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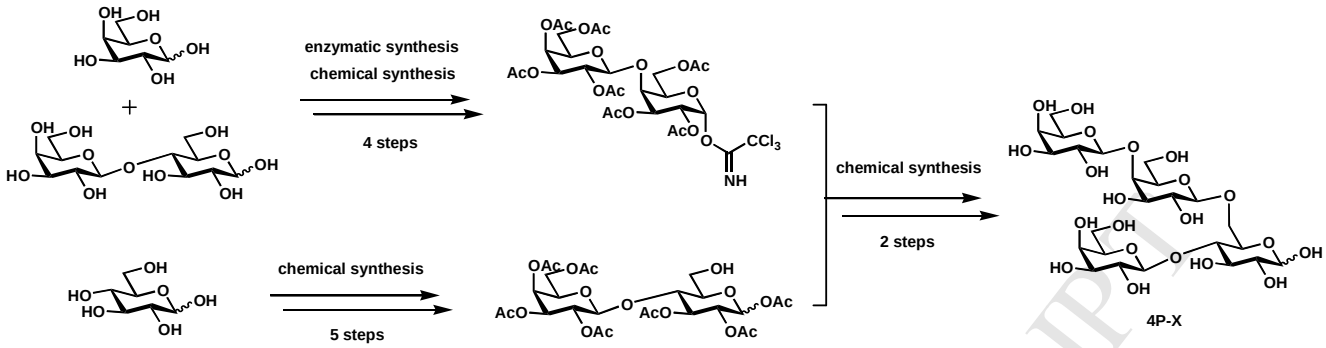

Synthesis of an allergy inducing tetrasaccharide "4P-X"

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Abstract

4P-X (β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-[β-D-galactopyranosyl-(1→4)]-β-D-glucopyranose) is included in galacto-oligosaccharides (GOSs) produced by β-galactosidase derived from *Bacillus circulans*. 4P-X has been known to induce particularly strong allergies. High purity 4P-X is essential for use as a standard to quantify the amount of 4P-X in GOSs; however, the isolation of high purity 4P-X has never been reported. In this study, we achieved the synthesis of 4P-X by a combination of organic and enzymatic chemical syntheses in a short time. This is the first report of isolated, high purity 4P-X.

Keywords: Galacto-oligosaccharides, 4P-X, allergy, oligosaccharide synthesis

Galacto-oligosaccharides (GOSs) is a general term for oligosaccharides that consist of a certain number of galactose units combined with lactose. GOSs exist naturally in human breast milk and bovine colostrum.[1] Various physiological functions of GOSs have been reported including regulating intestinal functions, promoting mineral absorption by improving the balance of enterobacterial flora, regulating the immune system, and preventing and improving inflammatory bowel disease.[2,3] GOSs have received particular attention for their prebiotic effects that promote the growth of *Bifidobacterium*, *Lactobacillus*, and other enteric bacteria.[4,5] Therefore, GOSs are commonly used in infant formula, beverages fermented by *Lactobacillus*, and yogurts. Some of these foods containing GOSs are certified as Food for Specified Health Uses by the Consumer Affairs Agency in Japan, and GOSs are certified as generally

recognized as safe (GRAS) substances by the U.S. Food and Drug Administration (GRAS Notices: GRN 233, 236, 285, 286, 334, 484, 489, 495, 518, and 569). In general, GOSs are produced by a transgalactosylating reaction with β-galactosidase (EC.3.2.1.23). β-Galactosidase is produced in many microorganisms such as *Bacillus circulans, Aspergillus oryzae, Kluyveromyces marxianus, Kluyveromyces fragilis, Sporobolomyces singularis*, and *Lactobacillus fermentum*.[6–11] As far as we know, β-galactosidases differ in their three-dimensional structures, resulting in stereo- and regioselectivity of glycosidic bonds. For example, fungal species such as *Aspergillus* regioselectively produce β1-6 bond (6′-GOS), while bacteria such as *Bacillus* regioselectively produce β1-4 bond (4′-GOS).[12] Moreover, β-galactosidase produced by *B. circulans* possesses particularly strong transglycosidation activity, and thus, GOS prepared by *B. circulans* are sold worldwide as Vivinal® GOS by FrieslandCampina (Netherlands).

Recently, several reports of allergy symptoms caused by GOSs have been published.[13–15] The first was a 1996 report from Japan that determined allergy symptoms among workers at an oyster farm in Hiroshima Prefecture after they consumed a lactobacillus beverage containing 6′-GOS produced by fungal β-galactosidase.[16] This report indicated a correlation between the allergies with sea-squirt asthma, and the authors suspected there may be some commonalities between 6′-GOS and sea-squirt antigens. Allergy symptoms have also been observed with 4′-GOS produced by bacterial β-galactosidase. In 2014, Kaneko *et al.* observed that GOS produced by β-galactosidase derived from *B. circulans* induced particularly strong allergic reactions compared with GOSs produced by other microorganisms[17] and revealed that the allergies were caused by two tetrasaccharides [Gal β1-4 (Gal β1-4 Gal β1-6) Glc, Gal β1-4 Gal β1-4 Gal β1-3 Glc]. Most notably, the former branched tetrasaccharide, commonly referred to as 4P-X, is seen as the most problematic. In that report, 4P-X was separated and quantitated by pyridylamination of a GOS sample followed by the use of three types of HPLC columns (Shodex KS-802, Shimadzu STR-ODS-II, Shimadzu ODS); however, this analysis system was extremely complex. Alternatively, Van Leeuwen *et al.* reported that it was generally possible to isolate structural isomers of Vivinal® GOS by ion chromatography (Dionex ICS-3000)[18,19] and over 40 structures (over 99%) of Vivinal® GOS have been characterized, including release of their NMR spectra; however, 4P-X was mixed with another isomer, and high purity 4P-X is not yet available. Since 4P-X is a high-risk allergen and important oligosaccharide, it is essential to obtain high purity 4P-X for the sake of future investigations. The goal of this research is to establish a fast and accurate quantitative analysis system for 4P-X. This system will enable us to evaluate the allergy

risk of GOS-containing foods. To accomplish this, high purity 4P-X is needed for use as a reference standard. By combining organic and enzymatic chemical syntheses, we successfully prepared high purity 4P-X in a short time.

