Oligosaccharide Synthesis by Coupled *endo*-Glycosynthases of Different Specificity: A Straightforward Preparation of Two Mixed-Linkage Hexasaccharide Substrates of 1,3/1,4-β-Glucanases

Magda Faijes,[a] Jon K. Fairweather,[b] Hugues Driguez,*[b] and Antoni Planas*[a]

Abstract: Glycosynthases are engineered glycosidases which are hydrolytically inactive yet efficiently catalyse transglycosylation reactions of glycosyl fluoride donors, and are thus promising tools for the enzymatic synthesis of oligosaccharides. Two *endo*-glycosynthases, the E134A mutant of 1,3/1,4-β-glucanase from *Bacillus licheniformis* and the E197A mutant of cellulase Cel7B from *Humicola insolens*, were used in coupled reactions for the stepwise synthesis of hexasaccharide substrates of 1,3/1,4-β-glucanases. Because the two *endo*-glycosynthases show different specificity, towards laminaribiosyl and cellobiosyl donors, respectively, the target hexasaccharides were prepared by condensation of the corresponding disaccharide building blocks through sequential addition of the glycosynthases in a "one-pot" process. Different strategies were used to achieve the desired transglycosylation between donor and acceptor in each step, and to prevent unwanted elongation of the first condensation product and polymerization (self-condensation) of the donor: 1) selection of disaccharide donors differing in the configuration of the hydroxyl substituent normally acting as acceptor, 2) temporary protection of the polymerizable hydroxyl group of the donor, or 3) addition of an excess of acceptor to decrease the probability that the donor can act as an acceptor. The best procedure involved the condensation of α-lactosyl or 4^{II}-*O*-tetrahydropyranyl-α-cellobiosyl fluorides with α-laminaribiosyl fluoride, catalyzed by E197A Cel7B, to give tetrasaccharide fluorides, which were then the donors for in situ condensation with methyl β-cellobioside catalyzed by E134A 1,3/1,4-β-glucanase. After isolation, the final hexasaccharides Galβ4Glcβ4Glcβ3Glcβ4Glcβ4Glcβ-OMe and Glcβ4Glcβ4Glcβ3Glcβ4Glcβ4Glcβ-OMe were obtained in 70–80% overall yields.

Keywords: cellulase · glucanases · glycosylation · glycosynthases · oligosaccharides

Introduction

The conventional use of retaining glycosidases for the enzymatic synthesis of oligosaccharides involves reversal of the hydrolytic reaction of the wild-type enzyme for the formation of a glycosidic linkage, either by displacing the equilibrium with a large excess of acceptor (thermodynamically controlled synthesis) or by using activated glycosyl donors such as glycosyl fluorides or aryl glycosides (kinetically controlled process).[1] Organic co-solvents have often been used to reduce the hydrolytic reaction, but transglycosylation yields rarely exceed 50% because of hydrolysis of the newly formed glycosidic linkage.[2, 3]

A novel strategy, based on the redesign of the enzyme's catalytic machinery, is changing the outlook for the enzymatic synthesis of oligosaccharides. Withers and co-workers[4] have introduced the *glycosynthase concept* for the efficient synthesis of oligosaccharides; a specifically mutated retaining glycosidase lacking its catalytic nucleophile is combined with an activated glycosyl donor of the opposite anomeric configuration to the normal substrate. This approach provides an improvement on the synthetic yields obtained under kinetically controlled transglycosylation reactions with wild-type enzymes, because the nucleophile-less mutant cannot hydrolyze the reaction product. This methodology was initially developed with the *exo*-enzyme β-glucosidase of *Agrobacterium faecalis*,[4] and was subsequently extended to *endo*-glycosidases in our laboratories,[5, 6] opening new synthetic opportunities for the regiospecific assembly of large oligosaccharides.

