PERKIN 1

Transglycosylation activity of *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases. Enzymic synthesis of alternate 1,3,-1,4-β-D-glucooligosaccharides

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The title enzyme from *Bacillus licheniformis* has been shown to catalyse the effective autocondensation of β-laminaribiosyl fluoride, and lead to alternate 1,3-1,4-β-D-glucotetraose and -glucohexaose products. The transglycosylation using the same donor and methyl β-laminaribioside as acceptor leads to the methyl 4-*O*-β-laminaribiosyl-β-laminaribioside in 40% overall yield.

Introduction

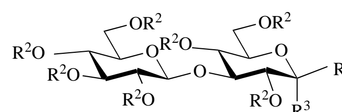
1,3-1,4-β-D-Glucan 4-glucanohydrolase (1,3-1,4-β-glucanase, E C 3.2.1.73) is an endo-glycosidase that hydrolyses β-glucans containing mixed β-1,3 and β-1,4 linkages, such as lichenin and cereal β-glucans. The enzyme has a strict cleavage specificity for β-1,4 glycosidic bonds in 3-*O*-substituted glucopyranose units.[1,2] The *Bacillus licheniformis* 1,3-1,4-β-glucanase is a retaining glycosidase[3] acting by general acid/base catalysis in a double-displacement mechanism. Glu[138] has been proposed as the proton-donor residue and Glu[134] as the catalytic nucleophile.[4,5] For a better knowledge of the topology of the active site of bacterial 1,3-1,4-β-glucanases, synthetic chromophoric substrates of the type [Glc*p*β(1→4)Glc*p*]$_n$β(1→3)Glc*p*β-MeUmb have been synthesized for kinetic studies,[6] as well as thiooligosaccharide inhibitors.[7] However, to gain more information on the acceptor part of the active site, new non-natural substrate analogues combining β-1,3 and β-1,4 linkages were required.

Since we and others have used the capability of retaining endo-glucanases, cellulases and α-amylases, to catalyse the enzymic synthesis of oligosaccharides starting from β-glycosyl (cellulase) and α-glycosyl (amylase) fluorides,[8–15] here we study the ability of the retaining *B. licheniformis* 1,3-1,4-β-glucanase to catalyse transglycosylation or condensation reactions using activated glycosyl donors. According to the specificity of the enzyme, the minimum structural requirement for a putative glycosyl donor is the laminaribiosyl core structure (G3G-) with an anomeric substituent with a β-configuration on the reducing end. If transglycosylation proceeds with the same high specificity as the enzyme does in the hydrolase activity, it would be a helpful strategy to synthesize oligomers containing mixed β-1,3 and β-1,4 linkages.

Results and discussion

Acetylated laminaribiose **1** was prepared by acid-catalysed acetolysis of Actigum CS6 containing 60–75% scleroglucan as reported.[7] It was transformed into the β-laminaribiosyl fluoride **4** by conventional synthesis: the acetylated α-bromide **2** was treated with AgF in anhydrous CH_3CN to yield acetylated β-fluoride **3**. De-O-acetylation of compound **3** was very sensitive to the experimental conditions. Under acidic conditions (HCl in anhydrous MeOH), hydrolysis of the C–F bond was the main reaction. Basic transesterification with sodium methoxide in MeOH afforded the desired unblocked fluoride **4** plus some methyl β-laminaribioside **5**. The ratio of products **4**:**5** was increased at low temperatures, to reach 85:15 at 0 °C. To avoid formation of the methyl glycoside, ammonia in anhydrous methanol was assayed,[16] but de-O-acetylation was not complete, yielding a monoacetylated laminaribioside derivative. The crude β-laminaribiosyl fluoride **4** obtained from MeO^-/MeOH treatment was immediately used in the enzymic condensation reactions.

p-Nitrophenyl β-laminaribioside **7** was prepared (28% yield) from the α-bromide **2** by phase-transfer glycosidation using a modification of the procedure already reported,[17] followed by de-O-acetylation with sodium methoxide in MeOH.



1 $R^1 = H, R^2 = Ac, R^3 = OAc$,
2 $R^1 = H, R^2 = Ac, R^3 = Br$
3 $R^1 = F, R^2 = Ac, R^3 = H$
4 $R^1 = F, R^2 = R^3 = H$
5 $R^1 = OMe, R^2 = R^3 = H$
6 $R^1 = OC_6H_4\text{-}4\text{-}NO_2, R^2 = Ac, R^3 = H$
7 $R^1 = OC_6H_4\text{-}4\text{-}NO_2, R^2 = R^3 = H$
8 $R^1 = OMe, R^2 = Ac, R^3 = H$

Initial attempts at polycondensation of laminaribiosyl fluoride were carried out in hydroorganic media as reported in previous studies on cellulases.[8–12] Acetonitrile–maleate buffer in 2.5:1 and 1.5:1 ratios gave oligosaccharides **9–11** in low yields, the di- and tetra-saccharides partly precipitating. The reaction did not proceed further because enzyme inactivation was faster than the condensation. When the amount of organic solvent was reduced to a 0.5:1 ratio with maleate, the reaction was even slower and fresh enzyme had to be added every 30 min in order to consume all the fluoride **4**. Other solvents such as MeOH and dimethyl sulfoxide (DMSO) were unsatisfactory for the same reason. Therefore we decided to study the reaction, by using HPLC and TLC analysis, without using an organic co-solvent. In 50 mmol dm^{-3} maleate buffer, the pH decreased from 7 to 4 in 15 min, and the reaction took 2 h for the fluoride **4** to be consumed with formation of products **9–11**. With a