Synthetic strategy for 4P-X (15). In order to efficiently prepare 4P-X, we used retrosynthetic analysis to reveal the convergent synthetic route shown in Figure 1. The tetrasaccharide is formed by coupling lactose **7** and galactobiose **13** derivatives.

The synthesis of the lactose derivative **7** has been reported. S. Tejima synthesized the lactose derivative **7** in short steps, starting from commercially available lactose monohydrate.[20] However, the use of highly toxic mercuric acetate was problematic. Therefore, we planned to synthesize the lactose derivative **7** by glycosylation of the glucose derivative **4** and the commercially available galactose derivative **5** without the use of highly toxic reagents.

The synthesis of the galactobiose derivative **13** has not been reported. We deduced that the synthesis of the galactobiose derivative **13** by the organic chemical synthesis requires multi-step processes. Therefore, in order to reduce the synthetic processes, we planned to adopt a combination of enzymatic and organic chemical methods. In particular, Galactobiose **10** is prepared by the enzymatic synthesis, and converted to the galactobiose derivative **13** by the organic chemical reactions.

Galactobiose **10** is not commercilally available, so we planned to prepare by transglycosylation of lactose and galactose using β-galactosidase. We selected Biolacta FN5 derived from *B. circulans*. It was known that β-galactosidase derived from *B. circulans* regioselectively produces β1-4 bond (4'-GOS),[21] we inferred that this is suitable for the preparation of Galactobiose **10**. We expected that this combination of enzymatic and chemical synthesis will result in the efficient synthesis of this galactobiose derivative.

Through the all processes, in order to simplify the deprotection process, we chose acetyl group as protecting groups as much as possible.

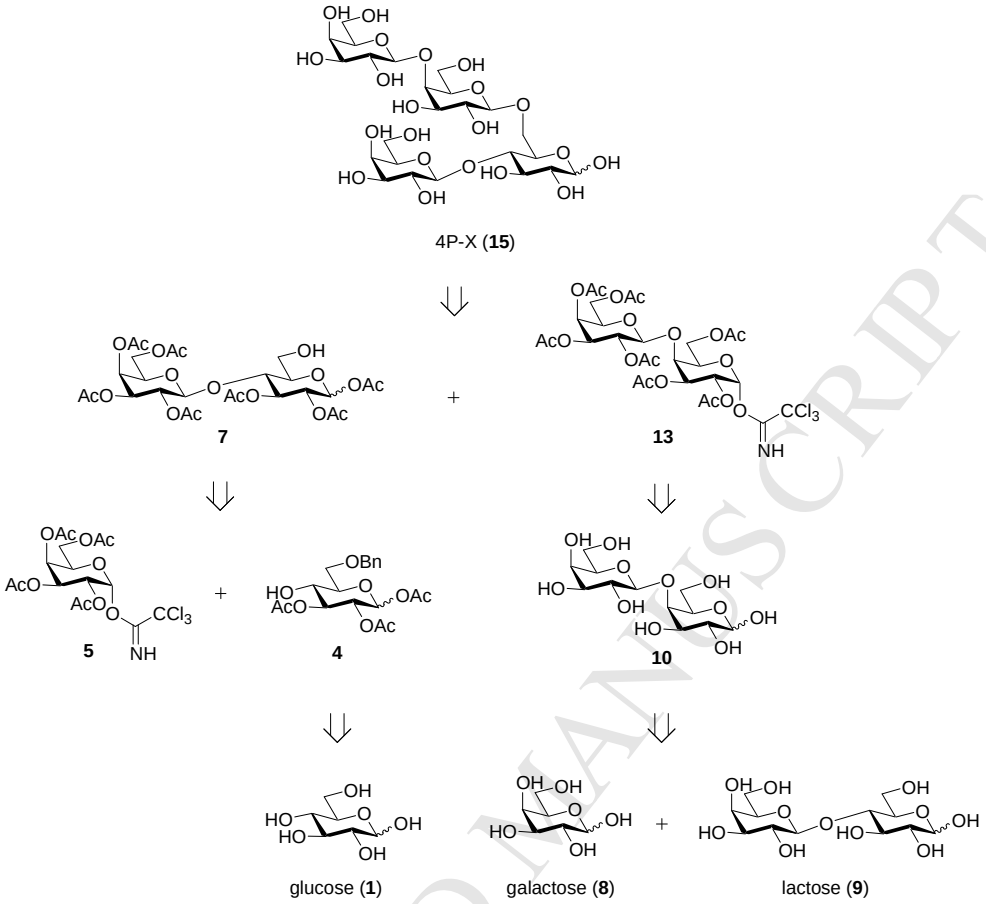
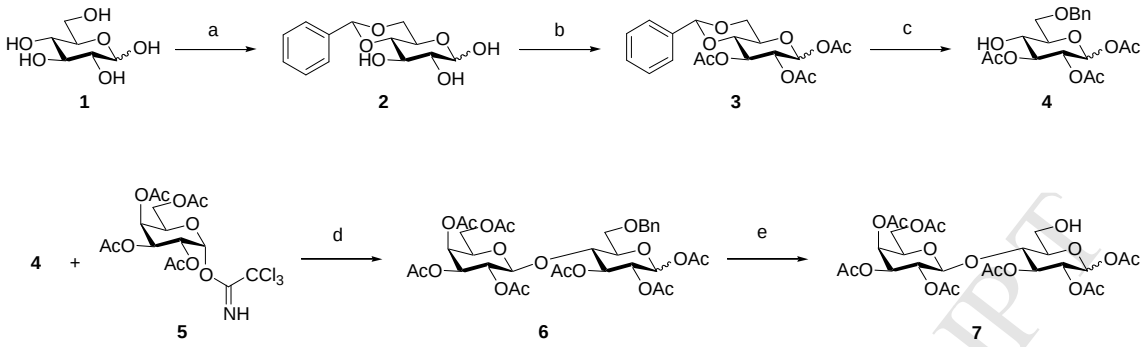