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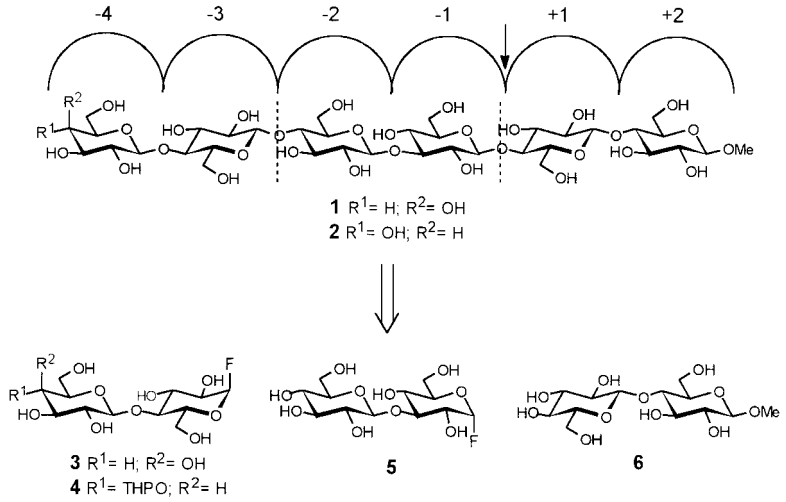
  0947-6539/01/0721-4651 $ 17.50+.50/0

Here we report the use of two *endo*-glycosynthases, the E134A mutant 1,3/1,4-β-glucanase from *Bacillus licheniformis*[5] and the E197A mutant cellulase Cel7B from *Humicola insolens*,[6] in coupled reactions. Both glycosynthases have been characterized with regard to their substrate specificity; this indicated that the minimal α-glycosyl fluoride donors were disaccharides and that both enzymes catalyze the regio- and stereospecific formation of a β-1,4 glycosidic bonds, following the same strict substrate specificity shown by their respective parent wild-type enzymes in glycoside bond hydrolysis (for reviews on structure-function studies, see ref. [7] for *Bacillus* 1,3/1,4-β-glucanases and ref. [8] for *Humicola* cellulases). The different donor specificities led us to propose the use of these glycosynthases in a stepwise (tandem) protocol or in coupled reactions, to provide a facile method for the synthesis or assembly of large oligosaccharides from readily available disaccharides.

The hexasaccharides **1** and **2** were chosen as target oligosaccharide substrates of 1,3/1,4-β-glucanases for our coupled glycosynthase methodology (Scheme 1). Depolymerization of 1,3/1,4-β-glucans, the major matrix polysaccharides of cereal endosperm cell walls, is an early event in the germination process.[9] These polysaccharides are efficiently hydrolyzed by endogenous or microbial 1,3/1,4-β-glucan 4-glucanohydrolase (EC 3.2.1.73, 1,3/1,4-β-glucanases), which specifically cleave 1,4-linkages when the glycosyl residue is a laminaribiosyl unit.[10–12] Because of its importance in glycobiotechnology, the *Bacillus* enzyme has been studied at the molecular level to elucidate its mechanism of recognition and action.[7] While protein–substrate interactions have been analyzed by mutational analysis[13] guided by the three-dimensional structure of a modeled E·S complex (based on the crystal structure of the free wild-type enzyme[14, 15]), the X-ray structure of a complex between an inactive mutant and a substrate spanning the -4 to $+2$ subsites is required to evaluate the fine structural details that define substrate specificity.



Scheme 1. Target hexasaccharides **1** and **2** are designed to span the six subsites (-4 to $+2$) of the active site of bacterial 1,3/1,4-β-glucanases; the arrow indicates the site cleaved by this class of enzyme. Dashed vertical lines indicate bond disconnections leading to the disaccharide building blocks **3**–**6**.