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100 mmol dm^{-3} buffer, the pH decreased to 6 in 30 min and remained constant at pH 5 after 1 h. The formation of products **9–11** was faster, but compound **11** was hydrolysed within 1 h and disappeared completely before all of the fluoride **4** had reacted. The reaction was then assayed at pH 4 using 100 mmol dm^{-3} acetate buffer, yielding the same result as with 50 mmol dm^{-3} maleate buffer. More acidic conditions than pH 4 resulted in a rapid hydrolysis of the fluoride, and only a small amount of tetrasaccharide **10** was formed. In conclusion, it is not necessary to use an organic co-solvent to observe polycondensation of the glycosyl fluoride **4** catalysed by the *B. licheniformis* 1,3-1,4-β-glucanase. The enzyme is rapidly inactivated in the presence of CH_3CN, MeOH or DMSO, and no larger excess of fluoride to speed up the condensation reaction can be used, due to solubility problems. A drop of the pH to 4–5 during the reaction seems to increase the yield of condensation products probably due to a decrease of the hydrolase activity on the newly formed oligosaccharides (the optimum pH for the glycosidase activity on natural substrates is 7.0).[18]

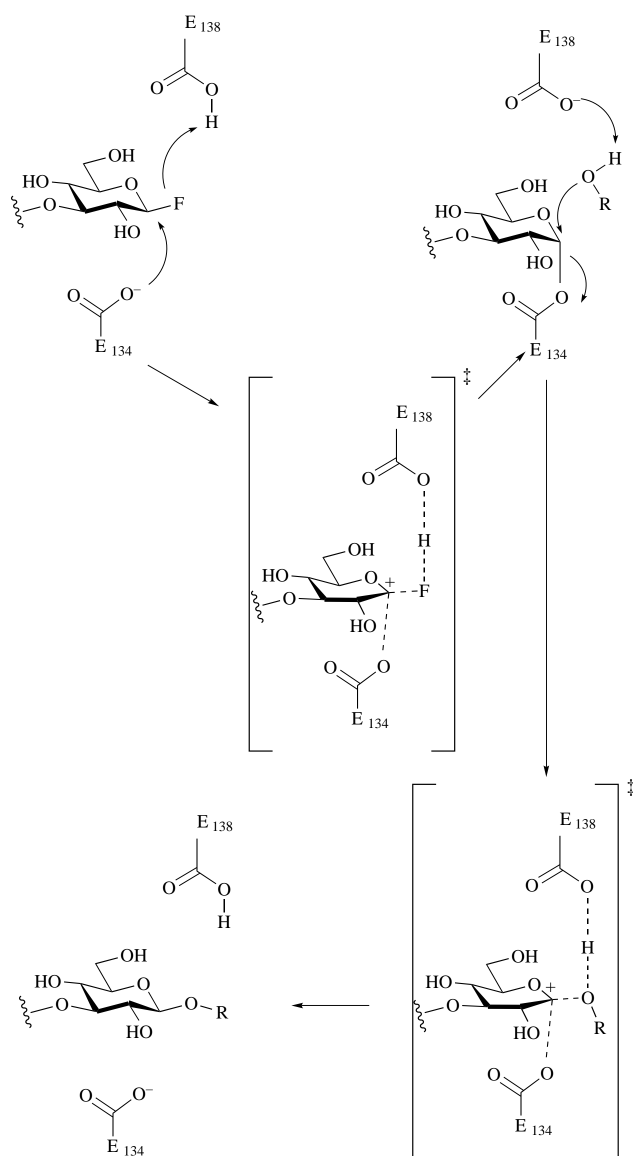
p-Nitrophenyl β-laminaribioside **7** was also tested as a substrate for the condensation. No polymeric products [degree of polymerization (DP) > 2] were detected under any conditions, only the hydrolysis product being formed. The result indicates that, for the less activated *p*-nitrophenyl glycosyl donor, hydrolysis is faster than transglycosylation. Being a retaining endo-enzyme, a glycosyl-enzyme intermediate was postulated with both donors (Scheme 1). Dissociation of HF may be faster than dissociation of nitrophenol and offers less steric hindrance to the acceptor attack which competes more efficiently with attack by water.

For the semi-preparative synthesis of the new oligosaccharides **10** and **11**, the reaction was pertformed in 50 mmol dm^{-3} maleate buffer at pH 7 (initial). Compounds **9** (30%), **10** (10%) and **11** (5%) were isolated after high-performance liquid chromatography (HPLC) purification. A different purification strategy was applied for larger-scale synthesis (Scheme 2). The crude mixture obtained at the end of the condensation was fully acetylated and was then purified by flash chromatography. Three fractions of DP2, DP4 and DP6 products were obtained. Each fraction contained the fully acetylated oligosaccharide and its corresponding methyl glycoside. The latter come from the small amount of methyl β-laminaribioside present in the initial fluoride donor and which competes as a glycosyl acceptor. After MeO$^-$/MeOH de-O-acetylation, chromatographic separation gave the free oligosaccharides **10** (11%) and **11** (3.6%), and the methyl glycosides **12** (3%) and **13** (0.6%).

