Carbohydrate Research, 249 (1993) 127–137
Elsevier Science Publishers B.V., Amsterdam

A facile enzymatic synthesis of cellooligosaccharide derivatives using β-lactosyl fluoride

Shin-ichiro Shoda, Tatsuya Kawasaki, Kei Obata and Shiro Kobayashi *

Department of Molecular Chemistry and Engineering, Faculty of Engineering, Tohoku University, Sendai 980 (Japan)

(Received January 4th, 1993; accepted April 2nd, 1993)

ABSTRACT

A convenient method for the preparation of cellooligosaccharide derivatives has been developed that uses β-lactosyl fluoride as the glycosyl donor. The reaction consists of the following enzymatic processes; (1) a cellulase-catalyzed regio- and stereo-selective lactosylation of a β-cellobioside as the glycosyl acceptor, utilizing the transglycosylating ability of an enzyme–substrate complex formed from β-lactosyl fluoride and cellulase; (2) β-D-galactosidase-catalyzed regioselective cleavage of the terminal D-galactose unit from the lactosylated product, giving rise to a β-cellotrioside derivative. A cellotetraoside derivative has successfully been prepared in a stereo- and regio-selective manner by repeating these enzymatic reactions and using the resulting β-cellotrioside as starting material.

INTRODUCTION

Cellooligosaccharides, glucose oligomers having a β-(1 → 4) glycosidic linkage, have become attractive substances for macromolecular scientists because these oligomers are intermediate between cellulose (polymer) and glucose (monomer) and, therefore, are useful for fundamental research on cellulose, especially for the elucidation of its intrinsic physical and chemical properties (crystal structure, morphology, biological activity, etc.).

Chemical approaches thus far reported for regioselective construction of the β-(1 → 4) glycosidic bond have always required blocking and deblocking of the hydroxyl group of a D-glucose derivative. In addition, complete stereocontrol of glycosidic bond-formation has not been achieved, despite numerous efforts for the development of new glycosylation reactions[1–3]. Recently, several oligomers, up to a cellooctamer derivative, have been synthesized starting from allyl 2,3,6-tri-*O*-benzyl-4-*O*-(*p*-methoxybenzyl)-β-D-glucoside by utilizing the imidate method[4]; how-

* Corresponding author.

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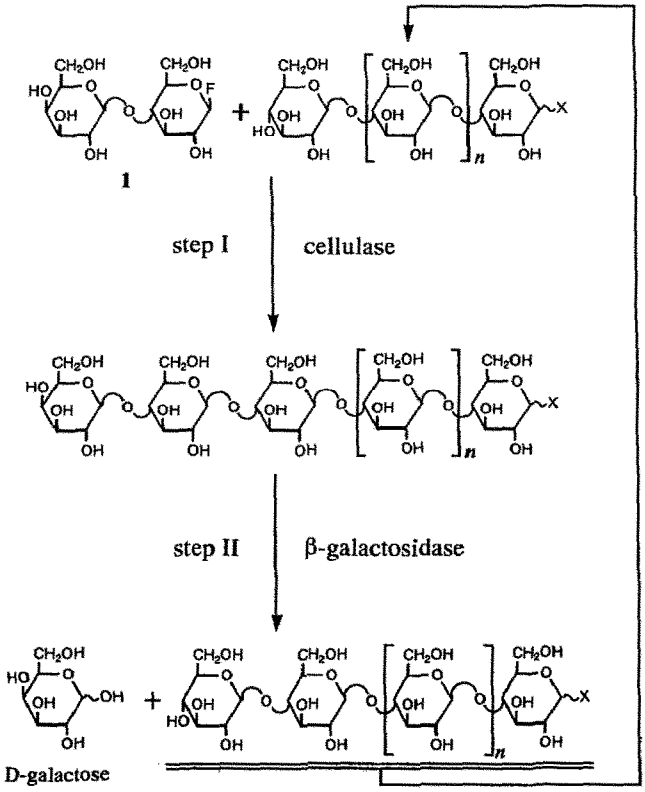
ever, elimination of the protecting group to the corresponding cellooligomers has not been achieved yet[5].