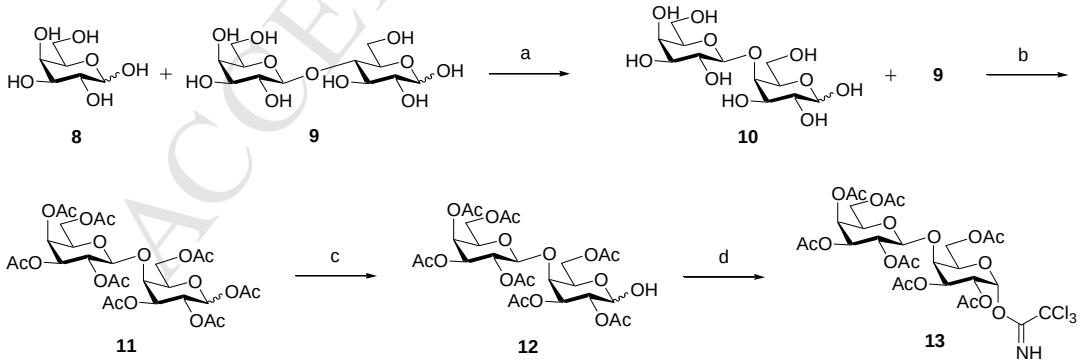
Figure 1. Retrosynthetic analysis of 4P-X (**15**).

Synthesis of lactose acceptor 7. The lactose acceptor **7** was synthesized as shown in Scheme 1. Glucose (**1**) was treated with benzaldehyde dimethyl acetal in the presence of a catalytic *p*-toluenesulfonic acid monohydrate (*p*-TsOH–H_2O) to give the benzyliden-protected glucose **2** (53.7%).[22] The protected glucose **2** was treated with acetic anhydride and 4-dimethylaminopyridine (DMAP) to afford the further acetylated glycoside **3** (93.8%). The benzylidene acetal in **3** was regioselectively cleaved by triethylsilane in the presence of trifluoroacetic acid (TFA) to afford the protected glycoside **4** (61.0%).[23] Glycosylation of glucose donor **4** and the commercially available galactose acceptor **5** in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and 4 Å molecular sieves afforded disaccharide **6** (53.1%). Deprotection of the benzyl group from disaccharide **6** was performed by palladium-catalyzed hydrogenation delivering disaccharide **7** (93.3%).



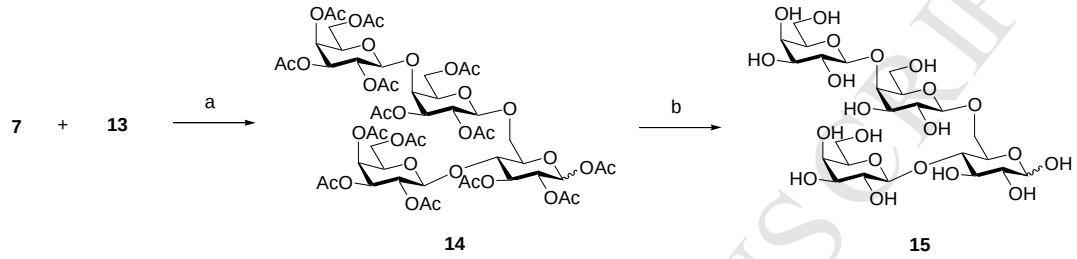
Scheme 1. Reagents and conditions: (a) $PhCH(OMe)_2$, *p*-TsOH–H_2O, DMF, 60 °C, 6 h, 54%; (b) Ac_2O, DMAP, pyr., rt, 1 h, 94%; (c) Et_3SiH, TFA, MS4Å, CH_2Cl_2, 0 °C to rt, 4.5 h, 61%; (d) TMSOTf, MS4Å, CH_2Cl_2, −40 °C, 1 h; −20 °C, 2 h, 53%; (e) H_2 gas, Pd/C, MeOH, rt, 22 h, 93%.

Synthesis of galactobiose donor 13. The galactobiose donor **13** was synthesized as shown in Scheme 2. Transglycosylation of galactose acceptor **8** and lactose donor **9** by Biolacta FN5® afforded GOSs containing galactobiose **10**; a mixture of galactobiose **10** and lactose (**9**) (3.3:1) was obtained by activated carbon purification. The mixture was acetylated to acetylated galactobiose **11** and acetylated lactose. This mixture was purified by silica gel column chromatography to afford pure acetylated galactobiose **11** (67.9%). The anomeric acetyl group was selectively deprotected from acetylated galactobiose **11** by piperidine,[24] resulting in disaccharide **12** (76.0%). Disaccharide **12** was treated with trichloroacetonitrile and 1,8-diazabicyclo[5,4,0]-7-undecane (DBU) to afford the trichloroimidate derivative **13** (86.1%).



Scheme 2. Reagents and conditions: (a) Biolacta FN5®, 50 °C, 24 h, 45% (**10**:**9** = 3.3:1); (b) Ac_2O, DMAP, pyr., rt, 21.5 h, 68%; (c) piperidine, THF, rt, 30 h, 76%; (d) CCl_3CN, DBU, CH_2Cl_2, rt, 4 h, 86%.

Synthesis of 4P-X (15). 4P-X was synthesized as shown in Scheme 3. Glycosylation of galactobiose donor **13** and lactose acceptor **7** in the presence of TMSOTf and 4 Å molecular sieves afforded tetrasaccharide **14** (43.4%). The acetyl groups were deprotected from tetrasaccharide **14** by NaOMe, resulting in disaccharide 4P-X (**15**) (81.9%). The structure of **15** was confirmed by ^{1}H-NMR (δ = 4.49 ppm, d, $J_{1,2}$ = 7.8 Hz, 0.6H, H-1″ and δ = 4.48 ppm, d, $J_{1,2}$ = 7.8 Hz, 0.4H, H-1″) and ^{13}C-NMR spectroscopy.



