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Chemoenzymatic Synthesis of Gal(β1-3)Gal(β1-4)Xyl(β)-L-Ser and Gal(β1-3)Gal(β1-4)Xyl(β)-MU by the Use of β-D-Galactosidase

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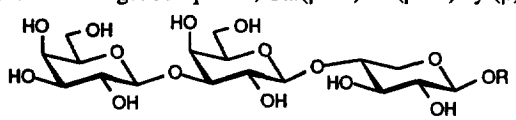
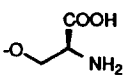
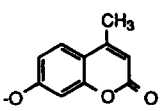
Abstract: The title trisaccharide-serine conjugate **1** constituting the linkage region between glycosaminoglycan and protein in proteoglycan, was synthesized via a trisaccharide *p*-nitrophenyl (PNP) glycoside prepared by stepwise enzymatic transglycosidation to Xyl-PNP with a β-D-galactosidase. In the second transglycosidation step, partial protection of the disaccharide intermediate, Gal-Xyl-PNP, furnished selective galactosylation at the 3'-position. Cleavage of the PNP group after peracetylation, chemical coupling with serine and final deprotection afforded **1**. Fluorescence labeled trisaccharide, Gal(β1-3)Gal(β1-4)Xyl(β)-MU (MU: 4-methylumbelliferyl) (**2**) was also synthesized in a similar way.

The synthesis of oligosaccharides has played an important role in the investigation of their biological functions. Enzymatic synthesis of oligosaccharides has been of much interest because complete stereoselective glycosidation can be achieved without the multistep reactions of selective protection and deprotection procedures.[1,2] Enzymatic glycosidation based on transglycosidation activity of glycosidases possesses obvious advantages in that both enzymes and substrates are readily available[2] and the specificity of enzymes for glycosyl acceptors is rather low thus allowing their reaction with a variety of compounds. One of the major issues in transglycosidation has been how to improve the regioselectivity which is not always high enough.[3]

Gal(β1-3)Gal(β1-4)Xyl(β) constitutes the linkage region between glycosaminoglycan (GAG) chains and protein parts in serine-linked connective tissue proteoglycans. In connection with the biosynthetic study of proteoglycan, we planned to synthesize the trisaccharide sequence linked to a L-serine moiety. For this purpose we applied a chemoenzymatic approach which is becoming more attractive as an alternative to pure chemical or enzymatic synthesis.[1,2b,t,u] A trisaccharide *p*-nitrophenyl glycoside, Gal(β1-3)Gal(β1-4)Xyl(β)-PNP, was first prepared by stepwise enzymatic transglycosidation to β-D-Xyl-PNP (**3**). The intermediate PNP glycosides were efficiently purified by reversed phase HPLC with the aid of the PNP group which renders the molecules UV-active and sufficiently lipophilic. After selective cleavage of the PNP glycoside by a previously reported method,[4] the trisaccharide was chemically coupled with a protected serine residue and final deprotection afforded the target compound, Gal(β1-3)Gal(β1-4)Xyl(β)-Ser (**1**).[5]

