PERKIN 1

Chemoenzymic synthesis of (1→3,1→4)-β-D-glucooligosaccharides for subsite mapping of (1→3,1→4)-β-D-glucan endohydrolases

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A series of unsubstituted (1→3,1→4)-β-D-glucooligosaccharides, designed for subsite mapping in which the number of glucosyl-binding subsites and the subsite-binding/transition state activation affinities at individual subsites of plant and bacterial (1→3,1→4)-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73) can be determined, has been synthesised through chemical and enzymic procedures. A recombinant (1→3,1→4)-β-D-glucan 4-glucanohydrolase from *Bacillus licheniformis* has been used in organic media to catalyse the condensation of 3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl fluoride (Glcβ3GlcβF, compound **1**) with cellobiose (Glcβ4Glc, **2**), cellotriose (Glcβ4Glcβ4Glc, **3**), cellotetraose (Glcβ4Glcβ4Glcβ4Glc, **4**) and cellopentaose (Glcβ4Glcβ4Glcβ4Glcβ4Glc, **5**), to produce the (1→3,1→4)-β-D-glucooligosaccharides, Glcβ3Glcβ4Glcβ4Glc **6**, Glcβ3Glcβ4Glcβ4Glcβ4Glc **7**, Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glc **8**, Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glcβ4Glc **9**. Synthesised oligosaccharides **6**–**9** were isolated in yields of 15–45%, compared with compound **1**. In a second series of syntheses, a cellodextrin phosphorylase (EC 2.4.1.49) from *Clostridium thermocellum* was used to sequentially transfer glucosyl residues from α-D-glucopyranosyl phosphate **10** to the 4-position of the non-reducing terminus of the trisaccharide Glcβ3Glcβ4Glc **11**, to generate the (1→3,1→4)-β-D-glucooligosaccharides, Glcβ4Glcβ3Glcβ4Glc **12**, Glcβ4Glcβ4Glcβ3Glcβ4Glc **13**, Glcβ4Glcβ4Glcβ4Glcβ3Glcβ4Glc **14** in 14, 10 and 5% yield, respectively, from compound **11**.

Introduction

Polymeric (1→3,1→4)-β-D-glucans are major constituents of cell walls in monocotyledonous plants of the Order Poales, which include commercially valuable pasture grasses and cereal species. The (1→3,1→4)-β-D-glucans consist of unbranched chains of 1000 or more β-D-glucosyl residues, polymerised through both (1→3)- and (1→4)-glycosidic linkages.[1–3] In (1→3,1→4)-β-D-glucans from walls of the starchy endosperm of barley (*Hordeum vulgare*), single (1→3)-β-D-glucosyl residues are usually separated by blocks of two or three adjacent (1→4)-β-D-glucosyl residues.[3] Longer blocks of up to 14 contiguous (1→4)-β-D-glucosyl residues also occur, but these account for less than 10% of the polysaccharide overall.[3]

During the mobilisation of endosperm reserves in germinated barley grain, (1→3,1→4)-β-D-glucan endohydrolases (EC 3.2.1.73) play a central role in the depolymerisation of cell wall (1→3,1→4)-β-D-glucans. These enzymes hydrolyse (1→4)-β-glucosyl linkages on 3-*O*-substituted glucopyranosyl residues.[1,4,5] The major products of (1→3,1→4)-β-D-glucanase action on this polysaccharide are therefore the trisaccharide 3-*O*-β-D-cellobiosyl D-glucose (Glcβ4Glcβ3Glc) and the tetrasaccharide 3-*O*-β-cellotriosyl D-glucose (Glcβ4Glcβ4Glcβ3Glc).

As part of a broader programme aimed at defining in detail the molecular basis of barley (1→3,1→4)-β-D-glucanase substrate specificity, the mechanism of binding of substrate to the enzyme, and the molecular mechanism of catalysis, we wish to use subsite mapping procedures to determine subsite affinities for individual β-glucosyl residues of the substrate and to define the disposition of catalytic amino acids in the substrate-binding region of the enzyme. Considerable effort is also being directed towards understanding the molecular mechanisms of catalysis of related (1→3,1→4)-β-D-glucanases from *Bacillus* spp.[6–9] The process of subsite mapping is based on observations that polysaccharide hydrolases bind individual glucosyl residues in their substrates *via* a series of tandemly arranged subsites.[10,11] Kinetic analyses of the hydrolysis of well defined oligosaccharides and the determination of initial products of the reactions allow a precise definition of the number of individual glucosyl binding subsites involved in the enzyme–substrate association to be made, they allow the position of the catalytic site to be defined in relation to the glycosyl-binding subsites, and they enable binding energies at each subsite to be calculated.