Scheme 3. Reagents and conditions: (a) TMSOTf, MS4Å, CH_2Cl_2, −40 °C, 2 h, 43%; (b) NaOMe, MeOH–H_2O, rt, 6 h, 82%.

In summary, we have succeeded in the synthesis of high purity 4P-X. Isolation and purification of 4P-X from a GOS prepared from lactose by incubation with β-galactosidase is difficult and, as a result, this is the first time high purity 4P-X has been reported. In future, we plan to use the synthesized 4P-X as a standard material to establish a fast and accurate analysis method for its quantification in the GOS-containing foods. This analysis method will enable us to evaluate the allergy risk of GOS-containing foods.

1. Experimental

1.1. General methods

All reagents and solvents were obtained from commercial suppliers and used without further purification. The production of galactobiose was accomplished with Biolacta FN5® (Amano Enzyme Inc.). Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates 60 F_{254} (Merck). Silica gel column chromatography was performed on silica gel 60N (63–210 µm) (Kanto Chemical Co., Inc.) or silica gel 60 (40–100 µm) (Kanto Chemical Co., Inc.). Carbon column chromatography was performed on activated carbon (Wako Pure Chemical Industries, Ltd.). Optical rotations were measured with a JASCO DIP-1000 Digital Polarimeter. IR

spectra were recorded on a JASCO FT/IR-4200 Fourier transform infrared spectrophotometer. ^{1}H- (400 and 600 MHz) and ^{13}C-NMR spectra (100 and 150 MHz) were recorded on JEOL JNM-ECX-400P and Varian NMR System 600 spectrometers, respectively. For ^{1}H-NMR spectra, chemical shifts (δ) are referenced using TMS (0.00 ppm) in $CDCl_3$ and acetone (2.23 ppm) in D_2O as internal standards. For ^{13}C-NMR spectra, chemical shifts (δ) are referenced using $CDCl_3$ (77.0 ppm) and acetone (31.1 ppm) in D_2O as internal standards. High-resolution mass spectra were recorded on JEOL JMS-T100LP AccuTOF LC-plus4G.

1.2. 4,6-*O*-Benzyliden-D-glucopyranose (2)

A suspension of D-glucose (**1**) (1.00 g, 5.55 mmol) in DMF (11 mL) was heated to 60 °C to dissolve the solid. Benzaldehyde dimethyl acetal (1.24 mL, 8.33 mmol) and *p*-toluenesulfonic acid monohydrate (10 mg) were added. The resulting solution was stirred at 60 °C for 6 h, depressurizing 10 min for each hour in order to remove MeOH. The mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (MeOH/AcOEt = 1:10, v/v) to give glucopyranose **2** (799.5 mg, 2.98 mmol, 53.7%) as a white solid.

2: $[\alpha]_D^{20}$ +12.9 (*c* 0.20, MeOH); ^{1}H-NMR (400 MHz, CD_3OD): δ 7.46–7.42 (m, 2H), 7.38–7.35 (m, 3H), 6.31 (d, *J* = 4.1 Hz, 0.5H), 5.79 (d, *J* = 7.8 Hz, 0.5H), 5.60 (t, *J* = 9.9 Hz, 0.5H), 5.52 (s, 0.5H), 5.51 (s, 0.5H), 5.37 (t, *J* = 9.2 Hz, 0.5H), 5.17–5.10 (m, 1H), 4.39 (dd, *J* = 10.3, 4.6 Hz, 0.5H), 4.32 (dd, *J* = 10.5, 5.0 Hz, 0.5H), 4.08–4.00 (m, 0.5H), 3.80–3.63 (m, 2.5H), 2.19 (s, 1.5H), 2.11 (s, 1.5H), 2.08 (s, 1.5H), 2.06 (s, 1.5H), 2.05 (s, 1.5H), 2.04 (s, 1.5H); ^{13}C-NMR (100 MHz, CD_3OD): δ 139.2, 139.1, 129.9, 129.0, 127.5, 103.0, 102.9, 99.0, 94.7, 83.1, 82.4, 77.2, 74.7, 74.4, 71.8, 70.3, 69.8, 67.7, 63.5; HRMS (ESI): calcd for $C_{13}H_{16}O_6Na$ ($[M+Na]^+$): 291.0845, found: 291.0842.

1.3. Acetyl 2,3-di-*O*-acetyl-4,6-*O*-benzyliden-D-glucopyranoside (3)

To a solution of glucopyranose **2** (1.42 g, 5.28 mmol) in pyridine (26 mL) was added acetic anhydride (7.5 mL, 79.2 mmol) and 4-dimethylaminopyridine (193 mg, 1.58 mmol). The resulting solution was stirred at rt for 1 h. The mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 1:1, v/v) to give glucopyranoside **3** (1.95 g, 4.96 mmol, 93.8%) as a white solid.

3: $[\alpha]_D^{20}$ +16.8 (*c* 0.20, CH_2Cl_2); IR (CH_2Cl_2): 1756, 1370, 1261, 1071 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.46–7.42 (m, 2H), 7.38–7.35 (m, 3H), 6.31 (d, *J* = 4.1 Hz, 0.5H),

5.79 (d, *J* = 7.8 Hz, 0.5H), 5.60 (t, *J* = 9.9 Hz, 0.5H), 5.52 (s, 0.5H), 5.51 (s, 0.5H), 5.37 (t, *J* = 9.2 Hz, 0.5H), 5.17–5.10 (m, 1H), 4.39 (dd, *J* = 10.3, 4.6 Hz, 0.5H), 4.32 (dd, *J* = 10.5, 5.0 Hz, 0.5H), 4.08–4.00 (m, 0.5H), 3.80–3.63 (m, 2.5H), 2.19 (s, 1.5H), 2.11 (s, 1.5H), 2.08 (s, 1.5H), 2.06 (s, 1.5H), 2.05 (s, 1.5H), 2.04 (s, 1.5H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 169.93, 169.90, 169.86, 169.5, 169.1, 168.8, 136.64, 136.56, 129.2, 129.1, 128.2, 126.10, 126.06, 78.6, 77.9, 71.7, 71.2, 69.8, 68.7, 68.5, 68.2, 67.0, 64.9, 20.9, 20.8, 20.7, 20.6, 20.5; HRMS (ESI): calcd for $C_{19}H_{22}O_9Na$ ($[M+Na]^+$): 417.1162, found: 417.1165.

