{-1}, gradient: 20—20—50% (0—5—35 min), detection: UV at 300 nm, retention time: 20.3 min] to give **8** as a colorless powder after lyophilization. Yield: 15.9 mg (15.8%); $[\alpha]_D^{25}$ −18.9 (*c* 1.43, DMF); ^1H NMR (500 MHz, D_2O) $\delta = 7.65$ (1H, d, $J = 7.0$ Hz, H-5: MU), 7.04—7.01 (2H, m, H-6 and H-8: MU), 6.18 (1H, s, H-3: MU), 5.12 (1H, d, $J = 7.5$ Hz, H-1: Xyl), 4.52 (1H, d, $J = 7.6$ Hz, H-1: Gal), 4.48 (1H, d, $J = 7.8$ Hz, H-1: Gal), 4.22—4.19 (2H, m, H-6: Gal_A), 4.12 (1H, d, $J = 5.2$ Hz, H-4: Gal_A), 4.12 (1H, dd, $J = 5.7$, 10.0 Hz, H-5eq: Xyl), 3.89—3.82 (2H, m, Gal-5, H-4: Xyl), 3.82 (1H, d, $J = 3.0$ Hz, H-4: Gal_B), 3.74 (1H, dd, $J = 3.5$, 9.9 Hz, H-3: Gal_A), 3.68—3.43 (9H, m), 2.35 (3H, s, CH_3: MU), 2.04 (3H, s, Ac). MALDI-MS. Found: *m/z* 697.1561. Calcd for $C_{29}H_{38}O_{18}Na$: (M+Na), 697.1956.

Recovery of **7**: 55.2 mg (72.0%).

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-β-D-Galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside (2). The trisaccharide **8** (41.4 mg, 0.614 mmol) was dissolved in a saturated methanolic K_2CO_3 solution (5 ml). The mixture was stirred at room temperature for 2 h and the reaction was stopped by addition of AcOH (1 ml). The solution was filtered and then concentrated. The residue was purified by HPLC [column A, solvent: H_2O–MeCN (16%), flow rate: 8 ml min^{-1}, detection: UV at 320 nm, retention time: 17.3 min] to give **2** as a colorless powder after lyophilization. Yield: 34.0 mg (85.0%); $[\alpha]_D^{25}$ −33.9 (*c* 1.65, H_2O); ^1H NMR (500 MHz, D_2O) $\delta = 7.58$ (1H, d, $J = 8.0$ Hz, H-5: MU), 6.97—6.94 (2H, m, H-6 and H-8: MU), 6.11 (s, 1H, H-3: MU), 5.06 (1H, d, $J = 7.0$ Hz, H-1: Xyl), 4.47 (1H, d, $J = 7.5$ Hz, H-1: Gal_B), 4.42 (1H, d, $J = 8.6$ Hz, H-1: Gal_A), 4.08—4.06 (2H, m, H-4: Gal_A, H-5eq: Xyl), 3.86—3.81 (1H, m, H-4: Xyl), 3.77 (1H, d, $J = 3$ Hz, H-4: Gal_B), 3.70—3.44 (13 H, m), 2.29 (3H, s, CH_3: MU); ^{13}C NMR (125 MHz, D_2O) $\delta = 178.48$, 166.69, 165.41, 160.05, 157.06, 154.75, 134.49, 127.51 (C-5: MU), 116.25 (C-6: MU), 114.70 (C-3: MU),

112.17 (C-8: MU), 105.09, 104.39, 102.21, 100.84, 82.82 (C-3: Gal_A), 76.91 (C-4: Xyl), 75.86, 75.77, 74.43, 73.29, 73.27, 71.82, 70.63, 69.35, 63.94 (C-5: Xyl), 61.86 (C-6: Gal), 61.74 (C-6: Gal), 18.74 (CH_3: MU); MALDI-MS. Found: *m/z* 655.1260. Calcd for $C_{27}H_{36}O_{17}Na$: (M+Na), 655.1850. On direct HPLC comparison with a corresponding biosynthetic MU-labeled trisaccharide isolated from cells fed with Xyl–MU,[1c] synthetic **2** showed identical retention time of 23.0 min [Shodex OHpak SB-803 column, solvent: 0.2 M aqueous NaCl, flow rate: 1 ml min^{-1}, detection: fluorescence (Ex. 320 nm, Em. 380 nm)].