Results and Discussion

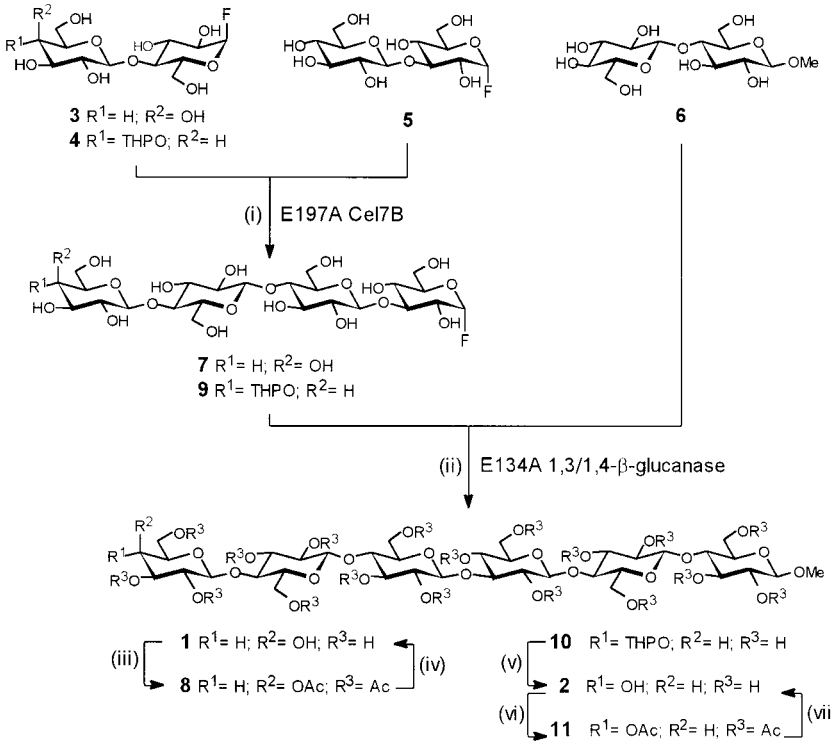
Kinetic studies on *Bacillus* 1,3/1,4-β-glucanase[7, 16] have led us to postulate that hexasaccharide **2** (Scheme 1), in which two cellotriosyl units are connected through a β-1,3-glycosidic linkage, should be the best substrate for this class of enzyme. Hexasaccharide **1**, which contains a D-galactosyl unit at the nonreducing end, should also be a good substrate, since molecular modeling indicated that no strong protein–ligand interactions (hydrogen-bond contacts) exist with a saccharide residue in subsite -4.

Scheme 1 delineates the retro-synthetic analysis on which the syntheses are based. Disconnection at the indicated bonds leads to the key disaccharide building blocks **3**–**6**. The specificity of each of the glycosynthases to be used was known from previous work. In the case of E197A mutant of Cel7B from *H. insolens*, α-cellobiosyl fluoride is an efficient donor but is readily polymerized. To prevent polymerization in coupling reactions, α-lactosyl fluoride (**3**)[6] or 4^{II}-*O*-tetrahydropyranyl-α-cellobiosyl fluoride (**4**)[17] may be used as donors in the presence of various acceptors. Quantitative yields of coupled compounds were obtained when β-cellobioside and β-laminaribioside were used as acceptors. On the other hand, α-laminaribiosyl fluoride is the donor for the E134A mutant of 1,3/1,4-β-glucanase from *B. licheniformis*, and both β-cellobiosides and β-laminaribiosides are good acceptors for efficient coupling.[5] Two routes are therefore proposed for preparation of the hexasaccharides **1** and **2**. In route 1, E197A Cel7B-catalyzed condensations of fluoride donors **3** or **4** with α-laminaribiosyl fluoride (**5**) will give tetrasaccharide fluorides, which in turn are donors for E134A 1,3/1,4-β-glucanase-catalyzed condensation with methyl β-cellobioside (**6**). Alternatively, in route 2, E134A 1,3/1,4-β-glucanase-catalyzed condensation of **5** with acceptor **6** will yield a stable methyl tetrasaccharide, which should then be an acceptor for the E197A Cel7B-catalyzed reaction with fluoride donor **3**.

Preliminary model studies were conducted with each enzyme to find the most appropriate experimental conditions compatible with both glycosynthases for a "one-pot" reaction (optimal conditions were phosphate buffer pH 7.0, 0.1 mM $CaCl_2$, 35 °C).

The first route for the synthesis of **1** is outlined in Scheme 2. The E197A Cel7B-catalyzed coupling of an equimolar amount of α-lactosyl fluoride (**3**) with α-laminaribiosyl fluoride (**5**) gave tetrasaccharide **7** as the sole product after 12 hours (TLC monitoring). Methyl β-cellobioside (**6**) and E134A 1,3/1,4-β-glucanase were then added to the reaction mixture. An excess of **6** (5 equiv) was used to saturate the