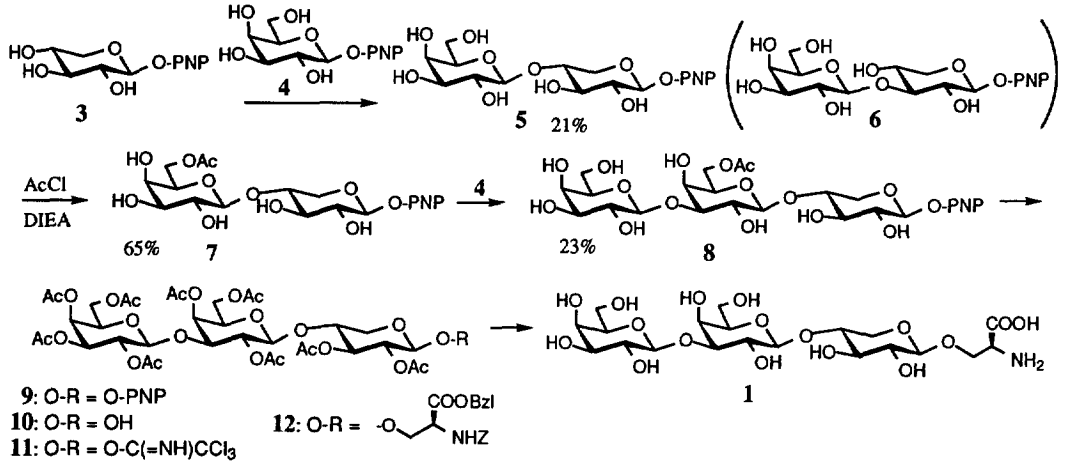


1: OR = 

2: OR = 

Transglycosidation to β-D-Xyl-PNP (**3**) with β-D-Gal-PNP (**4**) (1.5 eq.) and β-D-galactosidase (EC 3.2.1.23; *E. coli*) was carried out in a phosphate buffer at 32°C to give Gal(β1-4)Xyl(β)-PNP (**5**) in 21% yield

with substantially high regioselectivity (**5** : **6** = 9 : 1).[2g,h,6] The β(1-4) and regioisomeric β(1-3) structures of **5** and **6** were confirmed ^{1}H-NMR spectrometrically after peracetylation (acetic anhydride and pyridine). Regioselective galactosylation of the disaccharide was investigated next. Direct transglycosidation to **5** with the galactosidase seemed to cause the following two problems. First, the acceptor **5** itself is a good substrate of the enzymatic hydrolysis. Second, glycosidation at the more reactive 6'-position would occur preferentially. These problems were overcome by modification of the substrate through selective protection of the 6'-hydroxyl group.[7] Acetylation of **5** with acetyl chloride (1.5 eq.) and ethyldiisopropylamine (10 eq) in DMF gave the desired 6-*O*-acetylated **7** in 65% yield. Regioselective transglycosidation to the 3'-position was effected by the use of **4** (8.4 eq.) in a phosphate buffer (0.05 M, pH 7.3) at 32°C for 7 h under supersaturated conditions of **7**.[8] The desired trisaccharide **8** was successfully obtained in 23% yield with 73% recovery of **7**. Neither hydrolysis of the substrate **7** nor formation of any other glycosylated byproducts was observed by this transglycosidation. The structure of the trisaccharide **8** was confirmed by ^{1}H-NMR (COSY) after peracetylation.

