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Note

Expeditious synthesis of a new hexasaccharide using transglycosylation reaction catalyzed by *Bacillus* (1→3),(1→4)-β-D-glucan 4-glucanohydrolase

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Abstract

Enzymatic hydrolysis of barley (1→3),(1→4)-β-D-glucan using a recombinant (1→3),(1→4)-β-glucanase from *Bacillus licheniformis* gives Glcβ4Glcβ3Glc isolated after acetylation in 49% yield. Conventional treatment produced the corresponding β-fluoride which was carefully de-*O*-acetylated. A transglycosylation reaction with this substrate, catalyzed by the title enzyme, gave Glcβ4Glcβ3Glcβ4Glcβ4Glcβ3Glc in 20% yield.

Keywords: Enzymatic synthesis; Transglycosylation; (1→3),(1→4)-β-glucanase; Glycosyl fluoride; Hexasaccharide

To obtain more information on the substrate specificity of enzymes acting on (1→3),(1→4)–β-D-glucans, the major cell wall constituents of barley and oat endosperm, novel oligoglucoside substrate analogues with alternating β-(1→3)- and β-(1→4)-linkages were required. It has been shown that SIII pneumococcal polysaccharide composed of repeating cellobiuronic acid units linked through β-(1→3)-linkages gave, after reduction, a potent substrate for diagnosing β–glucanhydrolase specificity [1]. It proved difficult, however, to isolate the target soluble oligosaccharides with alternating β-(1→3)- and β-(1→4)-linkages from the partial enzymatic hydrolysate of this insoluble polymer, except for the laminaribiose end product. This problem was overcome using a recently developed and efficient strategy in which laminaribiosyl units are joined through β-(1→4)-links by enzymatic autocondensation of β-laminaribiosyl fluoride using the retaining *Bacillus licheniformis* (1→3)-(1→4)-β-glucanase [2]. The tetra- and hexasaccharides obtained are the shortest oligosaccharides that mimic the alternating β-(1→3) and β-(1→4)-linkage

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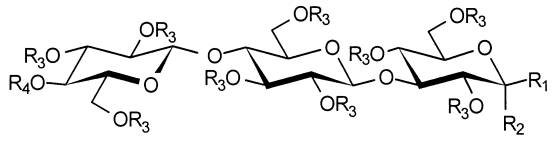
arrangement in the reduced pneumococcal polysaccharide. However, one of the best substrates for (1→3),(1→4)-β-glucanases, the water-soluble, barley (1→3),(1→4)-β-D-glucan does not exhibit this strict alternation of linkages, therefore, oligosaccharides mimicking the native sequences would be preferred as substrate analogues.

In barley (1→3),(1→4)-β-D-glucan up to 90% of each chain consists of cellotriosyl and cellotetraosyl residues joined by single β-(1→3)-glycosidic linkages [1,3]. Both bacterial and plant (1→3),(1→4)-β-glucanases EC 3.2.1.73 (family 16 and family 17 glycosyl hydrolases [4]) hydrolyse the polysaccharide to give the trisaccharide, 3-*O*-β-cellobiosyl-D-glucose, and the tetrasaccharide, 3-*O*-β-cellotriosyl-D-glucose, end products. The structure of these products define the enzyme's specificity for cleavage at β-(1→4)-glycosidic linkages on 3-*O*-substituted glucopyranose units [3]. Since it is known that in the binding site groove of bacterial (1→3),(1→4)–β-glucanases 5-7 glucosyl binding subsites are present [5], the hexasaccharide **1** shown in Scheme 1 represents the shortest oligomer that can mimic the polysaccharide substrate.

To obtain this hexasaccharide, it was postulated, based on our previous results [2], that a transglycosylation reaction catalyzed by (1→3),(1→4)-β-glucanases using the fluoride **2** would be a straightforward approach. Furthermore, the trimeric fluoride **2** would be a better substrate than the laminaribiosyl fluoride since the binding of a 4-*O*-β-D-glucosyl unit in subsite -III has the largest effect to transition state stabilisation [5].