1.4. Acetyl 2,3-di-*O*-acetyl-6-*O*-benzyl-D-glucopyranoside (4)

To a solution of glucopyranoside **3** (1.01 g, 2.57 mmol) in dry CH_2Cl_2 (26 mL) was added activated 4 Å molecular sieves, the resulting mixture was stirred at rt for 10 min, then triethylsilane (4.09 mL, 25.7 mmol) was added. The resulting mixture was cooled to 0 °C then TFA (1.97 mL, 25.7 mmol) was added dropwise over 5 min. The resulting mixture was stirred at rt for 4.5 h, then cooled to 0 °C and quenched with trimethylamine (4 mL) and H_2O (40 mL). The solution was separated into CH_2Cl_2 and aqueous layers. The aqueous layer was extracted with AcOEt (50 mL × 2). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 1:1, v/v) to give glucopyranoside **4** (622 mg, 1.57 mmol, 61.0%) as a colorless oil.

4: $[\alpha]_D^{20}$ +50.2 (*c* 0.30, CH_2Cl_2); IR (CH_2Cl_2): 2873, 1754, 1371, 1221, 1083, 1044 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.38–7.27 (m, 5H), 6.30 (d, *J* = 3.7 Hz, 0.6H), 5.69 (d, *J* = 7.8 Hz, 0.4H), 5.33 (t, *J* = 9.9 Hz, 0.6H), 5.13–5.00 (m, 1.4H), 4.63–4.52 (m, 2H), 3.97–3.91 (m, 0.6H), 3.86–3.76 (m, 2H), 3.76–3.62 (m, 1.4H), 2.15 (s, 1.8H), 2.10 (s, 1.8H), 2.088 (s, 1.2H), 2.086 (s, 1.2H), 2.03 (s, 1.2H), 2.01 (s, 1.8H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.3, 171.1, 169.9, 169.5, 169.1, 137.40, 137.35, 128.4, 127.9, 127.8, 127.7, 91.8, 89.3, 75.4, 74.9, 73.8, 73.7, 72.5, 72.4, 70.3, 70.0, 69.9, 69.3, 69.2, 20.88, 20.85, 20.78, 20.77, 20.55, 20.46; HRMS (ESI): calcd for $C_{19}H_{24}O_9Na$ ($[M+Na]^+$): 419.1318, found: 419.1320.

1.5. Acetyl ,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzyl-D-glucopyranoside (6)

A mixture of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl 2,2,2-trichloroacetimidate (**5**) (374 mg, 0.759 mmol) (Tokyo Chemical Industry Co., Ltd.) and glycosyl donor **4**

(201 mg, 0.506 mmol) was co-evaporated with toluene (2 mL) and dissolved in dry CH_2Cl_2 (2 mL). Activated 4 Å molecular sieves were added and the resulting mixture was stirred at rt for 10 min. TMSOTf in CH_2Cl_2 (51 mM, 1.0 mL, 51 μmol) was added dropwise and the mixture was stirred at −40 °C for 30 min after which TMSOTf in CH_2Cl_2 (51 mM, 2.0 mL, 102 μmol) was added dropwise and the mixture was stirred at −40 °C for 30 min. The mixture was then heated to −20 °C and stirred for 2 h. Then, the mixture was diluted with CH_2Cl_2 (10 mL) and stirred for 30 min. The mixture was poured into sat. $NaHCO_3$ aq. (20 mL) at 0 °C and was separated into CH_2Cl_2 and aqueous layers. The aqueous layer was extracted with AcOEt (50 mL × 2). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 1:1 to 2:1, v/v) to give disaccharide **6** (195 mg, 0.269 mmol, 53.1%) as a white solid.

6: $[\alpha]_D^{20}$ +26.9 (*c* 0.25, CH_2Cl_2); IR (CH_2Cl_2): 1754, 1370, 1223, 1049 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.43–7.34 (m, 5H), 6.30 (d, *J* = 3.6 Hz, 0.7H), 5.63 (d, *J* = 8.2 Hz, 0.3H), 5.40 (t, *J* = 9.9 Hz, 0.7H), 5.29 (d, *J* = 2.7 Hz, 0.7H), 5.26 (d, *J* = 2.8 Hz, 0.3H), 5.17 (t, *J* = 9.4 Hz, 0.3H), 5.10–4.95 (m, 2H), 4.81–4.73 (m, 2H), 4.47 (d, *J* = 11.9 Hz, 0.7H), 4.40–4.33 (m, 1H), 4.12–3.97 (m, 3.3H), 3.85 (d, *J* = 10.1 Hz, 0.7H), 3.77 (d, *J* = 2.8 Hz, 0.3H), 3.74 (d, *J* = 2.3 Hz, 1.0H), 3.68–3.56 (m, 2H), 2.17 (s, 2.1H), 2.14 (s, 2.1H), 2.13 (s, 0.9H), 2.10 (s, 0.9H), 2.08 (s, 3H), 2.03 (s, 2.1H), 2.023 (s, 0.9H), 2.017 (s, 0.9H), 2.00 (s, 2.1H), 1.963 (s, 2.1H), 1.957 (s, 0.9H), 1.94 (s, 2.1H), 1.93 (s, 0.9H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.4, 170.2, 170.0, 169.9, 169.5, 169.1, 168.8, 168.7, 137.4, 128.7, 128.3, 128.2, 100.3, 100.2, 91.9, 89.3, 75.2, 74.2, 74.0, 73.8, 73.7, 72.7, 72.5, 71.0, 70.9, 70.5, 70.4, 69.51, 69.48, 69.1, 69.0, 66.8, 66.7, 66.6, 21.0, 20.8, 20.74, 20.66, 20.63, 20.5; HRMS (ESI): calcd for $C_{33}H_{42}O_{18}Na$ ($[M+Na]^+$): 749.2269, found: 749.2257.