Subsite mapping of the (1→3,1→4)-β-D-glucanases requires a series of oligomeric substrates that contain both (1→3)- and (1→4)-β-glucosyl residues (see Scheme 3). Although the protein folds of the *Bacillus* (classified in glycosyl hydrolase family 16[12]) and the barley (family 17[12]) (1→3,1→4)-β-glucanases differ, both enzymes have deep clefts 30–40 Å in length that extend over their surfaces. These clefts are long enough to accommodate 6–9 individual β-glucosyl residues.[13–15] This suggests that there may be up to nine β-glucosyl-binding subsites on the enzymes and it has been necessary to design oligomeric substrates to map regions on both the non-reducing (donor) and reducing (acceptor) terminal side of the linkage that is hydrolysed (Scheme 3). Some of the oligosaccharides shown in Scheme 3 could be purified from enzymic hydrolysates of the barley (1→3,1→4)-β-D-glucan, but many are not easily obtained. Furthermore, to ensure that binding of the substrates would most accurately mimic (1→3,1→4)-β-D-glucan binding to the enzyme, it was considered essential to avoid substrates

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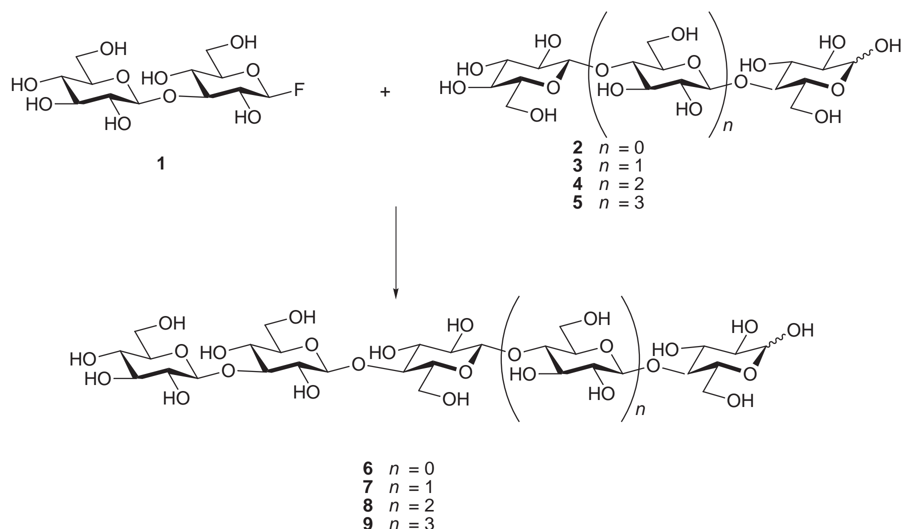
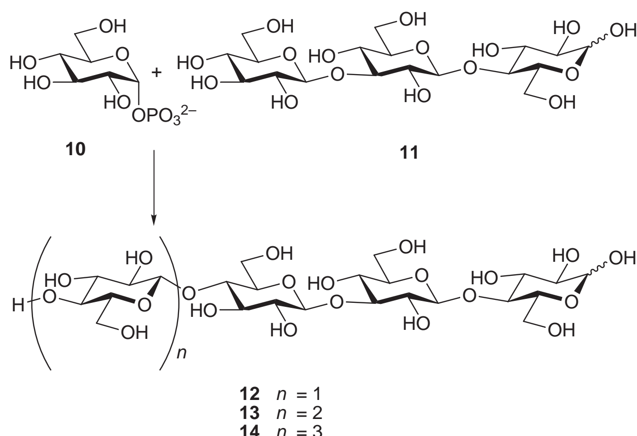


Scheme 1 Preparative condensation reaction catalysed by the (1→3,1→4)-β-D-glucanase from *B. licheniformis*. Condensation of the glucosyl donor Glcβ3GlcβF **1** and (1→4)-β-D-linked glucooligosaccharides of DP 2-5 (compounds **2**–**5**), which serve as acceptor molecules: (1→3,1→4)-β-D-glucanase 0.1% w/v, 50 mmol l^{-1} sodium maleate buffer, pH 7.0, containing 1 mmol l^{-1} $CaCl_2$, 40 °C, 20 min, 3–7 molar excess of compounds **2**–**5**.