0947-6539/01/0721-4652 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 21

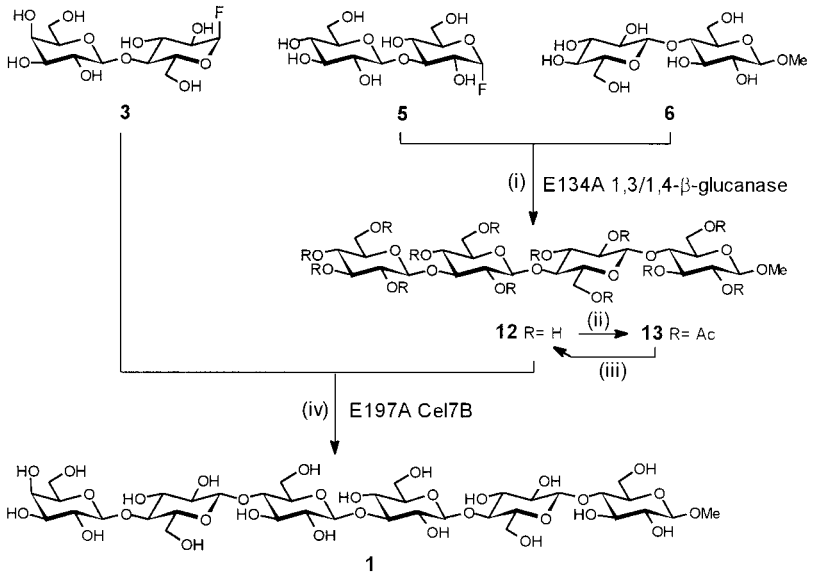


Scheme 2. Synthesis of hexasaccharides **1** and **2** by route 1. i), ii) phosphate buffer pH 7.0, $CaCl_2$ 0.1 mM, 35 °C; iii) Ac_2O/pyridine, 24 h, RT (80 % from **3**); iv) MeONa/MeOH, 4 h, RT (90 %); v) 1M HCl, 30 min, RT; vi) Ac_2O/pyridine, 12 h, RT (75 % from **4**); (vii) MeONa/MeOH, 3 h, RT (90 %).

acceptor subsites and increase the rate of transglycosylation over hydrolysis of **7**. Mass spectrometry confirmed the formation of a hexasaccharide product (*m/z* 1027 $[M+Na]^+$). Removal of the excess **6** and enzymes was achieved by acetylation, and chromatographic separation of the reaction mixture then gave the peracetylated hexasaccharide **8** in 80 % overall yield. Minor tetrasaccharide side-products were also isolated; peracetylated Glcβ3Glcβ4Glcβ4Glcβ-OMe, (arising from condensation of acceptor **6** with traces of unreacted **5** from the first glycosynthase reaction), and an inseparable mixture of peracetylated tetrasaccharides corresponding to Glcβ3Glcβ-4Glcβ3Glc (from self-condensation of **5**) and Galβ-4Glcβ4Glcβ3Glc (from hydrolysis of **7**). De-*O*-acetylation of **8** with sodium methoxide in methanol gave the target hexasaccharide **1** in 90 % yield (75 % yield from **3**).

Hexasaccharide **2** was prepared using the analogous route (Scheme 2). E197A Cel7B-catalyzed coupling of the THP-protected (THP = tetrahydropyranyl) cellobiosyl fluoride **4** with **5** gave tetrasaccharide fluoride **9** in quantitative yield after 14 hours. A fivefold excess of methyl β-cellobioside (**6**) and E134A 1,3/1,4-β-glucanase were then added to give hexasaccharide **10**, as the sole reaction product (not isolated), after 24 hours. The THP group did not seem to affect binding to the donor subsites of the enzyme; this prevented self-condensation in the second glycosynthase reaction [the corresponding unprotected tetrasaccharide, 3-*O*-β-cellotriosyl-α-glucosyl fluoride, undergoes a fast polymerization reaction catalyzed by E134A 1,3/1,4-β-glucanase (data not shown)]. Once the oligosaccharide chain was assembled, the THP group was removed by acid hydrolysis (1M HCl) to give hexasaccharide **2**, which had to be isolated as the acetate **11**. De-*O*-acetylation afforded **2** in excellent yield (70 % from fluoride **4**)

Scheme 3 outlines the second route for the preparation of the hexasaccharide **1**. The preparation of methyl tetrasaccharide **12** by the E134A 1,3/1,4-β-glucanase-catalyzed coupling of fluoride **5** with methyl β-cellobioside (**6**) was investigated first. Thus, reaction of equimolar amounts of donor **5** (unprotected at 4^{II}OH) with β-cellobioside **6**, followed by acetylation of the mixture, gave tetrasaccharide **13** in low yield (44 %), along with undesired side-products from the self-condensation of **5** or elongation of the newly formed tetrasaccharide **12**.