Enzymatic depolymerisation of barley β-glucan by recombinant (1→3),(1→4)-β-glucanase from *B. licheniformis* followed by complete acetylation of the reaction mixture gave acetylated 3-*O*-β-cellobiosyl-D-glucopyranose **3** and the tetrasaccharide **4** in about 50 and 30% yield respectively (Chart 1). As compared to our previous procedure [6], **3** was obtained in improved yield when the enzymatic depolymerization mixture from medium viscosity

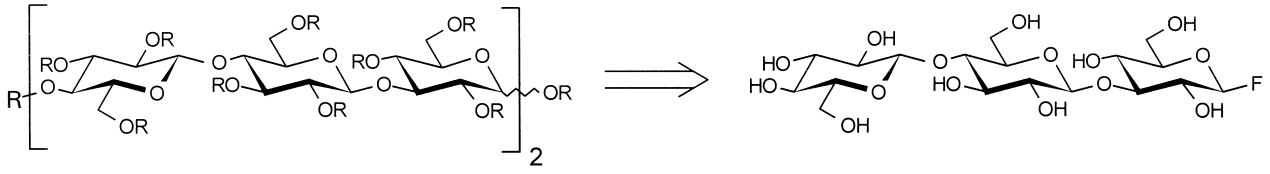


3	R_1,R_2=OAc,H	R_3, R_4=Ac	
4	R_1,R_2=OAc,H	R_3=Ac	R_4=β-Glc*p*
5	R_1=H	R_2=Br	R_3, R_4=H
6	R_1=F	R_2=H	R_3, R_4=Ac
7	R_1=OMe	R_2=H	R_3, R_4=H
8	R_1,R_2=OH,H	R_3, R_4=H	
10	R_1=4NP	R_2=H	R_3, R_4=H
11	R_1=3,4DNP	R_2=H	R_3, R_4=H

Chart 1.

barley β–glucan was directly acetylated without removal of residual high molecular weight oligosaccharides by acetone precipitation.

Compound **3** was transformed into acetylated 3-*O*-β-cellobiosyl-β-D-glucopyranosyl fluoride **6** by treatment of the acetylated α-glycosyl bromide **5** with AgF in anhydrous CH_3CN, using similar experimental conditions as previously reported for the synthesis of β-laminaribiosyl fluoride [2]. De-*O*-acetylation of **6** was very sensitive to experimental conditions. Whereas the C–F bond was proved to be very labile under acidic conditions (HCl in anhydrous methanol) [7], transesterification with sodium methoxide in methanol gave the unblocked fluoride **2**, but excess of sodium methoxide, reaction at room temperature, and long incubation times led to the formation of side products. A typical run gave a mixture of β-glycosyl fluoride **2** (75%), β-methyl glycoside **7** (15%), the free trisaccharide **8** (5%), and partially acetylated β-glycosyl fluoride (5%), as judged by ^{1}H NMR of the final reaction mixture. To minimize these side reactions, de-*O*-acetylation was carried out at 0 °C, and reaction time was carefully controlled by TLC monitoring, under these conditions **2** was obtained in 85% yield.



1 R=H
9 R=Ac

2

Scheme 1.

The β-glycosyl fluoride **2** underwent autocondensation under the conditions used for β–laminaribiosyl fluoride [2] (Scheme 1): maleate buffer pH 7.0, 40 °C, 0.1% (w/w) enzyme relative to the glycosyl fluoride. The hexasaccharide **1** was the major product formed and no higher oligomers were detected on TLC and mass spectrometry.