Enzymatic synthesis of oligo- or poly-saccharides, on the other hand, is a promising methodology for regio- and stereo-selective glycoside formation without using any protecting groups. There are many possibilities concerning the combination of a glycosyl donor, a glycosyl acceptor, and an enzyme catalyst. From the time that a glycosyl fluoride was found to be recognized by a glycosidase[6], numerous studies on the interaction of glycosyl fluorides and enzymes have been investigated[7–10]. Recently, we reported the first in vitro synthesis of cellulose by the "enzymatic polymerization"[11] of β-cellobiosyl fluoride (Glc-Glc-F) in a mixed system of organic solvent–acetate buffer, using cellulase as catalyst[12]. During these studies, we found that β-lactosyl fluoride (Gal-Glc-F, **1**), the 4′-epimer of β-cellobiosyl fluoride, is smoothly hydrolyzed to lactose (Gal-Glc-OH) by the action of cellulase catalyst. This result indicates that **1** is also recognized as a substrate for cellulase and capable of forming a reactive glycosyl–enzyme intermediate (Gal-Glc-Enz). It was, therefore, postulated that a condensation reaction of the intermediate with an appropriate glycosyl acceptor having a D-glucose moiety would be possible, leading to a regio- and stereo-selective β-(1 → 4)-glycosidic bond formation between the lactose unit of **1** and the D-glucose unit of the acceptor. The present paper proposes a novel synthetic strategy for the preparation of cellooligosaccharide derivatives having a definite degree of polymerization via a one glucose-unit elongation, as shown in Scheme 1[13]. The reaction involves a cellulase-catalyzed, highly selective lactosylation process (step I) followed by enzymatic cleavage of the D-galactose unit from the nonreducing end of the resulting condensation products by the action of β-D-galactosidase (step II).

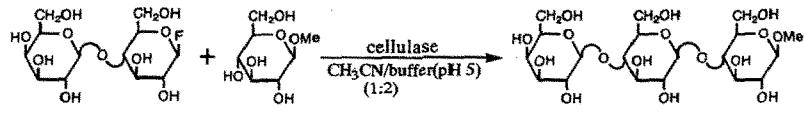
RESULTS AND DISCUSSIONS

A novel trisaccharide, methyl *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside (Gal-Glc-Glc-OMe, **3**), has been synthesized by enzymatic lactosylation of methyl β-D-glucoside (Glc-OMe, **2**) as glycosyl acceptor. The reaction was performed in a mixed solvent system of acetonitrile and acetate buffer (0.05 M, pH 5) in order to favor the condensation reaction over hydrolysis of the substrate **1** to lactose. The high performance liquid chromatographic (HPLC) recording of the mixture showed rapid consumption of the starting material **1** and the appearance of a new peak due to the product trisaccharide **3**. The mixture was heated for 10 min at 100°C after adding an excess amount of acetonitrile in order to deactivate the enzyme at the point of maximum formation of the condensation product **3** (Scheme 2). The yield of the product trimer was determined to be 51% after 30 min by comparing the peak area of the product with those of the other sugar units. The resulting crude product was purified by preparative HPLC and the structure of the resulting trisaccharide determined by means of ^{1}H and ^{13}C NMR spectroscopy.



Scheme 1.

The ^{1}H NMR spectrum showed a singlet peak at δ 3.59 ppm for the methyl protons at the reducing terminal. Three doublet peaks with coupling constants of 7.7–8.0 Hz at δ 4.54, 4.47, and 4.42 ppm are attributable to the anomeric protons (c, d, and b, respectively in Fig. 1), indicating the regio- and stereo-selective formation of a β-(1 → 4) linkage. The respective anomeric protons were assigned on the basis of the reported chemical shift of methyl β-cellobioside and lactose[14,15]. The ^{13}C NMR spectrum also supported the structure of the trisaccharide **3** (Fig. 2). All peaks derived from the anomeric carbon atoms appeared near δ 103 ppm, characteristic of the (1 → 4)-β-D-glucosidic bond[16]. The 4″ carbon atom at the nonreducing end resonated at δ 69.4 ppm.