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-(β-D-Glucopyranosyluronic acid)-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside (10). To a solution of **1** (19.1 mg, 0.0406 mmol) and GlcA–PNP (**9**) (102 mg, 0.325 mmol) in citrate buffer (0.2 M, pH 5.0, 5.0 ml) was added a saturated $NaHCO_3$ solution (0.30 ml) to adjust the solution to pH 5. To the solution was added β-glucuronidase (4.5 kU,[13] bovine liver) and the mixture was stirred at 37 °C for 30 h. The reaction was stopped by heating the mixture at 100 °C for 3 min. The mixture was filtered and the filtrate concentrated in vacuo. The residue was purified by HPLC [column C, solvent: 0.1% aqueous TFA–MeCN (18%), flow rate: 8 ml min^{-1}, detection: UV abs 320 nm, retention time: 16.9 min] to give **10** as a colorless powder after lyophilization. Yield: 3.14 mg (12.1%); FAB-MS (negative, matrix: NBA) *m/z* 645.1 $[(M-H)^-]$; $[\alpha]_D^{25}$ −96.0 (*c* 1.25, H_2O); ^1H NMR (500 MHz, D_2O) δ = 7.59 (1H, d, *J* = 8.9 Hz, H-5: MU), 6.98 (1H, dd, *J* = 2.3, 8.7 Hz, H-6: MU), 6.94 (1H, d, *J* = 2.3 Hz, H-8: MU), 6.12 (1H, s, H-3: MU), 5.07 (1H, d, *J* = 7.6 Hz, H-1: Xyl), 4.62 (1H, d, *J* = 8.0 Hz, H-1: GlcA), 4.44 (1H, d, *J* = 7.7 Hz, H-1: Gal), 4.09 (1H, dd, *J* = 5.3, 11.7 Hz, H-5ex: Xyl), 4.03 (1H, d, *J* = 3.0 Hz, H-4: Gal), 3.88—3.83 (1H, m, H-4: Xyl), 3.82 (1H, d, *J* = 9.4 Hz, H-5: GlcA), 3.72—3.41 (11H, m), 3.31 (1H, dd, *J* = 8.1, 8.3 Hz, H-2: GlcA), 2.31 (3H, s, CH_3: MU); ^{13}C NMR (125 MHz, D_2O) 175.30 (C-6: GlcA), 162.60, 159.27, 154.62, 152.37, 126.75 (C-5: MU), 115.54, 113.93 (C-6: MU), 112.07 (C-3: MU), 103.63 (C-1: Gal), 101.44 (C-1: GlcA), 100.01 (C-1: Xyl), 82.34 (C-3: Gal), 76.19 (C-4: Xyl), 75.18, 75.04, 73.65, 73.04, 72.48, 71.51, 69.79, 68.22, 63.16 (C-6: Gal), 61.09 (C-5: Xyl), 17.94 (CH_3: MU).

Recovery of **1**: 10.4 mg (54.5%).