1.6. Acetyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3-di-*O*-acetyl-D-glucopyranoside (7)

To a solution of a disaccharide **6** (151 mg, 0.207 mmol) in MeOH (4.1 mL) was added 5% Pd/C (151 mg). The suspension was stirred at rt for 20 h under a hydrogen atmosphere. The mixture was filtered over Celite by elution with MeOH (10 mL × 4) and AcOEt (10 mL × 4). The combined filtrates were concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 2:1 to 4:1 to 1:0, v/v) to give disaccharide **7** (123 mg, 0.193 mmol, 93.3%) as a colorless oil.

7: $[\alpha]_D^{20}$ +26.6 (*c* 0.10, CH_2Cl_2); IR (CH_2Cl_2): 2993, 1754, 1605, 1370, 1223, 1059

cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.26 (d, *J* = 4.1 Hz, 0.7H), 5.68 (d, *J* = 8.7 Hz, 0.3H), 5.48 (t, *J* = 9.8 Hz, 0.7H), 5.36 (d, *J* = 3.7 Hz, 1H), 5.24 (t, *J* = 9.4 Hz, 0.3H), 5.16–5.09 (m, 1H), 5.05–4.97 (m, 1.7H), 4.96 (d, *J* = 3.7 Hz, 0.3H), 4.63 (d, *J* = 8.2 Hz, 0.7H), 4.62 (d, *J* = 8.2 Hz, 0.3H), 4.17–4.05 (m, 2H), 4.03–3.72 (m, 4.7H), 3.54 (d, *J* = 9.6 Hz, 0.3H), 2.17 (s, 2.1H), 2.16 (s, 2.1H), 2.15 (s, 0.9H), 2.10 (s, 0.9H), 2.07 (s, 4.2H), 2.06 (s, 3H), 2.052 (s, 0.9H), 2.047 (s, 0.9H), 2.03 (s, 0.9H), 2.02 (s, 2.1H), 1.972 (s, 2.1H), 1.969 (s, 0.9H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.5, 170.24, 170.15, 170.05, 169.8, 169.6, 169.2, 169.11, 169.06, 101.2, 101.0, 91.8, 89.3, 77.8, 75.7, 74.6, 74.4, 73.1, 71.1, 71.0, 70.8, 70.7, 69.8, 69.7, 69.4, 69.3, 66.9, 61.0, 60.1, 60.0, 21.0, 20.94, 20.87, 20.8, 20.7, 20.6; HRMS (ESI): calcd for $C_{26}H_{36}O_{18}Na$ ($[M+Na]^+$): 659.1799, found: 659.1795.

1.7. Acetyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-D-glactopyranoside (11)

Galactobiose **10** was synthesized by the transglycosylation reaction of β-galactosidase [accepter:galactose (**8**), donor:lactose (**9**)]. β-Galactosidase was obtained from Biolacta FN5® (Amano Enzymes) and derived from *B. circulans.* The transglycosylation reaction was performed at 50 °C. The buffer employed was 100 mM sodium phosphate (10 mL), pH 6.5. 0.01% of enzyme was added to the substrate solution containing 25% (w/v) lactose (2.50 g, 7.30 mmol) and 25% (w/v) galactose (2.50 g, 13.9 mmol), and was incubated for 24 h with shaking. The reaction was stopped by boiling (5 min at 100 °C). After boiling, the supernatant was collected by centrifugation (14,000 rpm, 5 min). The supernatant was loaded onto a charcoal column (φ 4.6 cm × 20 cm; solvent, H_2O), and monosaccharides were eluted with H_2O. After that, disaccharides that adhered to the charcoal were eluted stepwise with EtOH. The 7.5 % (v/v) of EtOH fraction gave a mixture of galactobiose **10** and lactose (**9**) (1.13 g, 3.29 mol, 45.0%) as a yellowish white solid. The ratio of galactobiose **10** and lactose (**9**) was 3.3:1, confirmed by HPLC analysis (CARBOSep CHO-620CA column, Transgenomic®; φ 6.5 mm × 300 mm).

To a solution of the mixture of galactobiose **10** and lactose (**9**) (555 mg, 1.62 mmol, **10**:**9** = 3.3:1) in pyridine (16 mL) was added acetic anhydride (2.3 mL, 24.3 mmol) and 4-dimethylaminopyridine (19.5 mg, 0.16 mmol). The resulting solution was stirred at rt for 3 h. Then, acetic anhydride (2.3 mL, 24 mmol) and DMAP (19.5 mg, 0.16 mmol) were added and the solution was stirred for 18.5 h. The mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane =

1:1.5 to 1:1 to 1:0 to MeOH/AcOEt/ = 1:10, v/v) to give disaccharide **11** (748 mg, 1.10 mmol, 67.9%) as a white solid.