Scheme 2.

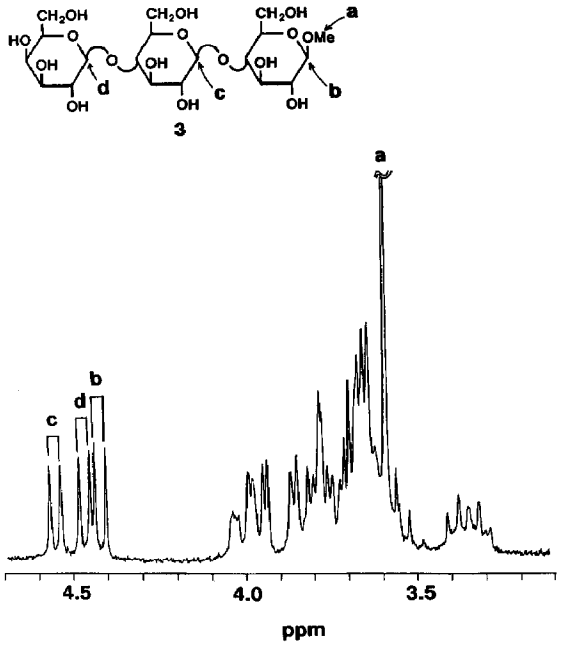


Fig. 1. ^{1}H NMR spectrum of trisaccharide (Gal-Glc-Glc-OMe) **3** in deuterium oxide.

The ^{13}C NMR spectrum of the crude mixture showed no signal for the methylene carbon atom (C-6) adjacent to the β-(1 → 6) glycosidic bond ~ 69 ppm, which is normally observed when an isomeric β-(1 → 4) linkage is formed[16].

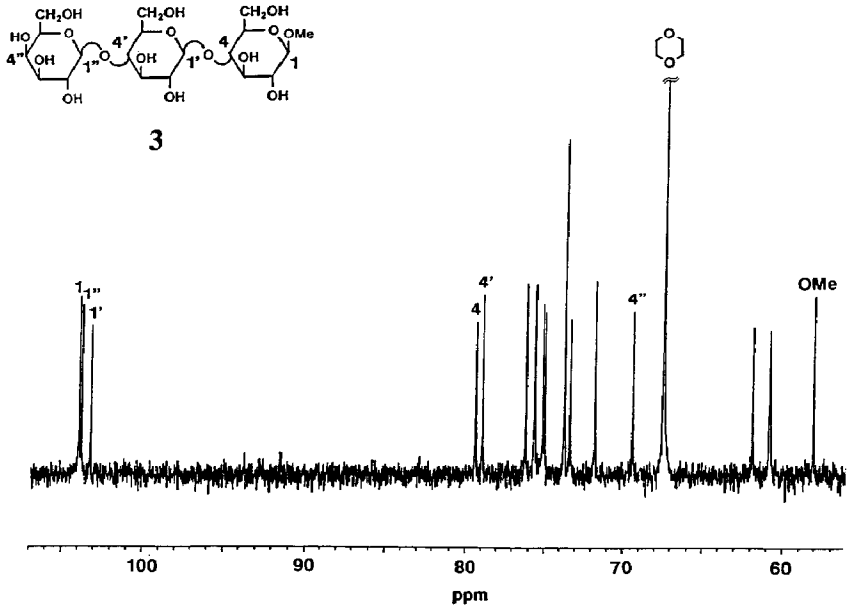


Fig. 2. ^{13}C NMR spectrum of trisaccharide (Gal-Glc-Glc-OMe) **3** in deuterium oxide.