Scheme 3. Synthesis of hexasaccharide **1** by route 2. i) Phosphate buffer pH 7.0, $CaCl_2$ 0.1 mM, 35 °C, 24 h; ii) Ac_2O/pyridine, 24 h, RT (87 % from **5**); iii) MeONa/MeOH, 4 h, RT (95 %); iv) phosphate buffer pH 7.0, 35 °C, 24 h (87 %).

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Although the use of a THP-protected compound as an alternative donor could be envisaged, an additional deprotection step of the first condensation product would still be required before it could be used as an acceptor in the second glycosynthase reaction. Alternatively, side-reactions could be minimized by using an excess of acceptor **6**. Indeed, the peracetylated tetrasaccharide **13** was obtained in 87% yield when a fivefold excess of **6** was employed.

De-*O*-acetylation of **13** gave **12** in 95% yield, which then acted as the acceptor in the E197A Cel7B-catalyzed coupling with α-lactosyl fluoride (**3**). Reaction of equimolar amounts of **12** and **3** afforded the target hexasaccharide **1** in 87% yield after purification by reversed-phase chromatography. The overall yield of **1** (72%) is similar to that obtained previously (Scheme 2); however the latter route required an additional purification step between the two glycosynthase reactions to remove the excess of acceptor **6**, which may otherwise compete in the second glycosynthase-catalyzed coupling.

Both hexasaccharides **1** and **2** have been assayed as substrates of the wild-type 1,3/1,4-β-glucanase from *Bacillus licheniformis*; methyl β-cellobioside (**6**) and the corresponding tetrasaccharides were the only hydrolysis products observed. In agreement with the strict substrate specificity of the wild-type enzyme, this result provides additional proof of the stereochemistry of the hexasaccharides.

Conclusion

This work shows that the sequential use of glycosynthases of different substrate specificity is a rapid method for the assembly of oligosaccharides.

The use of two *endo*-glycosynthases required the modulation of the three possible reactions: the desired transglycosylation between donor and acceptor, the unwanted elongation of the first condensation product, and the polymerization (self-condensation) of the donor. We have used different strategies to avoid or reduce the undesired polymerization and elongation reactions: 1) selection of a disaccharide donor differing in the configuration of the hydroxyl substituent normally acting as acceptor, 2) use of a temporary protecting group on the polymerizable hydroxyl group of the donor, or 3) by addition of an excess of acceptor to decrease the probability that the donor can act as an acceptor. The hexasaccharides **1** and **2** obtained here are invaluable as substrates for kinetic and structural studies of wild-type and mutated 1,3/1,4-β-glucanases.

Experimental Section

General: NMR spectra were recorded on a Bruker AC300 or Varian Gemini-300 in solvents as specified. ^{1}H and ^{13}C chemical shifts (δ in ppm) were referenced to TMS and to the solvent signal, respectively. Full NMR spectra are provided in the supporting material. High-resolution mass spectra (HRMS) were recorded on VG ZAB and low-resolution (MS) on a Nermag R-1010C spectrometer in the fast-atom bombardment (FAB) mode; *m/z* for the peaks (100%) corresponding to $[M+Na]^+$ are given. Optical rotations were measured at 20 °C with a Perkin–Elmer 241 polarimeter. Melting points were determined on a Büchi 535 apparatus and are uncorrected. TLC was performed on silica gel 60 F_{254} aluminium plates with detection by development with H_2O/MeOH/H_2SO_4 (9:9:1, v/v/v) and heating at 125 °C. Flash chromatography was performed with Merck silica gel 60 (0.040–0.063 nm) and reversed-phase chromatography on C18 Sep-Pak Plus cartridges (Waters) with eluents as specified.

Enzymes and substrates: Mutant E134A 1,3/1,4-β-glucanase from *Bacillus licheniformis* was expressed and purified as previously reported,[5, 18] as was Mutant E197A endoglucanase Cel7B from *Humicola insolens*.[6] Both enzymes were freeze-dried for storage (at −20 °C) and dissolved in the reaction buffer prior to use. Disaccharide donors and acceptors were prepared as previously reported: α-lactosyl fluoride (**3**),[6] 4^{II}-*O*-tetrahydropyranyl-α-cellobiosyl fluoride (**4**),[17] α-laminaribiosyl fluoride (**5**),[5] and methyl β-cellobioside (**6**).[19]