The purification strategy for the preparative scale synthesis followed a three step procedure: the reaction mixture was fully acetylated with Ac_2O/pyr, the per-acetylated hexasaccharide **9** purified by flash chromatography, and then de-*O*-acetylated with sodium methoxide in methanol to afford **1** in 20% overall yield (from **2**). De-*O*-acetylation of **9** was achieved in 71% yield, probably due to some β-elimination of the terminal 3-*O*-linked glucopyranose unit. The structure of the hexasaccharide was determined by its hydrolysis products. Treatment with (1→3),(1→4)-β–glucanase in citrate–phosphate buffer pH 7.2 at 55 °C gave the trisaccharide **8** as the only hydrolysis product. Since the enzyme has a strict specificity for cleavage of β-(1→4) glycosidic bonds on 3-*O*–substituted Gl*cp* units [1,3], this result indicates that the condensation product contains a new β-(1→4) glucosidic bond. Final evidence for structure **1** was provided by mass spectrometry (*m*/*z* 991 $[M+H]^+$) and NMR spectroscopy. Complete assignment from 2D NMR experiments (DQF-COSY, TOCSY, NOESY and HQMC) are shown in Tables 1 and 2.

Nitrophenyl glycosides have been used as substrates in transglycosylation reactions catalyzed by various glycosidases [8,9]. However, with 4-nitrophenyl and 3,4-dinitrophenyl trisaccharides **10** and **11** [10] as substrates in maleate buffer pH 7.0, 40 °C, and 0.1% (w/w) enzyme relative to the glycoside, transglycosylation was not observed but release of the aryl aglycon occurred. Since the hydrolase activity of (1→3),(1→4)-β-glucanase is markedly decreased at acidic pHs [11] and transglycosylation is less affected, as shown in the autocondensation of β–laminaribiosyl fluoride [2], the enzymatic autocondensation of the 4-nitrophenyl glycoside **10** was attempted at pH 4. Again no condensation products were detected.

The chemoenzymatic synthesis reported here provides quick access to a hexasaccharide that mimics the structure of cereal (1→3),(1→4)-β-D-glucans. The methodology is currently being extended to different glycosyl acceptors (methyl β-(1→4)-oligoglucosides) to obtain a family of substrates that will be useful for binding and specificity studies of bacterial and plant β-glucanases.

1. Experimental

Optical rotations were measured at 20 °C with a Perkin–Elmer 241 polarimeter. Melting points were measured on a Büchi 535 apparatus. Elemental analyses were performed on a CHNS-O Carlo Erba EA1108 analyzer. Mass spectra were performed on a Nermag R-1010C spectrometers in the fast atom bombardment (FAB) mode. NMR spectra were recorded on a Bruker 300 AC, Varian Gemini-300 or Varian VXR-500 spectrometers. Proton chemical shifts (δ in ppm) were referenced to internal Me_4Si for solutions in $CDCl_3$ and to an external reference for solutions in D_2O or d_6–Me_2SO; ^{13}C chemical shifts were referenced to the solvent signal. TLC was performed on Silica Gel 60 F_{254} aluminium plates with detection by charring with H_2SO_4/MeOH/H_2O (1:15:15 v/v/v) and heating at 125 °C. Flash chromatography was performed with Merck Silica Gel 60 (0.040–0.063 mm). Barley (1→3),(1→4)-β-glucan was from Megazyme (225 kDa; viscosity of 1% w/v solution 21.1 cSt measured on Ostwald C-type viscosimeter

Table 1
^1H NMR chemical shifts (δ in ppm) for compound **1** in D_2O solution at 25 °C

Unit[a]	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
3-*O*-β-D-Glc*p* Aα[b]	5.25	3.58	3.84	3.60	3.61	3.97	3.89
Aβ	4.66	3.30	3.65	3.57	3.62	4.00	3.84
4-*O*-β-D-Glc*p* B	4.78	3.40	3.67	3.69	3.63	4.01	3.85
4-*O*-β-D-Glc*p* C	4.56	3.37	3.66	3.70	3.64	3.99	3.82
3-*O*-β-D-Glc*p* D	4.56	3.54	3.78	3.52	3.64	3.95	3.78
4-*O*-β-D-Glc*p* E	4.78	3.40	3.67	3.69	3.63	4.01	3.85
β-D-Glc*p* F	4.52	3.34	3.52	3.51	3.48	3.94	3.76

[a] The residues are labeled A (reducing end) to F (non-reducing end).[b] The molar ratio of $\alpha\beta$ anomers was 1:2.