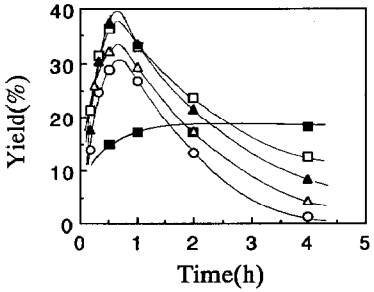


Fig. 3. Effect of acetonitrile concentration and reaction time on cellulase-catalyzed lactosylation of methyl β-D-glucoside (**2**). The reaction was carried out at 30°C: [**1**] = [**2**] = 2.5×10^{-2} mol/L, cellulase 5.0 wt% for **1**. ○ buffer:MeCN = 1:1; △, buffer:MeCN = 5:1; ▲, buffer:MeCN = 3:1; □, buffer:MeCN = 2:1; ■, buffer:MeCN = 1:1.

To optimize reaction conditions, the effect of acetonitrile concentration on the lactosylation of **2** was investigated (Fig. 3). When mixed solvents having acetate buffer–acetonitrile ratios of 1:0, 5:1, 3:1, and 2:1 were used, the yield of the products increased to a maximum at 0.5–1.0 h from the initiation, and decreased as the reaction proceeded. This behavior may be explained as resulting from further hydrolysis of a glycosidic bond in the resulting condensation product to a smaller saccharide by the action of the catalyst cellulase. The best result in terms of maximal yield was obtained when 2:1 or 3:1 acetate buffer–acetonitrile was used as solvent. The yields after 4 h are proportional to the amount of acetonitrile, indicating that the rate of the hydrolysis of the product was lowered by the addition of acetonitrile.

The substrate concentration also affected the yield of the product (Fig. 4). The yield of product increased as the substrate concentration increased in the region of 0.01 to 0.15 M. When the reaction was performed at a higher substrate concentration, the reaction system became heterogeneous. All of the lactosylation reactions

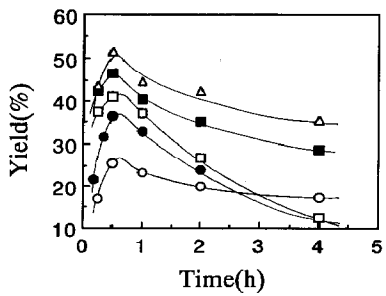
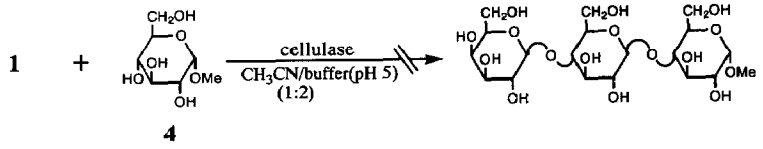


Fig. 4. Effect of substrate concentration of β-lactosyl fluoride **1** and reaction time on enzymatic lactosylation of methyl β-D-glucoside (**2**) as catalyzed by cellulase. The reaction was carried out at 30°C in acetate buffer (0.05 M, pH 5):MeCN = 2:1, cellulase 5.0 wt% for **1**. ○, 0.01 mol/L; ●, 0.25 mol/L; □, 0.05 mol/L; ■, 0.1 mol/L; △, 0.15 mol/L.



Scheme 3.

described in this paper, therefore, were performed at a substrate concentration of 0.15 M.

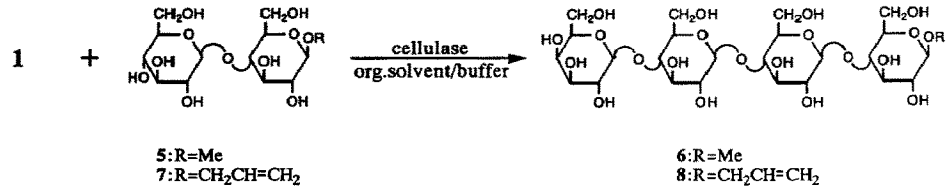
Other solvents, such as methanol and ethanol were also found effective for promotion of the lactosylation. The condensation reaction did not take place effectively in an acetate buffer (pH 0.05 M, pH 5) solution without using organic solvents; hydrolysis through the cleavage of the C–F bond of **1** occurred predominantly because of the preferential attack of a water molecule on the enzyme–substrate complex, affording lactose as the main product.