Methyl *O*-β-D-galactopyranosyl-(1→4)-*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-glucopyranoside (1)

Route 1 (Scheme 2): α-Lactosyl fluoride (**3**; 30 mg, 1 equiv, 0.09 mmol) and α-laminaribiosyl fluoride (**5**; 30 mg, 1 equiv, 0.09 mmol) were incubated with E197A Cel7B (3 mg) in phosphate buffer (0.5 mL, Na_2HPO_4 100 mM, $CaCl_2$ 0.1 mM, pH 7.0) at 35 °C. After 12 h, TLC (acetonitrile/water 7:3) indicated that the reaction was complete. Methyl β-cellobioside **6** (155 mg, 5 equiv, 0.44 mmol) and E134A 1,3/1,4-β-glucanase (1 mg) were dissolved in phosphate buffer (0.5 mL), then added to the reaction mixture and incubated at 35 °C for 2 days. After lyophilization, the reaction mixture was treated with Ac_2O/pyridine (6 mL, 1:1 v/v) at RT for 24 h. The mixture was poured into ice-water and then extracted with CH_2Cl_2. The organic layer was washed with 20% aqueous $KHSO_4$, saturated aq. $NaHCO_3$, and water. After drying over Na_2SO_4 and evaporation of the solvent, the residue was purified by flash chromatography (ethyl acetate/petroleum ether 2:1 v/v) to afford, in order of elution: peracetylated methyl β-cellobioside (226 mg) (MS: *m/z* 673 $[M+Na]^+$); an inseparable mixture of two peracetylated tetrasaccharides (34 mg) corresponding to Glcβ3Glcβ4Glcβ4GlcβOMe (MS: *m/z*: 1249 $[M+Na]^+$), and Glcβ3Glcβ4Glcβ3Glc and/or Galβ4Glcβ4Glcβ3Glc (MS: *m/z*: 1277 $[M+Na]^+$); and peracetylated hexasaccharide **8** (124 mg, 80% (from **3**)) which was crystallized from ethanol: m.p. 137–141 °C; $[\alpha]_D^{20} = -29.1$ ($c = 0.25$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 25 °C): $\delta = 5.30-3.50$ (m, 42H; H-1^{I-VI}, H-2^{I-VI}, H-3^{I-VI}, H-4^{I-VI}, H-5^{I-VI}, H-6a^{I-VI}, H-6b^{I-VI}), 3.43 (s, 3H; OCH_3), 2.20–1.80 (19s, 57H; 19CH_3CO); ^{13}C NMR ($CDCl_3$, 25 °C): $\delta = 170.5-168.4$ (CH_3CO), 101.4–100.5 (C-1^{I-VI}), 78.7 (C-3^{III}), 76.4–75.6 (C-4^{I,II,IV,V}), 72.9–67.8 (C-2^{I-VI}, C-3$^{I,II,IV-VI}$, C-4^{III}, C-5^{I-VI}), 66.5 (C-4^{VI}), 62.2–60.7 (C-6^{I-VI}), 56.9 (OCH_3), 20.8–20.3 (CH_3CO); HRMS: *m/z*: calcd for $C_{75}H_{102}O_{50}Na$: 1825.5337; found: 1825.5312 $[M+Na]^+$.

Freshly prepared sodium methoxide (1 M in methanol, 400 μL) was added to a stirred solution of **8** in anhydrous methanol (40 mL), and the mixture was allowed to react at RT for 4 h. The mixture was then neutralitzed with Amberlite IR 120 (H^+) resin, filtered, and lyophilized to give **1**: m.p. 203–208 °C; $[\alpha]_D^{20} = -12.1$ ($c = 0.25$ in H_2O); ^{1}H NMR (D_2O, 30 °C): $\delta = 4.78$ (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{IV}), 4.52 (3d, $J(1,2) = 7.8$ Hz, 3H; H-1^{II,III,V}), 4.45 (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{VI}), 4.40 (d, $J_{1,2} = 7.8$ Hz, 1H; H-1^{I}), 3.58 (s, 3H; OCH_3), 4.10–3.20 (m, 36H; H-2^{I-VI}, H-3^{I-VI}, H-4^{I-VI}, H-5^{I-VI}, H-6a^{I-VI}, H-6b^{I-VI}); ^{13}C NMR (D_2O, 30 °C): $\delta = 103.7-103.0$ (C-1^{I-VI}), 84.4 (C-3^{III}), 79.2–78.7 (C-4^{I,II,IV,V}), 76.2–71.6 (C-2^{I-VI}, C-3$^{I,II,IV-VI}$, C-5^{I-VI}), 69.2, 68.6 (C-4^{VI}, C-4^{III}), 61.7–60.6 (C-6^{I-VI}), 57.9 (OCH_3); HRMS: *m/z*: calcd for $C_{37}H_{64}O_{31}Na$: 1027.3329; found: 1027.3331 $[M+Na]^+$.