Scheme 2 Preparative transglycosylation reaction catalyzed by cellodextrin phosphorylase from *Cl. thermocellum*. G-1P (compound **10**) served as the glucosyl donor and Glcβ3Glcβ4Glc (compound **11**) was the acceptor: 0.4 U cellodextrin phosphorylase in 100 mmol l^{-1} MOPS buffer, pH 7.0, containing 4 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 25 °C, 22 h, 10-molar excess of compound **10**.

which carried any chemical substituents or were chemically distinct in any other way from the native oligosaccharides.

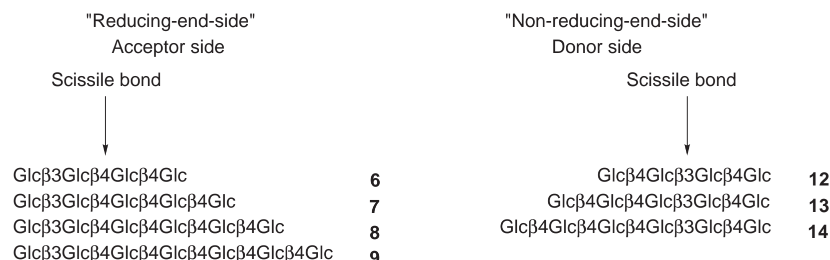
Here we describe the synthesis, purification and characterisation of eight (1→3,1→4)-β-D-glucooligosaccharides which will allow the future characterisation of (1→3,1→4)-β-D-glucanases by subsite-mapping procedures. The oligosaccharides required for mapping the reducing terminal region of the enzyme–substrate complex (acceptor subsites) (Scheme 3) were synthesised through transglycosylation reactions between laminarabiosyl fluoride **1** and various acceptors in reactions catalysed in acetonitrile–water by a highly stable (1→3,1→4)-β-D-glucanase from *B. licheniformis* (see Scheme 1). Oligosaccharides required to map the non-reducing terminal region (donor subsites) (Scheme 3) were synthesised from 4-*O*-β-D-laminarabiosyl-D-glucose (Glcβ3Glcβ4Glc) using a cellodextrin phosphorylase (EC 2.4.1.49) from *Clostidium thermocellum* and α-glucopyranosyl phosphate (G-1P) (see Scheme 2). This reaction results in the transfer of glucose units from the glucose 1-phosphate to the 4-position on the non-reducing end glucosyl residue of the primer oligosaccharide, Glcβ3Glcβ4Glc.

Results and discussion

The primary objective of the present study was to synthesise a series of unsubstituted (1→3,1→4)-β-D-glucooligosaccharides which could subsequently be used for subsite mapping of (1→3,1→4)-β-D-glucan endohydrolases (EC 3.2.1.73). To avoid possible complications in kinetic analyses imposed by multiple hydrolytic events during a single enzyme–substrate encounter (multiple attack), each substrate was synthesised with only one scissile linkage; these enzymes have an absolute requirement for adjacent (1→3)- and (1→4)-linked β-D-glucosyl residues (Scheme 3). Similar oligosaccharides have been synthesised in the past, but usually carry methyl, 4-nitrophenyl or 4-methylumbelliferyl groups at their reducing termini.[8,16–21] Although the substituted oligosaccharides are bound and hydrolysed by (1→3,1→4)-β-D-glucan endohydrolases, and have been used for subsite mapping of bacterial (1→3,1→4)-β-D-glucanases,[8,17,21] the methyl, 4-nitrophenyl and 4-methylumbelliferyl substituents are likely to influence substrate-binding affinities during subsite-mapping experiments, particularly the affinities of glucosyl-binding subsites near the reducing end of the substrate, where substrate-stacking interactions might occur.[22,23]