Transglycosylation with a different acceptor was investigated to see if we could obtain a single reaction product in higher yield. Methyl glycosides have been shown to be good substrates for 1,3-1,4-β-glucanases,[19] and therefore we chose methyl β-laminaribioside **5** as glycosyl acceptor for the enzymic transglycosylation in order to obtain a novel substrate for kinetic studies.

Since a large excess of acceptor was used to avoid autocondensation of fluoride **4**, a hydroorganic medium was chosen. Under these conditions (1:7 mol equiv. of subtrates **4**:**5** in 50 mmol dm^{-3} maleate buffer–CH_3CN 2:3 at 40 °C) the reaction was faster than enzyme inactivation, the donor being consumed in 15 min. The tetrasaccharide **12** was the only transglycosylation product formed. The reaction mixture was fully acetylated (Scheme 2), and product **16** was purified by flash chromatography and obtained in 48% yield. De-O-acetylation gave the free methyl tetrasaccharide **12** in 40% overall yield.

The alternate structure of compounds **10** and **11** has been determined by their hydrolysis product. Treatment of oligosaccharides **10** and **11** with 1,3-1,4-β-D-glucanase from *B. licheniformis* in 6.5 mmol dm^{-3} citrate/85 mmol dm^{-3} phosphate buffer (pH 7.2) afforded laminaribiose **9** as the final product. Likewise, the newly formed β-1,4 linkage in compound **12** was demonstrated by the obtention of disaccharides **5** and **9** upon enzymic hydrolysis, and was confirmed by NMR spectroscopy: the signals at δ_C 87.6 and 87.7 are assigned to C-3 of 3-*O*-substituted Glc units, and the signal at δ_C 80.7 to the 4-*O*-substituted Glc unit. Furthermore, compound **12** was resistant to hydrolysis after treatment with *Trichoderma reesei* cellulase in phosphate buffer (30 mmol dm^{-3}, pH 7.0).



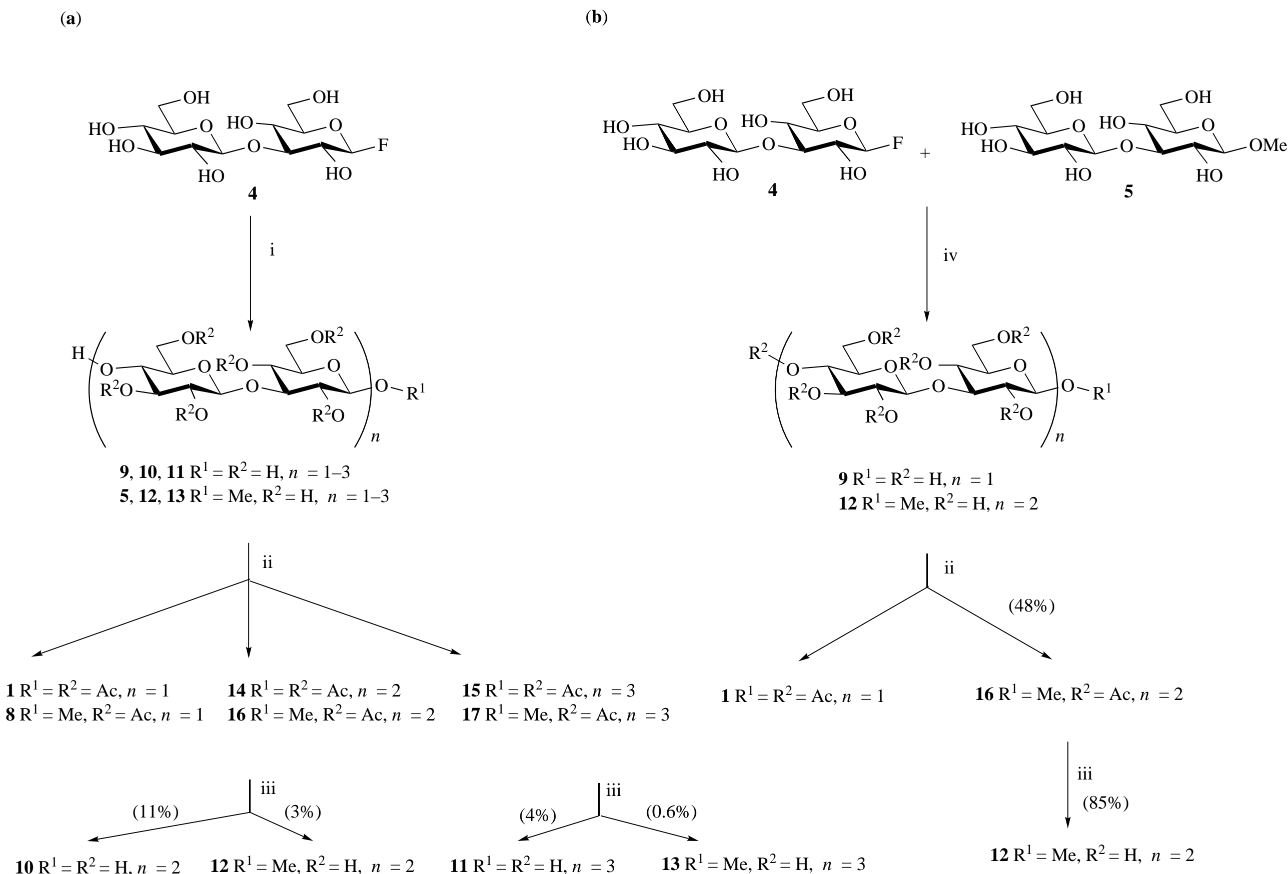
Scheme 1 Proposed mechanism of glycosyl transfer to an acceptor catalysed by β-1,3-1,4 glucanases

Kinetic studies and their application to an analysis of the active-site topology of bacterial 1,3-1,4-β-glucanases will be described in due course.