Route 2 (Scheme 3): α-Laminaribiosyl fluoride (**5**; 30 mg, 0.09 mmol), methyl β-cellobioside (**6**; 31 mg, 0.09 mmol) and E134A 1,3/1,4-β-glucanase (0.5 mg) were dissolved in phosphate buffer (0.5 mL, pH 7.0) and left for 3 days at 37 °C. After lyophilization and treatment with Ac_2O/pyridine (6 mL, 1:1 v/v) for 24 h, the reaction mixture was purified by flash chromatography (ethyl acetate/petroleum ether 2:1 v/v) to afford, in order of elution: peracetylated methyl β-cellobioside (35 mg), peracetylated tetrasaccharide **13** (47 mg, 44% yield), and an inseparable mixture of peracetylated (Glcβ3Glcβ4)$_2$Glcβ4GlcβOMe (MS: *m/z*: 1825) and (Glcβ3Glcβ4)$_4$ (MS: *m/z*: 2401).

α-Laminaribiosyl fluoride (**5**; 30 mg, 1 equiv, 0.09 mmol) and methyl β-cellobioside (**6**; 155 mg, 5 equiv, 0.44 mmol) were incubated with E134A 1,3/1,4-β-glucanase (0.5 mg) in the phosphate buffer (1 mL, pH 7.0) at 35 °C for 24 h. The mixture was lyophilized and treated with Ac_2O/pyridine (10 mL, 1:1 v/v) at RT for 24 h before work-up and flash chromatography,

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as previously described, gave peracetylated methyl β-cellobioside (207 mg) and compound **13** (92 mg, 87 %), which was identical in all respects to the sample prepared in the previous experiment.

The tetrasaccharide **13** was treated with freshly prepared sodium methoxide (1M in methanol, 300 μL) in methanol (30 mL). After 4 h at RT, the solution was neutralized with Amberlite IR 120 (H^+) resin, filtered, and lyophilized to give **12** (49 mg, 95 % yield): ^{1}H NMR (D_2O, 30 °C): $\delta = 4.6$ (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{IV}), 4.43, 4.42 (2d, $J(1,2) = 7.8$ Hz, 2H; H-1^{II,III}), 4.29 (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{I}), 3.90–3.20 (m, 28H; H-2$^{I–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6a$^{I–IV}$, H-6b$^{I–IV}$), 3.45 (s, 3H; OCH_3); ^{13}C NMR (D_2O, 30 °C): $\delta = 103.4$, 103.1, 102.6, 102.6 (C-1$^{I–IV}$), 84.3 (C-3^{III}), 78.9, 78.7 (C-4^{I,II}), 76.3, 75.9, 75.9, 75.1, 75.1, 74.6, 74.4, 74.4, 73.8, 73.3, 73.3, (C-2$^{I–IV}$, C-3^{I,II,IV}, C-5$^{I–IV}$), 69.9, 68.4 (C-4^{III,IV}), 61.0, 60.9, 60.3, 60.2 (C-6$^{I–IV}$), 57.7 (OCH_3); MS: m/z: 703 $[M+Na]^+$.

The tetrasaccharide **12** (15 mg, 0.02 mmol) and α-lactosyl fluoride (**3**; 7.6 mg, 0.02 mmol) were incubated with E197A Cel7B (0.5 mg) in phosphate buffer (0.5 mL, pH 7.0) at 35 °C. After 24 h the reaction was complete, and the mixture subjected to reversed-phase chromatography (1–2.5 % methanol in water). The fractions containing the hexasaccharide **1** were pooled and lyophilized to yield a colorless powder (19.3 mg, 87 %). MS, ^{1}H NMR, and ^{13}C NMR spectra were identical to the product obtained in route 1.