Table 2
^{13}C NMR chemical shifts (δ in ppm) for compound **1** in D_2O solution at 25 °C

Unit[a]	C-1	C-2	C-3	C-4	C-5	C-6
3-*O*-β-D-Glc*p* Aα	93.5	71.1	83.4	69.6	71.1	62.2
Aβ	97.4	75.6	85.3	69.6	76.3	61.5
4-*O*-β-D-Glc*p* B	104.3	74.7	75.7	80.2	76.5	61.7
4-*O*-β-D-Glc*p* C	104.1	74.6	75.7	80.1	76.5	62.2
3-*O*-β-D-Glc*p* D	104.1	74.6	85.3	69.6	76.5	62.2
4-*O*-β-D-Glc*p* E	104.3	74.7	75.7	80.2	76.5	61.5
β-D-Glc*p* F	104.3	74.8	77.0	69.6	77.0	62.21

[a] The residues are labeled A (reducing end) to F (non-reducing end).

at 30 °C). Light petroleum refers to the 60–80 °C fraction. Recombinant *B. licheniformis* (1→3), (1→4)-β-glucanase was produced and purified as already described [11,12].

O-*(2,3,4,6-tetra*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl)-(1→3)-2,4,6,tri*-O-*acetyl*-D-*glucose* (**3**) *and* O-*(2,3,4,6-tetra*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl* D-*glucose* (**4**).—Recombinant (1→3),(1→4)-β-glucanase from *B. licheniformis* (65 μg) was added to a solution of barley β-glucan (10.0 g) in 0.65 mM citric acid/8.7 mM Na_2HPO_4 buffer (1 L) pH 7.2. After 16 h incubation at 55 °C, further enzyme was added (28 μg), and the mixture was incubated at 45 °C for 22 h. Evaporation of the solvent gave 19.4 g of crude mixture, which was fully acetylated with Ac_2O/pyridine (600 mL; 1:1 v/v) at room temperature. The resulting solution was poured into ice-water (1.5 L), the precipitated solid was removed by filtration, dissolved in $CHCl_3$, and the solution washed with saturated aq $NaHCO_3$ until neutral, followed by water. After drying over $MgSO_4$, the solvent was evaporated, and the residue (15.0 g) purified by flash chromatography ($CHCl_3$/AcOEt 2:1, 3:2, 1:1). Three main fractions were obtained. The first eluted was **3** (9.52 g, 49%), the second a mixture of **3** and **4** (0.39 g) and last per-acetylated tetrasaccharide **4** (5.68 g, 29%).

Compound **3**.—^{1}H NMR (300 MHz, $CDCl_3$): 1.96–2.19 (11s, 33H, CH_3CO), 3.60–5.16 (m, 20 H, H–1B, 1C, 2A–C, 3A–C, 4A–C, 5A–C, 6aA–C, 6bA–C), 5.61 (d, $J_{1,2}$ 8.4, 1/2 H, H-1Aβ), 6.23 (d, $J_{1,2}$ 3.5, 1/2 H, H-1Aα); ^{13}C NMR (75 MHz, $CDCl_3$): 20.0–20.4 (CH_3CO), 61.2–61.8 (C-6A–C), 67.0, 67.1, 67.3, 69.6, 70.8, 70.9, 71.2, 71.5, 71.7, 72.5 (C-2A–C, 3B, 3C, 4A, 4C, 5A–C), 75.8, 76.0, 78.8 (C–3Aα, 3Aβ,4B), 88.9 (C-1Aα), 91.4 (C-1Aβ), 100.6 (C-1B, 1C), 168.7–170.7 (CO).