4-Methyl-2-oxo-2*H*-chromen-7-yl *O*-(β-D-Glucopyranosyluronic acid)-(1→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside (3). To a solution of **2** (34.0 mg, 0.0538 mmol) and **9** (135 mg, 0.43 mmol) in citrate buffer (0.2 M, pH 5.0, 6.0 ml) was added a saturated $NaHCO_3$ solution (0.33 ml) to adjust the solution to pH 5. To the solution was added β-glucuronidase (6.7 kU,[13] bovine liver) and the mixture was stirred at 37 °C for 30 h. The reaction was stopped by heating the mixture at 100 °C for 3 min. The mixture was filtered and the filtrate concentrated in vacuo. The residue was purified by HPLC [column C, solvent: 0.1% aqueous TFA–MeCN (18%), flow rate: 8 ml min^{-1}, detection: UV at 320 nm, retention time: 15.4 min] and lyophilized to give **3** as a colorless powder. Yield: 8.58 mg (19.6%); $[\alpha]_D^{25}$ −51.2 (*c* 4.10, H_2O); ^1H NMR (500 MHz, D_2O) δ = 7.46 (1H, d, *J* = 7.0 Hz, H-5: MU), 6.90 (1H, dd, *J* = 2.3, 8.7 Hz, H-6: MU), 6.81 (1H, d, *J* = 2.3 Hz, H-8: MU), 6.02 (1H, s, H-3: MU), 5.02 (1H, d, *J* = 7.5 Hz, H-1: Xyl), 4.63 (1H, d, *J* = 7.8 Hz, H-1: GlcA), 4.56 (1H, d, *J* = 7.4 Hz, H-1: Gal_B), 4.45 (1H, d, *J* = 8.1 Hz, H-1: Gal_A), 4.22—4.05 (2H, m, H-5eq: Xyl; H-4: Gal_B), 4.03 (1H, d, *J* = 2.7 Hz, H-4: Gal_A), 3.88 (1H, d, *J* = 9.4 Hz, H-5: GlcA), 3.86—3.83 (1H, m, H-4: Xyl), 3.75—3.42 (15H, m), 3.33 (1H, dd, *J* = 7.8, 9.2 Hz, H-2: GlcA); ^{13}C NMR (125 MHz, D_2O) 173.70 (C-6: GlcA), 165.17, 160.15, 156.93, 154.60, 127.39 (C-5: MU), 116.03, 114.76 (C-6: MU), 112.07 (C-3: MU), 104.83 (C-8: MU), 104.50 (C-1: Gal_B), 104.25 (C-1: GlcA), 102.32 (C-1: Gal_A), 100.79 (H-1: Xyl), 83.13 (C-3: Gal_B), 82.86 (C-3: Gal_A), 77.06 (C-4: Xyl), 75.90, 75.84, 75.59 (C-5: GlcA), 75.41, 74.51, 73.33, (C-2: GlcA), 72.14, 71.02, 70.71, 69.33 (C-4: Gal_B), 69.04 (C-4: Gal_A), 63.98 (C-6: Gal), 61.94 (C-6: Gal) 61.76 (C-5: Xyl), 18.75 (CH_3: MU); MALDI-MS. Found: *m/z* 831.1576. Calcd for $C_{33}H_{44}O_{23}Na$: (M+Na), 831.2171.

Recovery of **2**: 24.1 mg (70.9%).

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References

1) a) K. Takagaki, A. Kon, A. Tanaka, S. Tamura, and M. Endo, *J. Biochem.*, **109**, 514 (1991); b) J. Izumi, K. Takagaki, T. Nakamura, S. Shibata, K. Kojima, I. Kato, and M. Endo, *J. Biochem.*, **116**, 524 (1994); c) S. Shibata, K. Takagaki, T. Nakamura, J. Izumi, K. Kojima, I. Kato, and M. Endo, *J. Biol. Chem.*, **270**, 13794 (1995); d) K. Takagaki, T. Nakamura, S. Shibata, T. Higuchi, and M. Endo, *J. Biochem.*, **119**, 697 (1996); e) A. Manizi, P. V. Salimath, R. C. Spiro, P. A. Keifer, and H. Freeze, *J. Biol. Chem.*, **270**, 9154 (1995); f) H. H. Freeze and J. R. Etchison, *Trends Glycosci. Glycotechnol.*, **8**, 65 (1996), and references therein.

2) K. W. Neumann, J. Tamura, and T. Ogawa, *Glycoconj. J.*, **13**, 933 (1996).

3) For some recent reviews see: a) C.-H. Wong, R. L. Halcomb, Y. Ichikawa, and T. Kajimoto, *Angew. Chem., Int. Ed. Engl.*, **34**, 521 (1995); b) M. M. Palcic and O. Hindsgaul, *Trends Glysosci. Glycotechnol.*, **8**, 37 (1996).