Methyl *O*-β-D-glucopyranosyl-(1→4)-*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-glucopyranoside (2): 4^{II}-Tetrahydropyranyl-α-cellobiosyl fluoride (**4**; 37.3 mg, 0.09 mmol) and fluoride **5** (30 mg, 0.09 mmol) were incubated with E197A Cel7B (3 mg) in phosphate buffer (0.5 mL, Na_2HPO_4 0.5 M, $CaCl_2$ 0.1 mM, pH 7.0) at 35 °C for 14 h. Methyl β-cellobioside **6** (155 mg, 5 equiv, 0.44 mmol) and E134A 1,3/1,4-β-glucanase (1 mg) were dissolved in phosphate buffer (0.5 mL) and were added to the reaction mixture, and the reaction mixture was left for 2 days at 35 °C. Finally, HCl (1M in methanol, 1 mL) was added, and stirring was continued for 30 min at RT. The solution was neutralized with triethylamine, lyophilized, and then treated with Ac_2O/pyridine (10 mL, 1:1 v/v) for 24 h at RT. Usual work-up and flash chromatography (ethyl acetate/petroleum ether 2:1 v/v) gave the peracetylated hexasaccharide **11** (112 mg, 75 %): ^{1}H NMR ($CDCl_3$, 25 °C): $\delta = 5.30–3.50$ (m, 42H; H-1$^{I–VI}$, H-2$^{I–VI}$, H-3$^{I–VI}$, H-4$^{I–VI}$, H-5$^{I–VI}$, H-6a$^{I–VI}$, H-6b$^{I–VI}$), 3.43 (s, 3H; OCH_3), 2.20–1.80 (19s, 57H; CH_3CO); HRMS: m/z: calcd for $C_{75}H_{102}O_{50}Na$: 1825.5337; found: 1825.5340 $[M+Na]^+$.

Compound **11** (76.5 mg) was de-*O*-acetylated in anhydrous methanol (10 mL) by treatment with freshly prepared sodium methoxide (1M in methanol, 400 μL) for 3 h at RT. The reaction mixture was neutralized with Amberlite IR 120 (H^+) resin, filtered, and lyophilized to give **2** (37 mg, 90 %): m.p. 190–192 °C; $[\alpha]_D^{20} = -12.1$ ($c = 0.26$ in H_2O); ^{1}H NMR (D_2O, 30 °C): $\delta = 4.78$ (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{IV}), 4.54, 4.52, 4.52, 4.50 (4 d, $J(1,2) = 7.8$ Hz, 4H; H-1^{II,III,V,VI}), 4.40 (d, $J(1,2) = 7.8$ Hz, 1H; H-1^{I}), 3.58 (s, 3H; OCH_3), 4.10–3.20 (m, 36H; H-2$^{I–VI}$, H-3$^{I–VI}$, H-4$^{I–VI}$, H-5$^{I–VI}$, H-6a$^{I–VI}$, H-6b$^{I–VI}$); ^{13}C NMR (D_2O, 30 °C): $\delta = 103.7–103.1$ (C-1$^{I–VI}$), 86.8 (C-3^{III}), 79.2–79.0 (C-4^{I,II,IV,V}), 76.6–73.5 (C-2$^{I–VI}$, C-3$^{I,II,IV–VI}$, C-5$^{I–VI}$), 70.1, 68.6 (C-4^{VI}, C-4^{III}), 61.2–60.5 (C-6$^{I–VI}$), 57.8 (OCH_3); HRMS: m/z: calcd for $C_{37}H_{64}O_{31}Na$: 1027.3329; found: 1027.3333 $[M+Na]^+$.

Acknowledgements

We thank Dr. Martin Schülein (Novozyme, Denmark) for supplying the enzyme E197A CelB cellulase from *Humicola insolens*, and Dr. Sebastien Fort (CERMAV) for providing the compounds **3** and **4** and for preliminary experiments with the Cel7B cellulase. This work was supported by Grants BIO97–0511-C02–02 and BIO2000–0647-C02–02 from the Ministerio de Ciencia y Tecnología, Spain, and 1999SGR0036 from the Generalitat de Catalunya (to A.P.). M.F. acknowledges a pre-doctoral fellowship from the Instituto Danone, and a travel grant from the Generalitat de Catalunya. CNRS is acknowledged for financial support (to H.D.) and a post-doctoral fellowship (to J.K.F.).

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Received: May 3, 2001 [F3234]