It may be noted that use of methyl α-D-glucopyranoside **4**, the anomer of **2**, as the glycosyl acceptor gave no condensation product; hydrolysis of **1** to lactose occurred quantitatively and **4** was recovered unchanged (Scheme 3). This result indicates that the configuration at C-1 of the glycosyl acceptor is an important factor which controls the course of lactosylation. This result may be explained by a steric repulsion between the anomeric methoxy group of **4** and an amino acid residue at the active site of the enzyme. The repulsion disturbs a suitable orientation of the 4-hydroxyl group of the glycosyl acceptor **4** toward the activated anomeric carbon atom of the lactose moiety.

A cellulase-catalyzed condensation reaction between **1** and methyl β-cellobioside (Glc-Glc-OMe, **5**) was performed in an aqueous methanol medium (4:1 MeOH–acetate buffer) giving rise to the corresponding tetrasaccharide, methyl *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside (Gal-Glc-Glc-Glc-OMe, **6**). The HPLC recording of the mixture after 6 h showed a significant decrease of the starting glycosyl donor **1** and a peak derived from the condensation product appeared. During the course of the reaction, the resulting oligomer precipitated from the reaction system because of its low solubility in the solvent (4:1 MeOH–acetate buffer). This behavior greatly simplified the isolation procedure. In fact, filtration of the product gave the desired tetrasaccharide **6** with > 90% purity in 36% yields (Scheme 4).

The structure of the oligomer was determined by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum showed four doublet peaks with coupling constants of 7.7–8.0 Hz at δ 4.56 (two peaks are overlapped), 4.47, and 4.42 ppm, indicating that all the glycosidic bonds in the tetrasaccharide are of the β type. In the ^{13}C NMR three peaks near 103 ppm due to the anomeric carbon atoms clearly indicates the regio- and stereo-selective formation of the β-(1 → 4) linkage.

Allyl β-cellobioside **7** was similarly lactosylated in an allyl alcohol–acetate



Scheme 4.

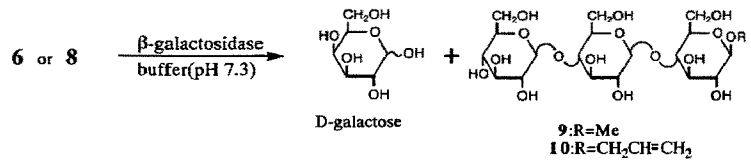
buffer (0.05 M, pH 5) solution giving rise to the corresponding tetrasaccharide (Gal-Glc-Glc-Glc-$OCH_2CH{=}CH_2$,**8**) whose structure was confirmed by ^1H and ^{13}C NMR spectroscopy.

The tetrasaccharide **6** was converted into the corresponding trisaccharide derivative, methyl cellotrioside (Glc-Glc-Glc-OMe, **9**) by a preferential cleavage of the glycosidic bond between the terminal D-galactose unit and the adjacent D-glucose unit by β-galactosidase. The structure of **9** was identified by comparing the ^1H and ^{13}C NMR spectra of the product with those reported in the literature[17]. Of the β-galactosidases screened, an enzyme from *E. coli* gave the most satisfactory result with regard to the regioselectivity. The reaction catalyzed by β-galactosidases from bovine liver, *S. fragilis*, and *A. oryzae* showed less regioselectivity; the glycosidic bond-cleavage between the second and the third glucose units also took place, affording the corresponding lactose and methyl β-cellobioside. The enzymatic degalactosylation reaction was also demonstrated by using the tetrasaccharide **8**, affording allyl β-cellotrioside **10**. The use of an allyl protecting group at the anomeric carbon of the glycosyl acceptor enabled us to convert the product into cellooligosaccharide (Scheme 5).