In conclusion, this paper reports the first easy access of alternate 1,3-1,4-β-D-glucooligosaccharides which were obtained as transient compounds during the hydrolysis of the reduced SIII pneumococcal polysaccharide.[1]

Experimental

Optical rotations were measured at 20 °C with a Perkin-Elmer 241 polarimeter, and $[a]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Mps were measured on a Büchi 535 apparatus and are uncorrected. Elemental analyses were performed by the microanalytical laboratories of the CNRS (Vernaisson) or at IQS on a CHNS-O Carlo Erba EA1108 analyser. Mass spectra were performed on a Nermag R-1010C spectrometer in the fast-atom bombardment (FAB) mode. NMR spectra were recorded



Scheme 2 Preparative transglycosidation reactions catalysed by β-1,3-1,4-glucanase from *B. licheniformis*. (**a**) Polycondensation of G3G-F **4**: i, 1,3-1,4-β-glucanase from *B. licheniformis* 0.1% (w/w), 50 mmol dm^{-3} maleate buffer (pH 7.0), 40 °C, 2 h; ii, Ac_2O–Py, 30 °C, 24 h, followed by chromatographic separation; iii, MeONa (20 mmol dm^{-3}) in MeOH, followed by chromatographic separation. (**b**) Condensation of G3G-F **4** with G3G-OMe **5**: iv, 1,3-1,4-β-glucanase from *B. licheniformis* 0.1% (w/w), MeCN–maleate buffer (50 mmol dm^{-3}; pH 7.0), 40 °C, 15 min. Py = pyridine.

on a Bruker 300, AC Varian Gemini-300 or Varian VXR 500 spectrometer. Proton chemical shifts (δ_H in ppm) were referenced to internal Me_4Si for solutions in $CDCl_3$ and to an external reference for solutions in D_2O or [$^2H_6$]DMSO, 2D experiments: DQF-COSY, TOCSY (100 ms), NOESY (100 and 500 ms) and HMQC. ^{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–water (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). Light petroleum refers to the 60–80 °C fraction. Actigum CS6 containing scleroglucan was a gift of Sanofi Bio-Industries (Paris, France). Recombinant *Bacillus licheniformis* 1,3-1,4-β-glucanase was produced and purified as already described.[18] The course of enzymic reactions was monitored on an analytical μ-Bondapak NH_2 column (Waters, Milford, MA, USA) with CH_3CN–water (70:30 v/v), and compounds **9–11** were purified by an HPLC μ-Bondapak NH_2 column (10 μm; 19 × 150 mm, Waters) using the same eluent.

2,4,6-Tetra-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl bromide 2

1,2,4,6-Tetra-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranose **1** was obtained by controlled acid-catalysed acetolysis of Actigum CS6 containing scleroglucan as already described.[7]

A suspension of compound **1** in HBr in AcOH (3 cm^3 per mmol, 4.1 mol dm^{-3}) was stirred at room temperature under anhydrous conditions until all the solid had dissolved (15 min). The resulting solution was poured into ice–water, the solid that precipitated was filtered off, and extracted with $CHCl_3$, and the organic solution was washed with saturated aq. $NaHCO_3$ until neutral, followed by water. After drying over Na_2SO_4, evaporation off of the solvent afforded title bromide **2** as an amorphous solid in 98% yield. The product was used immediately in the next glycosidation step without further purification. The purity of the crude product was checked by TLC and NMR analysis; $\delta_H(CDCl_3)$ 1.91–2.06 (21 H, 7 s, CH_3CO), 3.75 (1 H, ddd, $J_{4,5}$ 9.8, $J_{5,6a}$ 4.4, $J_{5,6b}$ 2.1, H-5^1 or -5^2), 4.09 (1 H, dd, $J_{5,6a}$ 2.0, $J_{6a,6b}$ 12.0, H-6a^1 or -6a^2), 4.16 (1 H, dd, $J_{1,2} = J_{2,3} = 9.3$, H-2^2), 4.19–4.29 (3 H, m, H-5^1 or -5^2, H-6a^2 or -6a^1, H-6b^1 or -6b^2), 4.39 (1 H, dd, $J_{5,6a}$ 4.2, $J_{6a,6b}$ 12.0, H-6b^2 or -6b^1), 4.69 (d, $J_{1,2}$ 8.1, H-1^2), 4.82 (1 H, dd, $J_{1,2}$ 4.2, $J_{2,3}$ 9.6, H-2^1), [4.91 (1 H, dd, J 8.1), 5.08 (1 H, dd, J 9.9) and 5.11 (1 H, dd, J 9.6): H-3^2, -4^1 and -4^2], 5.16 (1 H, dd, $J_{2,3} = J_{3,4} = 9.3$, H-3^1) and 6.47 (1 H, d, $J_{1,2}$ 4.0, H-1^1); $\delta_C(CDCl_3)$ 20.9–21.3 (CH_3), 61.7 and 62.2 (C-6^1 and -6^2), 67.2, 68.5, 72.0, 72.3, 72.9, 73.0 and 73.4 (C-2^1, -2^2, -3^2, -4^1, 4^2, -5^1 and -5^2), 78.0 (C-3^1), 87.9 (C-1^1), 101.3 (C-1^2) and 169.6–171.1 (CO).