Compound **4**.—^{1}H NMR (300 MHz, $CDCl_3$): 1.96–2.18 (14 s, 42 H, CH_3CO), 3.52–5.18 (m, 26H, H–1B–D, 2A–D, 3A–D, 4A–D, 5A–D, 6aA–D, 6bA–D), 5.60 (d, $J_{1,2}$ 8.1, 1/2H, H-1Aβ), 6.21 (d, $J_{1,2}$ 3.3, 1/2H, H-1Aα); ^{13}C NMR (75 MHz, $CDCl_3$): 20.4–20.7 (CH_3CO), 61.4–62.1 (C-6A–D), 67.3, 67.4, 67.6, 69.9, 71.1, 71.2, 71.3, 71.5, 71.7, 71.8, 72.0, 72.5, 72.6, 72.7, 72.8 (C-2A–D, 3B–D, 4A, 4D, 5A–D), 76.0, 76.1, 76.2, 79.0 (C-3Aα, 3Aβ, 4B, 4C), 89.0 (C-1Aα), 91.7 (C-1Aβ), 100.6, 100.8, 100.9 (C-1B–D), 168.6–170.6 (CO).

O-*(2,3,4,6-Tetra*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl*-β-D-*glucopyranosyl fluoride* (**6**).—The α-glycosyl bromide **5** was prepared as described in Malet et al. [6] by treatment of **3** with 4.1 M HBr in AcOH at room temperature for 30 min. After usual work-up, the product (amorphous solid, 98% yield) was used immediately in the next step without further purification. The purity of the crude product was controlled by TLC and ^{1}H NMR ($CDCl_3$; δ 6.5, d, 1H, $J_{1,2}$ 4 Hz, H-1A).

The bromide **5** (9.66 g, 9.52 mmol) was treated with silver fluoride (9.64 g, 75.96 mmol) in dry acetonitrile (450 mL). After stirring for 2.5 h at room temp, the reaction mixture was filtered through Celite and the solvent was evaporated off. The residue obtained was dissolved in $CHCl_3$ and purified by flash chromatography (AcOEt/light petroleum 1:1) to yield the acetylated fluoride **6** (6.80 g, 75%). m.p. (EtOEt/hexane): 123°C; $[\alpha]_D^{20}$: −12.6° (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): 1.91–2.13 (7 s, 21 H, CH_3CO), 3.59–3.64 (m, 2H, H-5A or 5C, 5B), 3.71 (dd, $J_{2,3}$ $J_{3,4}$ 8.7, 1 H, H-2B), 3.79 (m, 1 H, H-5C or 5A), 3.84 (dd, $J_{2,3}$ $J_{3,4}$ 8.2, 1 H, H-2C), 3.98–4.18 (m, 5 H, H-6aA–C, 6bA or 6bC, 6bB), 4.31 (dd, $J_{5,6b}$, 4.4, J_{gem} 12.5, 1 H, H-6bC or 6bA), 4.58 (d, $J_{1,2}$ 8.0, 1 H, H-1C), [4.80 (dd, *J* 9.5, 1 H), 4.86 (dd, *J* 8.0, 1 H), 4.97–5.12 (m, 5 H), H-2A, 3A–C, 4A–C], 5.08 (d, $J_{1,2}$ 9.1, 1 H, H-1B), 5.29 (d, $J_{1,2}$ 6.2, 1 H, H-1A); ^{13}C NMR (75 MHz, $CDCl_3$): 20.3–20.7 (CH_3CO), 61.5, 61.9, 62.0 (C-6A–C), 67.4, 67.8, 71.1, 71.6, 71.6, 72.0, 72.1, 72.6, 72.7, 72.8 (C-2A–C, 3B, 3C, 4A, 4C, 5A–C), 76.1 (C-4B), 77.2 (d, $J_{3,F}$ 8.4, C-3A), 100.5, 100.7 (C-1B, 1C), 106.3 (d, $J_{1,F}$ 220, C-1A), 168.9–171.1 (CO); Anal. Calcd for $C_{40}H_{53}O_{25}F$: C, 50.42; H, 5.61; F, 1.99. Found: C, 50.29; H, 5.81; F, 1.95.