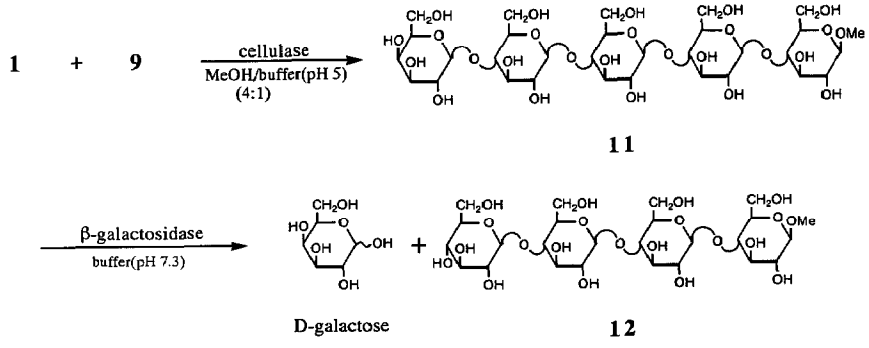
The resulting methyl β-cellotrisaccharide **9** was further subjected to condensation with **1** in an aqueous organic solvent to give the corresponding pentasaccharide (Gal-Glc-Glc-Glc-Glc-OMe, **11**). When a solvent of 4:1 MeOH–acetate buffer was used as solvent, the resulting product from the oligosaccharide precipitated as the condensation reaction proceeded. The resulting pentasaccharide was converted into the corresponding cellotetraoside (Glc-Glc-Glc-Glc-OMe, **12**) by enzymatic degalactosylation according to the same procedure as above (Scheme 6).

The regio- and stereo-selective formation of the β-(1 → 4) glycosidic linkage may be explained by assuming a double displacement mechanism[17].

The present new methodology, which involves a one glucose-unit elongation process, enables us to prepare cellooligosaccharide derivatives having a definite



Scheme 5.



Scheme 6.

degree of polymerization. In addition, the present methodology of using a glycosyl fluoride as substrate for a hydrolytic enzyme in an organic to water mixed solvent has opened a way to develop a new useful synthetic tool in glycotechnology. Further attempts for synthesis of longer cellooligomers and for elucidation of detailed reaction mechanism are now in progress.

EXPERIMENTAL

General methods.—^{1}H and ^{13}C NMR spectra were recorded on a 250 MHz Bruker AC250T NMR spectrometer. The positions of peaks are expressed from the Me_4Si signal in $CDCl_3$. Chemical shifts (ppm) are given using sodium 4,4-dimethyl-[2,2,3,3-$^2H_4$]-4-silapentanoate (0.00 ppm) and 1,4-dioxane (67.40 ppm) in D_2O. The chemical shift of ^{19}F NMR was determined from an external standard of hexafluorobenzene. HPLC analyses were performed by using a Hitachi 655A apparatus with a RI detector under the following conditions: Merck Lichrosorb RP-18 column with water eluent at a flow rate of 1.0 mL/min. For preparative HPLC, a Merck Lichrosorb RP-18-5 column (10 × 250 mm) was used. The silica gel used for column chromatography was Merck Kieselgel 60 (70–230 mesh). Solvents, MeCN and MeOH, were purified by distillation over CaH_2 and Mg, respectively. Methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, lactose, and cellobiose were purchased from Nakarai Chemicals (Japan) and used without purification. The substrate β-lactosyl fluoride was prepared by a procedure similar to that for the synthesis of β-cellobiosyl fluoride described in the literature[18–20]. ^{1}H NMR (D_2O) δ 5.18 ppm (dd, J_{1F} 52.9, J_{12} 7.1 Hz; 1H); ^{19}F NMR (D_2O) δ −9.22 ppm (dd, J_{1F} 52.9, J_{2F} 14.7 Hz). Methyl β-cellobioside and allyl β-cellobioside were prepared by the reaction of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiosyl bromide with excess of MeOH or allyl alcohol followed by deacetylation according to the literature[21,22]. Cellulase Onozuka R-10 from *Trichoderma viride* (activity with *O*-(carbomethoxy)cellulose: 6.5 units/mg solid, pH 4.5, 30°C) was obtained from Yakult company (Japan). β-Galactosidase from *Aspergillus oryzae* (standardized with starch, activity with lactose: 3.8 units/mg solid, pH 7.3, 37°C), bovine