3-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl fluoride 4

The bromide **2** (1.84 g, 2.63 mmol) was treated with silver fluoride (2.56 g, 20.15 mmol) in dry acetonitrile (130 cm^3). After being stirred for 2 h at room temperature, the reaction mixture was filtered through Celite. The residue obtained upon concentration of the filtrate was purified by flash chromatography (EtOAc–light petroleum 1:1 ⟶ 2:1) to yield *acetylated fluoride* **3** (1.44 g, 85%), mp 151 °C (from Et_2O–hexane) (Found: C, 48.80; H, 5.51, F, 2.94. $C_{26}H_{35}FO_{17}$ requires C, 48.91; H, 5.52; F, 2.98%); $[\alpha]_D$ −26 (c 0.44, $CHCl_3$); $\delta_H(CDCl_3)$ 1.91–2.13 (21 H, 7 s, CH_3CO), 3.62 (1 H, m, H-5^1 or -5^2), 3.79 (1 H, m, H-5^2 or -5^1), 3.86 (1 H, dd, $J_{1,2}$ 6.4, $J_{2,3}$ 8.3, H-2^2), 3.96–4.15 (3 H, m, H-6a^1,

-6a² and -6b¹ or -6b²), 4.28 (1 H, dd, $J_{5,6b}$ 4.3, $J_{6a,6b}$ 12.5, H-6b² or -6b¹), 4.57 (1 H, d, $J_{1,2}$ 8.0, H-1²), [4.83 (1 H, dd, *J* 8.7) and 4.97–5.12 (4 H, m): H-2¹, -3¹, -3², -4¹ and -4²] and 5.25 (1 H, d, $J_{1,2}$ 6.4, H-1¹); δ_C($CDCl_3$) 20.0–20.5 (CH_3), 61.4 and 61.7 (C-6¹ and -6²), 67.2, 67.8, 70.8, 71.5, 71.6, 71.8 and 72.5 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 76.9 (C-3¹), 100.3 (C-1²), 106.1 (d, $J_{1,F}$ 218, C-1¹) and 168.8–170.3 (CO).

To a stirred solution of compound **3** (1.87 g, 2.92 mmol) in anhydrous MeOH (40 cm³) was added freshly prepared NaOMe (1.2 mol dm^{-3}, 2.0 cm³) and the mixture was stirred for 2 h 15 min at 0 °C. The reaction mixture was neutralized with Amberlite IRN 77(H^+). The residue was filtered off and the filtrate was concentrated to dryness. The residue (1.12 g), composed of compounds **4** (approx. 85%) and **5** (minor product), was immediately used for enzymic condensations without further purification. The purity of the crude product was checked by TLC and NMR spectral analysis, δ_H([²H₆]DMSO) 3.00–3.77 (H-2¹, -2², -3¹, -3², -4¹, -4², -5¹, -5², -6a¹, -6a², 6b¹ and -6b²), 5.05 (1 H, d, $J_{1,2}$ 7.1, H-1²) and 5.23 (1 H, d, $J_{1,2}$ 5.2, H-1¹); δ_C([²H₆]DMSO) 60.5 and 61.1 (C-6¹ and -6²), 67.8, 70.2, 72.2, 73.8, 73.9, 76.1 and 76.9 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 86.5 (d, $J_{3,F}$ 11.5, C-3¹), 103.9 (C-1²) and 109.1 (d, $J_{1,F}$ 208, C-1¹).

4′-Nitrophenyl 3-*O*-β-D-glucopyranosyl-β-D-glucopyranoside 7

A solution of bromide **2** (1.40 g, 2.0 mmol) in dry CH_2Cl_2 (25 cm³) was added to a mixture of *p*-nitrophenol (410 mg, 4.51 mmol) and benzyltriethylammonium chloride (513 mg, 1.89 mmol) in aq. NaOH (5 cm³; 5%). The biphasic system was heated under reflux with vigorous stirring for 4 h before being diluted with water (10 cm³), and the aq. layer was extracted with CH_2Cl_2. The combined organic phases were washed with 5% aq. NaOH, neutralized with dil. HCl, washed with water, dried over $NaSO_4$, and evaporated. The reaction mixture was purified by flash chromatography (EtOAc–light petroleum 1:1) to yield *compound* **6** (672 mg, 45%) after recrystallization, mp 149–150 °C (from Et_2O–hexane) (Found: C, 50.67; H, 5.20; N, 1.83. $C_{32}H_{39}NO_{20}$ requires C, 50.73; H, 5.19; N, 1.85%); $[\alpha]_D$ −153 (*c* 0.59, $CHCl_3$); δ_H($CDCl_3$) 1.96–2.15 (21 H, 7 s, CH_3CO), 3.68 (1 H, m, H-5¹ or -5²), 3.80 (1 H, m, H-5² or -5¹), 3.95–4.15 (3 H, m, H-6a¹, -6a² and -6b¹ or -6b²), 4.29 (1 H, d, $J_{1,2}$ 8.0, H-1²), 4.35 (1 H, m, H-6b² or -6b¹), [4.63 (1 H, dd, *J* 8.5), 4.81 (1 H, dd, *J* 8.5), 5.05 (1 H, dd, *J* 8.5), 5.10 (1 H, dd, *J* 8.8) and 5.14 (1 H, dd, *J* 8.5): H-2¹, -2², -3², -4¹ and -4²], 5.11 (1 H, d, $J_{1,2}$ 8.0, H-1¹), 5.30 (1 H, dd, $J_{2,3} = J_{3,4}$ = 8.1, H-3¹), 7.04 (2 H, d, $J_{2',3'}$ 9.6, H-2′ and -6′) and 8.22 (2 H, d, H-3′ and -5′); δ_C($CDCl_3$) 20.9–21.4 (CH_3), 62.3 and 62.6 (C-6¹ and -6²), 68.7, 68.8, 71.1, 72.4, 72.7, 73.0 and 73.4 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 78.9 (C-3¹), 98.7 (C-1¹), 101.5 (C-1²), 117.1 (C-2¹ and -6¹), 126.3 (C-3¹ and -5¹), 143.5 (C-4¹), 161.5 (C-1¹) and 169.7–171.0 (CO).