11: $[\alpha]_D^{20}$ +48.0 (*c* 0.10, CH_2Cl_2); IR (CH_2Cl_2): 1752, 1371, 1225, 1068 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.29 (d, *J* = 3.7 Hz, 0.67H), 5.65 (d, *J* = 8.2 Hz, 0.33H), 5.38–5.36 (m, 1H), 5.35 (d, *J* = 4.1 Hz, 0.33H), 5.32 (d, *J* = 3.7 Hz, 0.33H), 5.29–5.18 (m, 1.67H), 5.00 (dd, *J* = 10.6, 3.7 Hz, 1H), 4.94 (dd, *J* = 10.1, 3.2 Hz, 0.33H), 4.44 (d, *J* = 7.8 Hz, 0.67H), 4.43 (d, *J* = 7.8 Hz, 0.33H), 4.41–4.33 (m, 1H), 4.24 (d, *J* = 2.8 Hz, 0.67H), 4.21–4.05 (m, 4.33H), 3.85 (t, *J* = 6.2 Hz, 1.33H), 2.19 (s, 1H), 2.17 (s, 2H), 2.15 (s, 1H), 2.14 (s, 2H), 2.13 (s, 2H), 2.12 (s, 1H), 2.113 (s, 2H), 2.110 (s, 1H), 2.07 (s, 3H), 2.050 (s, 2H), 2.047 (s, 1H), 2.02 (s, 1H), 2.002 (s, 3H), 1.998 (s, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.6, 170.5, 170.28, 170.25, 170.19, 170.1, 169.5, 169.4, 169.14, 169.11, 168.9, 101.9, 101.8, 91.8, 89.9, 74.5, 74.2, 73.1, 72.9, 70.72, 70.67, 70.63, 70.3, 70.0, 68.6, 68.5, 68.2, 66.8, 66.1, 63.4, 63.2, 61.3, 61.2, 20.89, 20.85, 20.82, 20.7, 20.63, 20.58, 20.50; HRMS (ESI): calcd for $C_{28}H_{38}O_{19}Na$ ($[M+Na]^+$): 701.1905, found: 701.1895.

1.8. 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-D-galactopyranose (12)

To a solution of disaccharide **11** (86.7 mg, 0.128 mmol) in THF (2.4 mL) was added piperidine in THF (2.0 M, 0.25 mL, 0.50 mmol). The resulting solution was stirred at rt for 30 h, then cooled to 0 °C and quenched with HCl aq. (1 N, 1 mL), H_2O (10 mL). The solution was extracted with AcOEt (2 × 30 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 2:1 to 4:1, v/v) to give disaccharide **12** (61.9 mg, 97.3 μmol, 76.0%) as a white solid.

12: $[\alpha]_D^{20}$ +43.1 (*c* 0.20, CH_2Cl_2); IR (CH_2Cl_2): 2941, 1750, 1370, 1227, 1057 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.40 (d, *J* = 3.6 Hz, 0.66H), 5.37 (d, *J* = 3.2 Hz, 1H), 5.31–5.17 (m, 2H), 5.03–4.89 (m, 2H), 4.61 (d, *J* = 7.3 Hz, 0.33H), 4.43–4.29 (m, 2.66H), 4.24–4.13 (m, 2H), 4.10 (d, *J* = 6.4 Hz, 2H), 3.85 (t, *J* = 6.6 Hz, 1H), 3.76 (dd, *J* = 7.1, 4.6 Hz, 0.33H), 2.17 (s, 1H), 2.16 (s, 2H), 2.15 (s, 1H), 2.12 (s, 5H), 2.085 (s, 4H), 2.081 (s, 2H), 2.05 (s, 3H), 2.004 (s, 1H), 1.997 (s, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.8, 170.6, 170.5, 170.4, 170.30, 170.26, 170.22, 170.15, 170.06, 169.7, 169.4, 101.9, 101.8, 95.6, 90.9, 75.3, 74.4, 72.7, 72.2, 71.2, 70.7, 70.5, 69.8, 68.7, 68.6, 67.8, 68.6, 67.85, 67.79, 66.9, 66.8, 63.9, 63.6, 61.4, 61.3, 20.9, 20.84, 20.77, 20.69, 20.64, 20.61, 20.58; HRMS (ESI): calcd for $C_{26}H_{36}O_{18}Na$ ($[M+Na]^+$): 659.1799, found:

659.1790.

1.9. 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-galactopyranosyl 2,2,2-trichloroacetimidate (13)

To a solution of disaccharide **12** (270 mg, 0.425 mmol) in CH_2Cl_2 (8.4 mL) was added Cl_3CCN (1.7 mL, 17.0 mmol) and DBU in THF (0.85 mM, 0.3 mL, 0.26 mmol). The resulting solution was stirred at rt for 4 h. The mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (0.5% Et_3N in AcOEt/Hexane = 2:1, v/v) to give disaccharide **13** (286 mg, 0.366 mmol, 86.1%) as a white solid.

13: $[\alpha]_D^{20}$ +75.1 (*c* 0.20, CH_2Cl_2); IR (CH_2Cl_2): 1751, 1370, 1226, 1070 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.64 (s, 1H), 6.51 (d, *J* = 3.7 Hz, 1H), 5.40–5.35 (m, 2H), 5.28 (dd, *J* = 11.0, 2.8 Hz, 1H), 5.20 (dd, *J* = 10.5, 7.8 Hz, 1H), 5.00 (dd, *J* = 10.5, 3.2 Hz, 1H), 4.43 (d, *J* = 8.2 Hz, 1H), 4.38 (dd, *J* = 11.9, 4.1 Hz, 1H), 4.31 (d, *J* = 3.2 Hz, 1H), 4.29 (d, *J* = 4.6 Hz, 1H), 4.15 (dd, *J* = 11.5, 7.3 Hz, 1 H), 4.10 (d, *J* = 6.4 Hz, 2H), 3.86 (t, *J* = 6.6 Hz, 1H), 2.17 (s, 3H), 2.14 (s, 3H), 2.12 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ ; 170.54, 170.49, 170.3, 170.2, 170.1, 169.5, 169.3, 160.8, 101.9, 93.8, 90.9, 74.5, 70.8, 70.6, 70.5, 70.2, 68.7, 66.8, 66.5, 63.5, 61.4, 20.9, 20.8, 20.7, 20.62, 20.57, 20.5; HRMS (ESI): calcd for $C_{28}H_{36}NO_{18}Cl_3Na$ ($[M+Na]^+$): 802.0896, found: 802.0876.