liver (lyophilized powder containing ~95% protein (Biuret) and ~5% buffer salts activity with *o*-nitrophenyl β-D-galactopyranoside: 0.14 units/mg solid, pH 7.3, 37°C), *S. fragilis* (partially purified preparation in 50% glycerol solution containing 5.7 mg protein/mL (Biuret), activity with *o*-nitrophenyl β-D-galactopyranoside: 9.4 units/mg protein, pH 7.2, 37°C), *E. coli* (lyophilized powder containing 73% protein (Biuret) activity with *o*-nitrophenyl β-D-galactopyranoside; 890 units/mg protein, pH 7.3, 37°C; balanced primarily by Tris buffer salts and $MgCl_2$) were purchased from Sigma Co.

Methyl O-β-D-*galactopyranosyl-(1 → 4)*-O-β-D-*glucopyranosyl-(1 → 4)*-β-D-*glucopyranoside* (**3**).—To a solution of β-lactosyl fluoride (**1**, 54 mg, 0.16 mmol) and methyl β-D-glucopyranoside (**2**, 32 mg, 0.16 mmol) in 0.05 M acetate buffer (pH 5, 0.5 mL) was added MeCN (0.3 mL). To this mixture was added a solution of cellulase (3.0 mg, 4.4 units, 5 wt% for the substrates) in 0.05 M acetate buffer (0.1 mL) and the resulting mixture was stirred for 30 min at 30°C. After adding 20 mL of MeCN, the mixture was heated for 10 min at 100°C to deactivate the cellulase. The solvent was evaporated to dryness and the residue dissolved in water. Water-insoluble material derived from the enzyme protein was filtered off and the filtrate was directly analyzed by HPLC (51%yield, determined by HPLC). A part of the solution was used for purification by preparative HPLC. ^1H NMR (D_2O): δ 4.54 (d, 1 H, *J* 8.0 Hz), 4.47 (d, 1 H, *J* 7.7 Hz), 4.42 (d, 1 H, *J* 8.0 Hz), 3.59 (s, 3 H, $-OCH_3$). ^{13}C NMR (D_2O): δ 103.9, 103.8, 103.2 (anomeric carbons), 79.4, 78.9 (C4 or C4′), 69.4 (C4″), and 58.0 (OCH_3). Anal. Calcd for $C_{19}H_{34}O_{16}$ $2H_2O$: C, 41.16; H, 6.91. Found: C, 41.16; H, 6.85.

Methyl O-β-D-*galactopyranosyl-(1 → 4)*-O-β-D-*glucopyranosyl-(1 → 4)*-O-β-D-*glucopyranosyl-(1 → 4)*-β-D-*glucopyranoside* (**6**).—The substrate **1** (213 mg, 0.62 mmol) and methyl β-cellobioside **5** (221 mg, 0.62 mmol) were dissolved in a mixture of 0.05 M acetate buffer (pH 5, 3.0 mL) and MeOH (20 mL). A 0.05 M acetate buffer (pH 5, 2.0 mL) solution of cellulase (11 mg, 17 units, 5 wt% for the substrate) was added and the resulting mixture was stirred for 6 h at 30°C. The white precipitate was filtered and dried in vacuo to afford 152 mg of **5** (34% yield). ^1H NMR (D_2O): δ 4.56 (d, 2 H, *J* 7.8 Hz, two anomeric protons were overlapped), 4.47 (d 1 H, *J* 7.6 Hz), 4.42 (d, 1 H, *J* 7.9 Hz), 3.60 (s, 3 H, $-OCH_3$). ^{13}C NMR (D_2O): δ 103.9, 103.8, 103.2 (anomeric carbons, two anomeric carbons are overlapped at 103 2 ppm), 58.1 (OCH_3). Anal. Calcd for $C_{25}H_{44}O_{21}$ $2H_2O$: C, 41.90; H, 6.75. Found: C, 42.06; H, 6.50.