To a stirred solution of compound **6** (616 mg, 0.81 mmol) in anhydrous MeOH (25 cm³) was added freshly prepared NaOMe (1 mol dm^{-3} in methanol; 0.75 cm³), and the mixture was stirred for 7 h at room temperature. The solid formed was filtered off, and washed with cold MeOH to yield *title compound* **7** (207 mg). An additional fraction of compound **7** was obtained from the mother liquors after neutralization with Amberlite IRN 77(H^+) resin, filtration, evaporation to dryness and crystallization from MeOH (23 mg, 62% overall yield), mp 237–238 °C (decomp.) (from aq. EtOH) (Found: C, 46.60; H, 5.48; N, 3.00. $C_{18}H_{25}NO_{13}$ requires C, 46.66; H, 5.44; N, 3.02%); *m/z* 486 [M + Na]⁺; $[\alpha]_D$ −40.0 (*c* 0.200, water); δ_H([²H₆]DMSO–D_2O) 3.28–3.84 (H-2¹, -2², -3¹, -3², -4¹, -4², -5¹, -5², -6a¹, -6a², -6b¹ and -6b²), 4.67 (1 H, d, $J_{1,2}$ 7.8, H-1²), 5.22 (1 H, d, $J_{1,2}$ 7.4, H-1¹), 7.24 (2 H, d, $J_{2',3'}$ 9.3, H-2′ and -6′) and 8.24 (2 H, d, $J_{2',3'}$ 9.3, H-3′ and -5′); δ_C([²H₆]DMSO–D_2O) 62.1 and 62.4 (C-6¹ and -6²), 70.0, 71.4, 74.0, 75.3, 77.3, 77.6 and 77.8 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 88.2 (C-3¹), 100.8 (C-1¹), 104.6 (C-1²), 118.3 (C-2′ and -6′), 127.8 (C-3′ and -5′), 144.0 (C-4′) and 166.2 (C-1′).

Methyl 3-*O*-β-D-glucopyranosyl-β-D-glucopyranoside 5

The bromide **2** was treated as already described.[20] Flash chromatography (EtOAc–light petroleum 1:1) of the reaction mixture afforded compound **8** (926 mg, 59%), mp 165 °C (from Et_2O–hexane); $[\alpha]_D$ −42.8 (*c* 1.0, $CHCl_3$) {(lit.,[20] mp 164–165 °C (from EtOH); $[\alpha]_D^{20}$ −46.0 (*c* 1.2, $CHCl_3$)}; δ_H($CDCl_3$) 1.99–2.14 (21 H, 7 s, CH_3CO), 3.48 (3 H, s, OCH_3), 3.64–3.71 (2 H, m, H-5¹ and -5²), 3.88 (1 H, dd, $J_{3,4} = J_{4,5}$ = 9.5, H-4¹), 4.30 (1 H, d, $J_{1,2}$ 8.1, H-1¹), 4.36, 4.41, 4.02 and 4.21 (4 H, m, H-6a¹, -6a², -6b¹ and -6b²), 4.59 (1 H, d, $J_{1,2}$ 8.1, H-1²) and 4.88–5.17 (5 H, m, H-2¹, -2², -3¹, -3² and -4²); δ_C($CDCl_3$) 20.9–21.4 ($COCH_3$), 56.6 (OCH_3), 61.7 and 62.1 (C-6¹ and -6²), 68.1, 68.3, 71.0, 71.7, 71.8, 72.6 and 72.9 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 78.9 (C-3¹), 100.9 (C-1¹), 101.5 (C-1²) and 160.8–170.7 (CO).

To a stirred solution of compound **8** (853 mg, 1.31 mmol) in anhydrous MeOH (19 cm³) was added freshly prepared NaOMe (0.4 mol dm^{-3} in methanol; 1 cm³), and the mixture was stirred for 20 h at room temperature. The reaction mixture was neutralized with Amberlite IRN 77(H^+) resin, filtered and evaporated to dryness. The residue was crystallized from MeOH to yield title compound **5** (465 mg, 100%), mp 163 °C (from aq. EtOH); $[\alpha]_D$ −29.3 (*c* 0.460, water) {lit.,[20] mp 164–165 °C (from EtOH–Et_2O); $[\alpha]_D^{20}$ −28.8 (*c* 1.8, water)}; δ_H([²H₆]-DMSO) 3.05–3.76 (H-2¹, -2², -3¹, -3², -4¹, -4², -5¹, -5², -6¹ and -6²), 3.45 (3 H, s, OCH_3), 4.20 (1 H, d, $J_{1,2}$ 7.8, H-1²) and 4.36 (1 H, d, $J_{1,2}$ 7.8, H-1¹); δ_C([²H₆]DMSO) 56.0 (OCH_3), 60.8 and 61.1 (C-6¹ and -6²), 68.4, 70.1, 72.7, 73.8, 76.1, 76.3 and 77.0 (C-2¹, -2², -3², -4¹, -4², -5¹ and -5²), 88.0 (C-3¹), 103.1 (C-1²) and 104.1 (C-1¹).