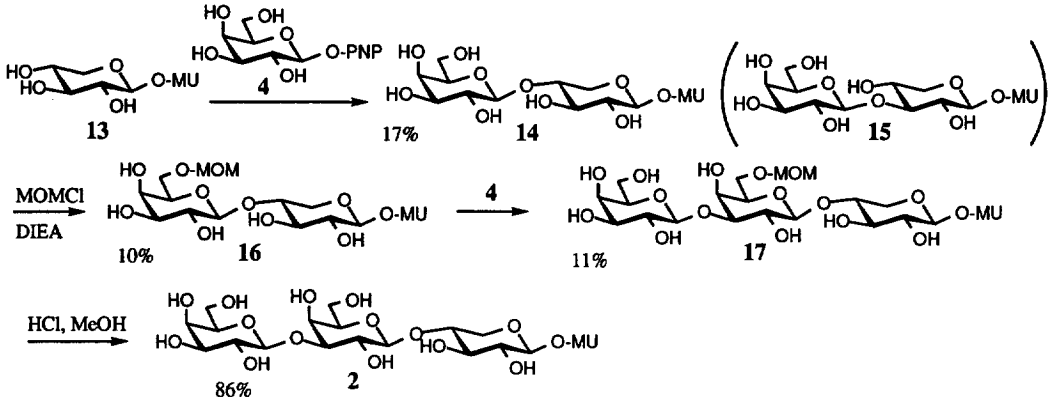


The PNP group of the peracetylated trisaccharide **9** was then converted to a *p*-acetamidophenyl group by catalytic hydrogenation at 7 kg/cm^2 in Ac_2O, and then cleaved by CAN oxidation to give the trisaccharide **10** (74% from **8**). Reaction of **10** with CCl_3CN in the presence of Cs_2CO_3[9] gave glycosyl trichloroacetimidate **11**, which was coupled with Z-L-Ser-OBzl by the use of trimethylsilyl triflate (TMSOTf) in 1,2-dichloroethane to give the protected serine conjugate **12** in 81% yield. Catalytic hydrogenolysis of **12** and subsequent hydrazinolysis[10] afforded the desired Gal(β1-3)Gal(β1-4)Xyl(β)-L-Ser (**1**) in 79% yield, whose structure was confirmed by ^{1}H-NMR and positive FAB-MS [m/z 562.2 [(M+H)$^+$]].

Biosynthesis of GAG chains in cultured cells was studied recently by the use of 4-methylumbelliferyl β-D-xyloside (Xyl-MU) (**13**) as a primer and Xyl-MU-initiated oligosaccharides were characterized.[11] The key trisaccharide glycoside, Gal(β1-3)Gal(β1-4)Xyl(β)-MU (**2**), was obtained as one of the intermediates. In the present study we also synthesized this fluorescence labeled trisaccharide derivative **2** by a similar transglycosidation procedure for further biosynthetic and related study of GAG chains.

Glycosylation at the 4-position of Xyl-MU (**13**) with Gal-PNP (**4**) was difficult as compared to the same reaction of Xyl-PNP (**3**) because of the poor solubility of **13** in a phosphate buffer (0.05 M, pH 7.3). When the transglycosidation reaction was carried out in a mixture of the same buffer and DMSO (3:2 v/v) by the use of 2 eq. of **4** at 30°C under supersaturated conditions of **13** (30 mg/ml, 0.098 M), the desired disaccharide,

Gal(β1-4)Xyl(β)-MU, (**14**) was obtained but only in 3.5% yield accompanied with an undesired regioisomer Gal(β1-3)Xyl(β)-MU (**15**) (2.3% yield). The structures of both **14** and **15** were confirmed by ^{1}H-^{1}H COSY of their peracetates.



The regioselectivity and yield were improved by the use of a detergent as follows. During our attempts to find better reaction conditions, the presence of Gal-PNP (**4**) was found to increase the solubility of Xyl-MU (**13**) in aqueous phases probably owing to a detergent-like effect of the former.[12] This observation brought us to use a detergent: thus, addition of 1% (v/v) of Triton X-100 was effective to obtain sufficient solubility of **13** where the amount of DMSO was reduced to as low as 3% (v/v). Under these reaction conditions, transglycosidation to **13** (30 mg/ml, 0.098 M) with **4** (2 eq.) and the β-galactosidase afforded the disaccharide **14** in 17% yield (**14** : **15** = 3:1).

For the introduction of the second Gal residue to the 3'-position of Gal(β1-4)Xyl(β)-MU (**14**), the 6'-hydroxyl group of the latter had to be protected as in the case of the corresponding PNP glycoside **5**. Owing to the presence of the highly hydrophobic MU residue in **14**, the use of an acetyl group was avoided and the more hydrophilic methoxymethyl (MOM) group was employed for 3'-protection.[13] Reaction of Gal(β1-4)Xyl(β)-MU (**14**) with MOMCl (1 eq.) and ethyldiisopropylamine (25 eq.) in DMF afforded 6'-momo-MOM derivative **16** (10%).[14] The structure of **16** was confirmed by the correlation of methylene protons of MOM with C'-6 on HMBC. Regioselective enzymatic galactosylation of **16** was successfully carried out by the use of 2.5 eq. of **4** in a phosphate buffer (0.05 M, pH 7.3) at 32°C to give the desired trisaccharide **17** in 11% yields with 83% recovery of **16**; no other regioisomers were observed on HPLC in this reaction either. The linkage position of the newly introduced Gal residue was confirmed by the observed low field shift of C'-3 (76.70 ppm in **16** → 82.63 ppm in **17**) on ^{13}C NMR. Finally, the MOM group of **17** was removed by 0.1% (v/v) aqueous conc. HCl in MeOH to give Gal(β1-3)Gal(β1-4)Xyl(β)-MU (**2**) in 86% yield, whose structure was confirmed by ^{1}H-NMR and positive FAB-MS [m/z 633.4 [(M+H)$^+$]].