Here, chemical and enzymic procedures have been used to synthesise the seven unsubstituted (1→3,1→4)-β-D-glucooligosaccharides shown in Scheme 3; the eighth oligosaccharide needed for subsite mapping (Glcβ3Glcβ4Glc) was isolated from hydrolysates of barley (1→3,1→4)-β-D-glucan. In all cases, reaction products were purified by high-performance liquid chromatography (HPLC) and analysed. Not only did this facilitate the isolation of highly purified oligosaccharides, it also enabled unchanged acceptor and donor molecules to be recovered for additional syntheses. Structures of these compounds were routinely confirmed by ^{13}C NMR spectroscopy, optical rotation and mass spectrometry. The NMR measurements were performed in mixtures of D_2O or mixtures of [$^2H_6$]DMSO and D_2O, and recorded at 350 MHz at 303 K, except for compounds **13** and **14** (Scheme 2), where significantly decreased or attenuated signals for C-3 glycosidic linkage were observed. However, by increasing the temperature to 333 K for compound **13** and 353 K for compound **14**, and by using dry [$^2H_6$]DMSO as a solvent, the relaxation of the C-3 signal improved and became fully detectable (results not shown).[24]

The first-order chemical shifts of the synthesised compounds were assigned from chromatograms by analysing the relative intensities of individual signals. For example, immediately after dissolving 10 mg of compound **11** in 350 μl D_2O and after 30 min of spectrum accumulation, we could detect 24 first-order chemical shifts. Compound **11** was predominantly in its β-anomeric form, as found by comparison of relative intensities of the C-1 signals (δ_C 92.60 and 96.55) of its respective α- and β-



Scheme 3 The two series of (1→3,1→4)-β-D-glucooligosaccharides required for subsite mapping of donor subsites (−5 to −2) and acceptor subsites (+2 to +5) of (1→3,1→4)-β-D-glucan endohydrolases. The glycosidic linkage hydrolysed by these enzymes is indicated by arrows.

reducing terminals. The C-3 singlet of the unit II was typically observed around δ_C 84.86 and the C-4 α- and β-anomeric signals were located at δ_C 79.56 and 79.42, respectively. The group of signals between δ_C 66.8 and 76.77 was assigned to atoms C-2 to C-5, and the group of signals between δ_C 60.74 and 61.49 belonged to C-6, which were not involved in glycosidic linkages. Similarly, the singlets of C-1 of units II and III, at δ_C 103.08 and 103.56, respectively, were fully resolved. Therefore, the fact that the compound **11** exhibited a single resonance for C-3 in unit II, which corresponded to that of β-laminarabiose (δ_C 86.0), indicated that this unit was 3-*O*-substituted. Thus, the compound was identified as Glcβ3Glcβ4Glc. Analogously, the ^{13}C NMR chemical shifts for compounds **6**–**9** and **12**–**14** were assigned and the data are listed in sections describing semi-preparative syntheses of the relevant compounds.

Different strategies were required for the synthesis of (1→3,1→4)-β-D-glucooligosaccharides that will be used to calculate binding energies of glucosyl residues bound on the reducing-end side or on the non-reducing-end side of the linkage that is hydrolysed (Scheme 3). For the 'reducing end' oligosaccharides, β-D-laminarabiosyl fluoride (Glcβ3GlcβF **1**), freshly prepared at 0 °C and under strictly anhydrous conditions, was used as the laminarabiosyl donor in condensation reactions with the acceptor molecules cellobiose, cellotriose, cellotetraose and cellopentaose. The reactions were effected with a highly stable (1→3,1→4)-β-D-glucanase from *B. licheniformis*, which retains activity in the presence of acetonitrile.[20,25] Although this enzyme normally acts as a hydrolase in aqueous media, its hydrolytic action can be reversed in solutions containing acetonitrile, in favour of the synthesis of higher oligosaccharides.[26–28] Furthermore, the enzyme provides the specificity that ensures that the laminarabiosyl residues are added only to the C-4 positions of acceptor cellodextrins.