Allyl O-β-D-*galactopyranosyl-(1 → 4)*-O-β-D-*glucopyranosyl-(1 → 4)*-O-β-D-*glucopyranosyl-(1 → 4)*-β-D-*glucopyranoside* (**8**).—The substrate **1** (23.7 mg, 0.069 mmol) and allyl β-cellobioside (**7**, 26.3 mg, 0.069 mmol) were dissolved in a mixture of 0.05 M acetate buffer (pH 5, 0.3 mL) and allyl alcohol (2.2 mL). To this mixture was added a 0.05 M acetate buffer (pH 5, 0.3 mL) solution of cellulase (1.2 mg, 1.8 units, 5 wt% for the substrate) and the resulting mixture was stirred for 6 h at 30°C. The yield of condensation product of allylated tetrasaccharide **8** was determined to be 13% by analytical HPLC. A part of the crude mixture was

purified by preparative HPLC for NMR measurements; ^{1}H NMR (D_2O): δ 5.27–5.42 (m, 3 H, alkenic protons), 4.54 (d, J 7.8 Hz, three anomeric protons were overlapped), 4.46 (d, 1 H, J 8.0 Hz). ^{13}C NMR (D_2O): 134.2, 119.6 (alkenic carbons of allyl group), 103.7, 103.3. 101.9 (anomeric carbons, two anomeric carbons of internal units were overlapped at 103.3 ppm).

Methyl O-β-D-galactopyranosyl-(1 → 4)-O-β-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside (**11**).—To a solution of **1** (45.8 mg, 0.13 mmol) and methyl β-cellotrioside **9** (49.8 mg, 0.096 mmol) in 0.05 M acetate buffer (0.5 mL, pH 5) was added 4.0 mL of MeOH. To this solution was added a solution of cellulase (2.3 mg, 3.5 units) in 0.05 M acetate buffer (0.5 mL, pH 5) and the mixture was stirred for 4 h at 30°C. The white precipitate was filtered and further purified by preparative HPLC using water as eluent, giving rise to 10 mg of pentasaccharide **11** (12% yield), ^{1}H NMR (D_2O): δ 4.56 (d, 3 H, J 8.2 Hz, three anomeric protons were overlapped), 4.48 (d, 1 H, J 7.5 Hz), 4.43 (d, 1 H, J 8.3 Hz), 3.60 (s, 3 H, $-OCH_3$). ^{13}C NMR (D_2O): δ 103.9, 103.8, 103.2 (anomeric carbons, three anomeric carbons are overlapped at 103.2 ppm), 58.1 (OCH_3).

Enzymatic degalactosylation.—A typical procedure for the degalactosylation of **6** by β-D-galactosidase is as follows: to a solution of **6** (124 mg, 0.18 mmol) in 0.05 M phosphate buffer (13 mL, pH 7.3) was added a 0.05 M phosphate buffer (1.0 mL, pH 7.3) solution of β-D-galactosidase (0.1 mL, 200 units) from *E. coli* and the mixture was shaken for 30 min at room temperature. After adding 40 mL of MeCN, the mixture was heated for 10 min at 100°C in order to deactivate the enzyme and the solvent was evaporated. The residue was dissolved in the minimum amount of water and water-insoluble material was filtered off. The filtrate was chromatographed by preparative HPLC to give 45 mg of methyl β-cellotrioside (Glc-Glc-Glc-OMe, **9**) in 48% yield.

ACKNOWLEDGMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (No. 03453114).

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