O-β-D-*Glucopyranosyl-(1→4)*-O-β-D-*glucopyranosyl-(1→3)*-O-β-D-*glucopyranosyl fluoride* (**2**) .—Freshly prepared NaOMe in MeOH (0.50 M, 6 mL) was added to a stirred solution of compound **6**(1.00 g, 1.07 mmol) in anhydrous MeOH (75 mL), and the mixture stirred for 45 min at 0 °C. The reaction mixture was neutralized with Amberlite® IRN 77 ($H^{\oplus}$) resin, filtered and the filtrate concentrated to dryness. The residue (550 mg), composed mainly of **2** (approx. 85%), was used immediately for enzymatic condensations without further purification. The purity of the crude mixture was checked by TLC and NMR spectroscopy. ^{1}H NMR (300 MHz, d_6-DMSO): 3.05–3.97 (m,

19H, H-1B, 2A–C, 3A–C, 4A–C, 5A–C, 6aA–C, 6bA–C), 5.05 (d, $J_{1,2}$ 7.3, 1 H, H-1C), 5.23 (d, $J_{1,2}$ 6.8, 1 H, H-1A); ^{13}C NMR (75 MHz, d_6-DMSO): 60.5, 60.9, 61.1 (C-6A–C), 64.9, 65.6, 67.4, 69.7, 70.8, 71.0, 72.0, 72.5, 74.0, 74.3 (C-2A–C, 3B, 3C, 4A, 4C, 5A–C), 77.6 (C-4B), 83.5 (d, $J_{3,F}$ 10.8, C–3A), 101.0, 101.3 (C-1B,1 C), 106.7 (d, $J_{1,F}$ 211, C-1A).

O-β-D-*Glucopyranosyl-(1→4)*-O-β-D-*glucopyranosyl-(1→3)*-O-β-D-*glucopyranosyl-(1→4)*-O-β-D-*Glucopyranosyl-(1→4)*-O-β-D-*glucopyranosyl-(1→3)*-O-D-*glucopyranose* (**1**).—The crude fluoride **2** (542 mg) dissolved in maleate buffer (17 mL, pH 7.0, 50 mM sodium maleate, 1 mM $CaCl_2$), was treated with recombinant (1→3),(1→4)-β-glucanase from *B. licheniformis* (500 μg) dissolved in the same buffer (1 mL), and the mixture was stirred at 40 °C for 2 h. After inactivating the enzyme by boiling for 5 min, the solvent was evaporated to dryness. The reaction mixture was fully acetylated with Ac_2O–pyridine (150 mL; 1:1 v/v) at 30 °C. After 24 h, MeOH (100 mL) was added at 0 °C. The solvent was evaporated, the residue was dissolved in $CHCl_3$, the solution was washed with saturated aq $NaHCO_3$ and water, dried over $MgSO_4$, and evaporated to dryness. Flash chromatography (gradient 1:1→5:1 EtOAc–light petroleum) of the reaction mixture yielded two main fractions.

Eluted first (410 mg) was the *per*-acetylated trisaccharide **3** (*m/z* 989 $[C_{40}H_{54}O_{27}+Na]^{\oplus}$); eluted last (144 mg) was the *per*-acetylated hexasaccharide **9** (*m/z* 1855 $[C_{76}H_{102}O_{51}+Na]^{\oplus}$).

Deacetylation of **9** (133 mg) was performed by treatment with 20 mM MeONa in MeOH (25 mL) at 30 °C for 20 h, then warmed up to 40 °C for 2 h. The reaction mixture was neutralized with Amberlite® IR-120 ($H^{\oplus}$) resin, filtered off, and the solvent was evaporated. The residue (74 mg) was crystallized from H_2O/EtOH to afford **1** (51 mg, 71%). m.p. (H_2O/EtOH): 190–194 °C (d); $[\alpha]_D^{20}$: −5.83° (*c* 0.600, H_2O); MS (FAB): *m/z* 991 $[C_{36}H_{62}O_3+H^{\oplus}]$. ^{1}H RMN and ^{13}C RMN are summarized in Tables 1 and 2. Chemical shifts have been assigned using 2D experiments (DQF-COSY, TOCSY, NOESY and HQMC).

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