4) a) K. G. I. Nilsson, *Carbohydr. Res.*, **188**, 9 (1989); b) G. C. Look and C.-H. Wong, *Tetrahedron Lett.*, **33**, 4253 (1992): c) G. F. Herrmann, Y. Ichikawa, C. Wandrey, F. C. A. Gaeta, J. C. Paulson, and C.-H. Wong, *Tetrahedron Lett.*, **34**, 3091 (1993); d) G. F. Herrmann, U. Kragl, and C. Wandrey, *Angew. Chem., Int. Ed. Engl.*, **32**, 1342 (1993); e) B. Sauerbrei and J. Thiem, *Tetrahedron Lett.*, **33**, 201 (1992); f) N. Taubken and J. Thieme, *Synthesis*, **1992**, 517; g) R. López and A. Fernández-Mayoralas, *Tetrahedron Lett.*, **33**, 5449 (1992); h) R. López and A. Fernández-Mayoralas, *J. Org. Chem.*, **59**, 737 (1994); i) T. Usui, T. Murata, Y. Yabuuchi, and K. Ogawa, *Carbohydr. Res.*, **250**, 57 (1993); j) Y. Matahira, K. Ohno, M. Kawaguchi, H. Kawagishi, and T. Usui, *J. Carbohydr. Chem.*, **14**, 213 (1995); k) K. G. I. Nilsson, *Carbohydr. Res.*, **180**, 53 (1988); l) M. Pozo and V. Gotor, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 1001; m) K. Ajisaka, H. Fujimoto, and M. Isomura, *Carbohydr. Res.*, **259**, 103 (1994); n) H.-J. Gais, A. Zeissler, and P. Maidonis, *Tetrahedron Lett.*, **29**, 5743 (1988); o) A. M. Blinkovsky and J. S. Dordick, *Tetrahedron: Asymmetry*, **4**, 1221 (1993); p) Y. Ooi, T. Hashimoto, H. Mitsuo, and T. Satoh, *Tetrahedron Lett.*, **25**, 2241 (1984); q) V. Křen, P. Sedmera, V. Havlíček, and A. Fišerová, *Tetrahedron Lett.*, **33**, 7233 (1992). r) S. Attal, S. Bay, and D. Cantacuzene,

Tetrahedron, **48**, 9251 (1992); s) S. Kobayashi, K. Kainuma, T. Kawasaki, and S. Shoda, *J. Am. Chem. Soc.*, **113**, 3079 (1991); t) W. H. Binder, H. Kählig, and W. Schmid, *Tetrahedron*, **50**, 10407 (1994); u) A. Trincone, E. Pagnotta, and G. Sodano, *Tetrahedron Lett.*, **35**, 1415 (1994); v) G. Vic, M. Scigelova, J. J. Hastings, O. W. Howarth, and D. H. G. Crout, *J. Chem.Soc., Chem. Commun.*, **1996**, 1473; w) T. Mori, S. Fujita, and Y. Okahata, *Chem. Lett.*, **1997**, 73.

5) In fact, NMR signals of aromatic protons of **5** shifted upfield by mixing with **4** in an aqueous solution, indicating mutual assembly through a face-to-face like interaction: Upfield shifts of 0.036 and 0.040 ppm were observed in the chemical shifts of the *m*- and *o*-protons of *p*-nitrophenyl group of **5** (26.5 mM), respectively, by adding **4** (0.5 mol amt. to **5**).

6) Look et al. previously described regioselective galactosylation to the 3-position of a glucal derivative protected with an acetyl group at the 6-position.[4b)]

7) K. Fukase, T. Yasukochi, Y. Suda, M. Yoshida, and S. Kusumoto, *Tetrahedron Lett.*, **37**, 6763 (1996).

8) Regioselective acetylation of carbohydrates by the use of lipase: a) W. J. Hennen, H. M. Sweers, Y.-F. Wang, and C.-H. Wong, *J. Org. Chem.*, **53**, 4939 (1988); b) M. Therisod and A. M. Klivanov, *J. Am. Chem. Soc.*, **108**, 5638 (1986); c) H. Waldmann, in "Enzyme Caralysis in Organic Synthesis," ed by K. Drauz and H. Waldmann, VCH, Weinheim (1995), Vol. 2, pp. 868—882.

9) Immobilization of lipase: T. Inagaki, J. Hiratake, T. Nishioka, and J. Oda, *J. Org. Chem.*, **57**, 5643 (1992).

10) Since the 6′-*O*-acetyl group in **7** was partially cleaved or migrated when the mixture was heated at 80 °C in a phosphate buffer (pH 7.3) to dissolve **7**, its supersaturated solution was prepared as described in the experimental section. The 6′-*O*-acetyl group in **7** was neither cleaved nor migrated under the enzymatic reaction conditions (pH 7.3, 37 °C).

11) T. Takao, Y. Tambara, A. Nakamura, K. Yoshino, H. Fukuda, M. Fukuda, and Y. Shimonishi, *Rapid Commun. Mass Spectrom.*, **10**, 637 (1996).

12) Unit Definition: One unit of the enzyme hydrolyzes 1.0 μmol of *o*-nitrophenyl β-D-galactopyranoside to *o*-nitrophenol and D-galactose per min at pH 7.3 and 37 °C.

13) Unit difinition: One modified "Fishman" unit of the enzyme liberates 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at pH 5.0 and 37 °C.