1.10. Acetyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→6)-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)]-2,3-di-*O*-acetyl-β-D-glucopyranoside (14)

A mixture of glycosyl acceptor **13** (47.9 mg, 61.4 μmol) and glycosyl donor **7** (26.0 mg, 40.9 μmol) was co-evaporated with toluene (2 mL) and dissolved in dry CH_2Cl_2 (1 mL). Activated 4 Å molecular sieves were added and the resulting mixture was stirred at rt for 10 min. TMSOTf in CH_2Cl_2 (4.1 mM, 1.0 mL, 4.1 μmol) was added dropwise and the mixture was stirred at −40 °C for 2 h. Then, the mixture was diluted with CH_2Cl_2 (10 mL) and stirred for 10 min. The mixture was poured to sat. $NaHCO_3$ aq. (30 mL) at 0 °C, and the solution was separated to CH_2Cl_2 layer and the aqueous layer. The aqueous layer was extracted with AcOEt (50 mL × 2). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (AcOEt/Hexane = 4:1 to 1:0, v/v) to give

tetrasaccharide **14** (22.3 mg, 17.8 µmol, 43.4%) as a colorless oil.

14: $[\alpha]_D^{20}$ +22.5 (*c* 0.41, CH_2Cl_2); IR (CH_2Cl_2): 1752, 1370, 1225, 1055 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.25 (d, *J* = 3.7 Hz, 0.8H), 5.62 (d, *J* = 8.2 Hz, 0.2H), 5.42 (dd *J* = 10.1, 8.2 Hz, 0.8H), 5.37 (t, *J* = 3.6 Hz, 2H), 5.20–5.05 (m, 4H), 5.03–4.97 (m, 2H), 4.89 (dd, *J* = 10.3, 3.2 Hz, 1H), 4.66 (d, *J* = 7.3 Hz, 0.8H), 4.60–4.56 (m, 1H), 4.50 (d, *J* = 12.1, 4.1 Hz, 0.8H), 4.42–4.38 (m, 1H), 4.21–4.05 (m, 6.4H), 4.00 (t, *J* = 6.9 Hz, 1H), 3.93–3.85 (m, 4.8H), 3.73 (dd, *J* = 6.4, 4.6 Hz, 1H), 2.19 (s, 3H), 2.16 (s, 3H), 2.149 (s, 3H), 2.146 (s, 3H), 2.13 (s, 3H), 2.11 (s, 3H), 2.09 (s, 3H), 2.06 (s, 6H), 2.054 (s, 3H), 2.046 (s, 3H), 2.00 (s, 6H), 1.96 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.7, 170.6, 170.4, 170.33, 170.27, 170.2, 170.1, 170.0, 169.95, 169.89, 169.7, 169.6, 169.51, 169.46, 169.39, 169.36, 169.2, 169.0, 102.2, 102.1, 100.8, 100.6, 100.4, 91.7, 89.2, 77.7, 75.5, 75.1, 74.8, 74.4, 73.1, 72.7, 72.4, 71.1, 71.0, 70.8, 70.7, 70.6, 70.5, 69.8, 69.6, 69.3, 68.5, 68.4, 68.1, 67.0, 65.6, 65.2, 63.7, 63.5, 61.3, 61.1, 61.0, 32.0, 29.8, 29.4, 22.8, 21.1, 21.0, 20.94, 20.89, 20.83, 20.71, 20.69, 20.66, 20.58; HRMS (ESI): calcd for $C_{52}H_{70}O_{35}Na$ ($[M+Na]^+$): 1277. 3595, found: 1277.3582.

1.11. β-D-Galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-[β-D-galactopyranosyl-(1→4)]-β-D-glucopyranose (15)

To a solution of tetrasaccharide **14** (21.9 mg, 17.4 µmol) in 45% MeOH aq. (1 mL) was added NaOMe in 45% MeOH aq. (2.9 mM, 1.0 mL, 2.9 µmol). The resulting solution was stirred at rt for 9 h. The solution was concentrated *in vacuo*. The residue was dissolved in MQ (2 mL) and poured to Cellulose Ester Membrane (Spectrum, MWCO: 0.1–0.5 kDa, FW: 31 mm, Dia: 20 mm, vol/L: 3.1 mL/cm) and dialyzed against MQ (1L) at rt for 6 h. The dialyzed solution was lyophilized to give tetrasaccharide **15** (9.50 mg, 14.3 µmol, 81.9%) as a white solid.

15: $[\alpha]_D^{20}$ +23.3 (*c* 0.19, D_2O); ^{1}H-NMR (600 MHz, D_2O): δ 5.23 (d, *J* = 4.2 Hz, 0.4H), 4.68 (d, *J* = 8.4 Hz, 0.6H), 4.59 (d, *J* = 7.8 Hz, 1H), 4.50 (d, *J* = 7.8 Hz, 1H), 4.49 (d, *J* = 7.8 Hz, 0.6H), 4.48 (d, *J* = 7.8 Hz, 0.4H), 4.28 (dd, *J* = 11.1, 1.8 Hz, 0.6H), 4.20 (dd, *J* = 11.1, 2.4 Hz, 0.4H), 4.18 (d, *J* = 3.6 Hz, 1H), 4.10–4.07 (m, 0.4H), 3.96 (dd, *J* = 11.4, 3.6 Hz, 0.4H), 3.93–3.52 (m, 20.6H), 3.30 (dd, *J* = 6.2, 5.2 Hz, 0.6H); ^{13}C-NMR (150 MHz, D_2O): δ 106.9, 105.8, 105.7, 105.52, 105.49, 98.6, 94.6, 80.5, 79.80, 79.76, 78.0, 77.8, 77.0, 76.9, 76.4, 76.3, 75.8, 75.4, 75.2, 74.1, 73.8, 73.6, 71.7, 71.30, 71.26, 70.3, 63.79, 63.76, 63.7, 63.2; HRMS (ESI): calcd for $C_{24}H_{42}O_{21}Na$ ($[M+Na]^+$): 689.2116, found: 689.2104.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://xxxxxxxx.

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- The total synthesis of 4P-X is achieved for the first time.
- A combination of enzymatic and organic chemical syntheses result in efficient synthesis of 4P-X.
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