During the preparation of acceptor cellodextrins, which were obtained by acid-catalysed acetolysis of cellulose followed by de-*O*-acetylation of the per-*O*-acetylated oligomeric products, a marked degradation of cellotriose was observed in the de-*O*-acetylation reaction, under conditions where yields of the other cellodextrins were relatively high. The reason for this is not clear, but good yields of cellotriose were eventually obtained by reducing the time of the de-*O*-acetylation, by ensuring that reaction mixtures remained free of water, and by keeping the temperature at 25 °C. Another critical factor in setting up the condensation reactions was the need to use both donor and acceptor molecules at close to saturating concentrations. Cellodextrins were used at 3 (cellopentaose)- to 7 (cellobiose)-times the concentration of Glcβ3GlcβF, on a molar basis, to prevent autocondensation of donor molecules. Under these conditions, molar yields of Glcβ3Glcβ4Glcβ4Glc **6**, Glcβ3-Glcβ4Glcβ4Glcβ4Glc **7**, Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glc **8** and Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glcβ4Glc **9** ranged from 15–45% from Glcβ3GlcβF **1**. To obtain these yields two successive additions of the (1→3,1→4)-β-D-glucanase to the reaction mixture were necessary; the second addition increased yields by 5–10%.

For the synthesis of the 'non-reducing end' oligosaccharides (Scheme 3), a different strategy was employed. The starting compound was Glcβ3Glcβ4Glc **11**, which was purified from hydrolysates generated by hydrolysis of barley (1→3,1→4)-β-D-glucan with a commercial (1→4)-β-D-glucanase (EC 3.2.1.4; 'endo-cellulase') preparation. Maximum yields of Glcβ3Glc-β4Glc were obtained after 3 h at 37 °C in 10 mM sodium acetate buffer, pH 5.0. As the incubation time was increased, yields of Glcβ3Glcβ4Glc decreased markedly and after 22 h only cellobiose (Glcβ4Glc) could be detected. The structure of Glcβ3Glcβ4Glc was confirmed by its ^{13}C NMR spectrum, which was identical with a previously published spectrum[29] and by the products formed following hydrolysis with purified barley β-D-glucosidase and β-D-glucan exohydrolase, both of which release glucose from the non-reducing terminus of oligomeric substrates.[30–32] In the enzymic assays cellobiose and glucose were observed initially, as expected for the hydrolysis of Glcβ3Glcβ4Glc from the non-reducing end, and both enzymes produced glucose as the final hydrolysis product.

Following the purification of Glcβ3Glcβ4Glc **11**, 4-linked β-glucosyl residues were sequentially added to its non-reducing terminus by a cellodextrin phosphorylase (EC 2.4.1.49) from *Cl. thermocellum*. This enzyme specifically transfers glucosyl residues from G-1P **10** to the C-4 atom of β-D-glucoside acceptors.[16] To avoid cellodextrin synthesis from dephosphorylated G-1P, glucose oxidase and catalase were added to destroy free glucose.[16,19] Time-course studies showed that maximum yields of the three target molecules were achieved after 22 h of incubation and these yields remained unchanged for an additional 24 h. Under these conditions, the three (1→3,1→4)-β-D-glucooligosaccharides Glcβ4Glcβ3Glcβ4Glc **12**, Glcβ4Glcβ4-Glcβ3Glcβ4Glc **13** and Glcβ4Glcβ4Glcβ4Glcβ3Glcβ4Glc **14** (Scheme 3) were obtained in 14%, 10% and 5% yield, respectively, from the initial acceptor molecule Glcβ3Glcβ4Glc **11**. Oligosaccharides with a degree of polymerisation higher than 6 were never observed.

In the course of these studies, attempts were made to synthesise Glcβ4Glcβ4Glcβ3Glcβ4Glc **13** *via* an alternative reaction in which the condensation of Glcβ4GlcF and Glcβ-3Glcβ4Glc was catalysed by a recombinant *Humicola insolens* (1→4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4).[33] Although this synthetic strategy was successful, yields of the Glcβ4Glc-β4Glcβ3Glcβ4Glc **13** were low and the approach was therefore abandoned (data not shown).