The combination of chemical and enzymatic methods described in this paper provides a facile overall procedure for synthesis of trisaccharide derivatives with minimum use of protecting groups. Although the yields of enzymatic transglycosidation are still generally moderate or low, this approach would give new alternative routes to preparation of complex glycoconjugates.

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Education, Science, Sports and Culture, Japan.

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3. In the case of glycosyltransferases, by contrast, the substrate specificity is always perfect so that complete regio- and stereospecific glycosidation can be achieved to give the respective specific product. This extremely high specificity, however, limits the applicability of transferases to general synthetic purposes.
4. Fukase, K.; Yasukochi, T.; Nakai, Y.; Kusumoto, S. *Tetrahedron Lett.* **1996**, *37*, 3343-3344.
5. Chemical synthesis of Gal(β1-3)Gal(β1-4)Xyl(β)-L-Ser (**1**) was reported previously: Ekborg, G. C.; Curenton, T.; Krishna, N. R.; Rodén, L. *J. Carbohydr. Chem.* **1990**, *9*, 15-37.
6. A typical transglycosidation procedure is as follows. To a mixture of Xyl-PNP (**3**) (722 mg, 1.55 mmol) and Gal-PNP (**4**) (1.23 g, 4.08 mmol) in a phosphate buffer (0.05 M, pH 7.3, 24 ml) was added β-D-galactosidase (EC 3.2.1.23; *E. coli* , 600U). After the mixture was allowed to stand at 32°C for 4 h, the reaction was stopped by heating at 100°C for 5 min. The mixture was filtered and then concentrated. The residue was purified by HPLC (column: Cosmosil 5C18 AR, 20 x 250 mm; eluent: 16% CH_3CN - aqueous 0.1% AcOH; flow rate: 10 ml/min; detection: UV at 320 nm; retention time: 16.9 min). Lyophilization afforded Gal(β1-4)Xyl(β)-PNP (**5**) as a colorless powder: Yield 243 mg (21%).
7. Look et al. previously described regioselective galactosylation to the 3-position of a simple glucal derivative protected with an acetyl group at the 6-position.[2b]
8. Since the 6'-*O*-acetyl group was partially removed or migrated in a phosphate buffer when the mixture was heated at 80°C to dissolve **7**, the supersaturated reaction solution was prepared as follows. Compound **7** (125 mg, 0.263 mmol) and 8.4 eq. of **4** (662 mg, 2.20 mmol) were dissolved in water (8.25 ml) at 60°C. After the solution was cooled to room temperature, the pH of the solution was adjusted to 7.3 by addition of 0.20 M phosphate buffer (2.75 ml).
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12. In fact, NMR signals of aromatic protons of both **4** and **13** shifted upfield in a mixed aqueous solution, indicating mutual assembly through a face-to-face like interaction. Such interaction was expected to increase by reducing the ratio of DMSO in the medium. The improved selectivity and yield of glycosidation described below may be due to similar assembly in the presence of Triton X-100.
13. As preliminary model experiments, three protected Gal derivatives, 4,6-*O*-isopropylidene-, 6-*O*-acetyl-, and 6-*O*-MOM-Gal-PNP, were tested as glycosyl acceptors. Among them, only the MOM derivative worked as an acceptor, giving a Gal(β1-3)Gal disaccharide in 13% yield. By contrast, 6-*O*-acetyl-Gal-PNP was not soluble in water. 4,6-*O*-Isopropylidene-Gal-PNP was soluble but no transglycosidation reaction proceeded at all probably owing to the steric hindrance of the isopropylidene function.
14. Total 65% of **14** was recovered by combining the material regenerated from other methoxymethylated byproducts.

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