Preliminary experiments in enzymic polycondensation of fluoride 4

β-1,3-1,4-Glucanase from *B. licheniformis* (2.5 mm³; 2 μg cm^{-3}) was added to a solution of fluoride **4** (75–125 mol dm^{-3}; 60 mm³) in an appropriate solvent (hydroorganic or buffer). The mixture was stirred at 40 °C and analysed by HPLC or TLC at different time intervals.

Preparative enzymic synthesis of oligosaccharides 10–13

The crude fluoride **4** (1.12 g) was dissolved in maleate buffer (17 cm³; pH 7.0; 50 mmol dm^{-3} sodium maleate, 1 mmol dm^{-3} $CaCl_2$), then 1,3-1,4-β-glucanase from *B. licheniformis* (1.05 mg) dissolved in the same buffer was added (3 cm³), and the mixture was stirred at 40 °C for 2 h, then was boiled for 2 min and the precipitated protein was removed by filtration. After evaporation of filtrate to dryness, the reaction mixture was fully acetylated by treatment with Ac_2O–pyridine (80 cm³; 1:1, v/v) at 30 °C. After 24 h, MeOH (50 cm³) was added at 0 °C. The mixture was evaporated, the residue was dissolved in CH_2Cl_2, the solution was washed successively with saturated aq. $NaHCO_3$ and water, dried with Na_sSO_4, and evaporated to dryness, and the residue was purified by flash chromatography (gradient 1:1 ⟶ 5:1 EtOAc–light petroleum). Three fractions were obtained.

Eluted first were the per-acetylated disaccharides (708 mg) **1** (*m/z* 701 [M + Na]⁺) and **8** (*m/z* 673 [M + Na]⁺); eluted second were the per-acetylated tetrasaccharides (368 mg) **14** (*m/z* 1277 [M + Na]⁺) and **16** (*m/z* 1249 [M + Na]⁺); eluted last were the per-acetylated hexasaccharides (202 mg) **15** (*m/z* 1855 [M + Na]⁺) and **17** (*m/z* 1826 [M + Na]⁺). Both mixtures of tetrasaccharides and hexasaccharides were separated after Zemplén transesterification as follows.

To a stirred solution of tetrasaccharides **14** + **16** (366 mg) in anhydrous MeOH (20 cm³) was added freshly prepared NaOMe (1 mol dm^{-3} in methanol; 0.4 cm³) and the mixture was stirred overnight at 30 °C. The reaction mixture was neutralized with Amberlite IRN 77(H^+) resin, filtered, evaporated to dryness and purified by flash chromatography (MeOH–CH_2Cl_2 1:2 ⟶ 1:1).

Compound **12** (28 mg, 3%), mp 176–178 °C (decomp.) (from aq. EtOH); *m/z* 703 [M + Na]$^+$; $[\alpha]_D$ −33 (*c* 0.79, water); $\delta_H(D_2O$; 80 °C) 3.25–3.87 (H-2^{1-4}, -3^{1-4}, -4^{1-4}, -5^{1-4}, -6a$^{1-4}$ and -6b$^{1-4}$), 3.44 (3 H, s, OCH_3), 4.27 (1 H, d, $J_{1,2}$ 7.8, H-1^4), 4.42 (1 H, d, $J_{1,2}$ 8.0, H-1^1) and 4.59 (1 H, d, $J_{1,2}$ 7.8) and 4.61 (1 H, d, $J_{1,2}$ 7.7) (together H-1^2 and -1^3); δ_H([$^2H_6$]DMSO + D_2O; 500 MHz) 4.25 (H-1^1), 4.36 (H-1^2), 4.40 (H-1^3), 4.13 (H-1^4), 3.02 (H-2^1), 3.18 (H-2^2), 3.10 (H-2^3), 3.14 (H-2^4), 3.34 (H-3^1), 3.23 (H-3^2), 3.37 (H-3^3), 3.19 (H-3^4), 3.04 (H-4^1), 3.32 (H-4^2), 3.28 (H-4^3), 3.15 (H-4^4), 3.13 (H-5^1), 3.36 (H-5^2), 3.32 (H-5^3), 3.35 (H-5^4), 3.39 (H-6a^1), 3.41 (H-6a^2), 3.54 (H-6a^3), 3.43 (H-6a^4), 3.67 (H-6b^1), 3.67 (H-6b^2), 3.78 (H-6b^3), 3.65 (H-6b^4) and 3.26 (OCH_3); δ_C([$^2H_6$]DMSO + D_2O) 104.0 (C-1^1), 103.6 (C-1^3), 103.1 (C-1^4), 102.8 (C-1^2), 87.6 and 87.7 (C-$3^{1,3}$), 80.7 (C-4^2), 76.9 (C-2^1), 76.3, 76.2, 76.0, 74.9, 74.4, 73.7, 73.5 and 72.1 (C-2^{2-4}, C-$3^{2,4}$ and C-5^{2-4}), 72.1 (C-5^1), 70.0 (C-4^3), 68.3 (C-4^1), 68.3 (C-4^4), 61.1, 60.8, 60.7 and 60.3 (C-6^{1-4}) and 55.9 (CH_3).