In conclusion, we have successfully used two distinct strategies to synthesise a series of specific (1→3,1→4)-β-D-gluco-oligosaccharides. The ^{13}C NMR spectroscopic data, together with mass spectrometric data and, in some instances, with enzymic analyses, were used to identify the structures of the compounds. The oligosaccharides will be used in the immediate future to explore the kinetics of hydrolysis and substrate binding by barley and *Bacillus* (1→3,1→4)-β-D-glucan endohydrolases, and to define binding/transition-state activation affinities of individual glucosyl-binding subsites in the substrate-binding regions of the enzymes. Furthermore, the newly synthesised oligosaccharides could prove useful in X-ray crystallographic studies on the disposition of amino acid residues that are

involved in substrate binding and catalysis. The three-dimensional structures of both the *Bacillus* and the barley (1→3,1→4)-β-D-glucanases have been solved.[6,13] Although they have quite different C^{α} folds, their overall molecular shapes are very similar.[6,13,15] However, it is not clear from the crystallographic data why k_{cat}-values for the *Bacillus* enzyme exceed those for the barley enzyme by more than 2 orders of magnitude (M. Hrmova, B. A. Stone and G. B. Fincher, unpublished data). The oligosaccharides synthesised in the present work might allow detailed comparisons of substrate binding between the two enzymes and could, in turn, offer structural and chemical explanations for the dramatic differences in their catalytic efficiencies.

Experimental

Materials

Silica gel 60, TLC plates and other chemicals of p.a. grade were obtained from Merck (Darmstadt, Germany). Cellulose powder (20 µm diameter) and cellobiose were from Aldrich (Milwaukee, WI, USA), Actigum CS6 containing scleroglucan was from System Bio-Industries (Paris, France), dithiothreitol (DTT), orcinol, α-D-glucopyranosyl phosphate (Glc-1P), glucose oxidase, catalase, Amberlite IRN 77(H^+) and MB-3 resin were from Sigma (St. Louis, MO, USA), Durapore HVLP 0.45 µm filters were from Millipore (Bedford, MA, USA) and D_2O and [$^2H_6$]DMSO were purchased from Peypin (Paris, France). The per-*O*-acetylated saccharides used as standards, α-D-glucopyranose pentaacetate and α-cellobiose octaacetate, were from Fluka (Buchs, Switzerland), barley (1→3,1→4)-β-D-glucan was from Megazyme International (Bray, Wicklow, Ireland) and the oligosaccharide standards, (1→4)-β-linked glucooligosaccharides (2–5) and laminarabiose were from Seikagaku Kogyo (Tokyo, Japan).

A recombinant *B. licheniformis* (1→3,1→4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73), which is referred to more simply as (1→3,1→4)-β-D-glucanase and had an activity of 510 U cm^{-3}, and a cellodextrin phosphorylase (EC 2.4.1.49) from *Cl. thermocellum*, with an activity of 1.2 U cm^{-3}, were partially purified as reported previously.[16,34] A crude *Aspergillus niger* β-D-glucanase enzyme preparation (Finizyme) with an activity of 500 U cm^{-3} was kindly provided by Dr Martin Schülein (Novo Nordisk, Copenhagen, Denmark).

General methods

^{13}C NMR spectra were recorded in D_2O and [$^2H_6$]DMSO with a Bruker 300 AC (350 MHz) spectrometer at 303, 333 or 353 K. Low-resolution mass measurements were performed in a Nermag R-1010C spectrometer in a fast-atom bombardment negative mode (LR-FAB-MS), using glycerol matrices. High-resolution mass measurements (HR-MS) were obtained in a VG ZAB spectrometer in time-of-flight (TOF) geometry, using MeCN–water (1:1, v/v) as a solvent. Optical rotations were measured at 20 °C in DMSO with a Perkin-Elmer 241 polarimeter, and $[a]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Flash and open-column chromatographies were performed on a silica gel 60 (230–400 mesh) matrix at flow rates of 10–20 ml min^{-1}. Light petroleum was the 60–80 °C fraction. Time courses of enzymic reactions and the purification of mixed-linkage oligosaccharides (compounds **6**–**9** and **12**–**14**) were performed on an HPLC system equipped with a refractometric detector (Waters, Milford, USA). Samples were eluted isocratically with MeCN–water (60–65:40–35, v/v) from a semi-preparative 250 × 21 mm Bondapak 8 µm NH_2 column. TLC was performed on Silica gel 60 plates with EtOAc–acetic acid–water (30:20:15, v/v) or EtOAC–MeOH–water (8:4:3, v/v), and mono- and oligo-saccharides were detected with the orcinol reagent[35] or by charring with 50% (v/v) sulfuric acid.