Compound **10** (98 mg, 11%), mp 179–180 °C (decomp.) (from aq. EtOH); *m/z* 689 [M + Na]$^+$; $[\alpha]_D$ +125 (*c* 0.79, water); $\delta_H(D_2O$; 80 °C) 3.23–3.91 (H-2^{1-4}, -3^{1-4}, -4^{1-4}, -5^{1-4}, -6a$^{1-4}$ and -6b$^{1-4}$), 4.44 (1 H, d, $J_{1,2}$ 8.1, H-1^4), 4.55 (2/3 H, d, $J_{1,2}$ 8.0, H-1β^1), 4.61 (1 H, d, $J_{1,2}$ 7.7, H-1^3), [4.62 (d, $J_{1,2}$ 8.0) and 4.63 (d, $J_{1,2}$ 8.0): 1 H, H-1^2] and 5.13 (1/3 H, d, $J_{1,2}$ 5.1, H-1α^1); $\delta_C(D_2O)$ 61.5, 62.1 and 62.2 (C-6^{1-4}), 69.6, 71.1, 72.5, 72.7, 74.5, 74.7, 74.9, 75.3, 75.6, 76.3, 77.0 and 77.5 (C-2^{1-4}, -$3^{2,4}$, -$4^{1,3,4}$ and -5^{2-4}), 83.7, 85.5 and 86.0 (C-$3^{1,3}$ and -4^2), 93.5 (C-1α^1), 97.1 (C-1β^1) and 103.8, 104.1 and 104.3 (C-1^{2-4}).

To a stirred solution of hexasaccharides **15** + **17** (124 mg) in anhydrous MeOH (9 cm^3) was added freshly prepared NaOMe (1 mol dm^{-3} in methanol; 180 mm^3), and the mixture was stirred overnight at 30 °C before being neutralized with Amberlite IRN 77(H^+) resin, filtered, and evaporated to dryness, and the residue purified by flash chromatography (MeOH–CH_2Cl_2 1 : 2 ⟶ 2 : 1).

Compound **13** (6 mg, 0.6%), mp 162 °C (decomp.) (from aq. EtOH); *m/z* 1027 [M + Na]$^+$; $[\alpha]_D$ −27 (*c* 0.28, water); $\delta_H(D_2O$; 25 °C) 3.19–3.90 (H-2^{1-6}, -3^{1-6}, -4^{1-6}, -5^{1-6}, -6a$^{1-6}$ and -6b$^{1-6}$), 3.40 (3 H, s, OCH_3), 4.30 (1 H, d, $J_{1,2}$ 7.5, H-1^6), 4.42 (1 H, d, $J_{1,2}$ 7.8, H-1^1) and 4.54–4.80 (4 H, m, H-1^{2-6}).

Compound **11** (35 mg, 3.6%), mp 176–177 °C (decomp.) (from aq. EtOH); *m/z* 1013 [M + Na]$^+$; $[\alpha]_D$ +167 (*c* 0.17, water); $\delta_H(D_2O$, 80 °C), 3.21–3.89 (H-2^{1-6}, -3^{1-6}, -4^{1-6}, -5^{1-6}, -6a$^{1-6}$, -6b$^{1-6}$), 4.43 (1 H, d, $J_{1,2}$ 7.9, H-1^6), 4.54 (2/3 H, d, $J_{1,2}$ 8.0, H-1β^1), [4.59 (1 H, d, $J_{1,2}$ 7.6), 4.60 (1 H, d, $J_{1,2}$ 7.2), 4.62 (1 H, d, $J_{1,2}$ 7.9) and 4.63 (1 H, d, $J_{1,2}$ 7.6): H-1^{2-6}] and 5.11 (1/3 H, d, $J_{1,2}$ 3.7, H-1α^1).

Methyl 4-*O*-laminaribiosyl-β-laminaribioside 12

The fluoride **4** (101 mg, 0.2 mmol) and glycoside **5** (481 mg, 1.35 mmol, 7 mol equiv.) were dissolved in a mixture of maleate buffer (2 cm^3; pH 7.0; 50 mmol dm^{-3} sodium maleate, 1 mmol dm^{-3} $CaCl_2$) and MeCN (3 cm^3). Then, 1,3-1,4-β-glucanase from *B. licheniformis* (500 mm^3, 200 μg cm^{-3}) in maleate buffer were added, and the mixture was stirred at 40 °C for 15 min. The protein was precipitated by boiling for 2 min and was removed by filtration. After evaporation of the mixture to dryness, the residue was fully acetylated by treatment with Ac_2O–pyridine (50 cm^3; 1 : 1, v/v) at 30 °C. After 24 h, MeOH (40 cm^3) was added at 0 °C. The reaction mixture was evaporated, the residue was dissolved in CH_2Cl_2, the solution was washed successively with saturated aq. $NaHCO_3$ and water, then was dried with Na_2SO_4 and evaporated to dryness, and the residue was purified by flash chromatography (gradient 1 : 1 ⟶ 4 : 1 EtOAc–light petroleum). Compounds **8** (768 mg) and **16** (120 mg, 48%) were isolated. Usual deacetylation and work-up as described above, followed by crystallization, afforded title compound **12** (71 mg, 85%).

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