3-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl fluoride or Glcβ3GlcβF, 1

Compound **1** was prepared as described previously[20] from 1,2,4,6-tetra-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-glucopyranose, which was obtained by controlled acid-catalysed acetolysis of Antigum CS6.[19] The molecular mass and purity of the *O*-acetylated form of Glcβ3GlcβF, together with the extent of de-*O*-acetylation of per-*O*-acetylated Glcβ3GlcβF, were routinely checked by LR-FAB-MS, ^{13}C NMR and TLC.

Cellobiose 2, cellotriose 3, cellotetraose 4 and cellopentaose 5

While cellobiose **2** is commercially available, the cellooligosaccharides **3**–**5** were prepared by a de-*O*-acetylation of their acetylated forms, which were obtained by controlled acid-catalysed acetolysis of cellulose.[17,36] The acetylated derivatives α-cellotriose undecaacetate, α-cellotetraose tetradecaacetate and α-cellopentaose heptadecaacetate were isolated by flash column chromatography using 1:1 ⟶ 1:1.5 (v/v) light petroleum–EtOAc gradients, and were de-*O*-acetylated at 25 °C, with constant vigorous stirring. Freshly prepared NaOMe (1 mol l^{-1}) was added to solutions of per-*O*-acetylated sugars (1.9–3.2 mmol) in anhydrous MeOH (150 ml), and the mixture was stirred for 1.5 h (per-*O*-acetylated cellotriose) or for 41 h (per-*O*-acetylated cellotetraose and cellopentaose). The free cellooligosaccharides precipitated and were recovered by filtration. Precipitates were filtered off, dissolved in water and neutralised with Amberlite IRN 77(H^+). The resin was removed by filtration and filtrates were concentrated and lyophilised. MS and ^{13}C NMR spectra and TLC analyses were in agreement with the proposed structures of compounds **3**–**5**.

Preliminary chemoenzymic syntheses of mixed-linkage (1→3,1→4)-β-D-glucooligosaccharides 6–9

To a freshly prepared solution of compound **1** (10 mg, 0.028 mmol) in 200 µl of 50 mmol l^{-1} sodium maleate buffer, pH 7.0, containing 1 mmol l^{-1} $CaCl_2$ and a 7–3 molar excess of (1→4)-β-linked oligoglucosides of DP 2–5 (compounds **2**–**5**) was added 26 µl of a 0.5 mg ml^{-1} (1→3,1→4)-β-D-glucanase solution (corresponding to 0.1% w/v of compound **2**) from *B. licheniformis* in the buffer described above, together with 300 µl of MeCN. The mixture was stirred vigorously for 10 min at 40 °C and, when a precipitate formed after 2–5 min, another 26 µl of (1→3,1→4)-β-D-glucanase solution was added. After a total of 20 min incubation time, the enzyme was inactivated in a boiling water-bath for 3 min, the reaction mixture desalted with Amberlite IRN 77(H^+), and the resin was removed by filtration. The supernatant was concentrated by evaporation and filtered through a 0.45 µm Millipore filter. The presence of (1→3,1→4)-β-D-glucooligosaccharides **6**–**9** was checked by TLC and HPLC. The (1→3,1→4)-β-D-glucooligosaccharides **6**–**9** were further purified by HPLC, as described above, and eluted with MeCN–water (65:35 v/v for compounds **6**, **7** or 60:40 v/v for compounds **8**, **9**).

Semi-preparative syntheses of Glcβ3Glcβ4Glcβ4Glc 6; Glcβ3Glcβ4Glcβ4Glcβ4Glc 7; Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glc 8; and Glcβ3Glcβ4Glcβ4Glcβ4Glcβ4Glcβ4Glc 9

The syntheses of (1→3,1→4)-β-D-glucooligosaccharides **6**–**9** were effected as described above, except that reaction mixtures were scaled up 10-fold. Yields of purified compounds **6**–**9** were 85, 78, 77 and 49 mg, respectively, corresponding to molar yields of 45, 33, 27 and 15% from compound **1** used in the condensation reactions.

Compound 6. (85 mg, 0.128 mmol, 45% from **1**). Analytical data: $[a]_D$ +37.65 (*c* 2.47, DMSO); HR-MS, Calc. for $C_{24}H_{42}O_{21}$ $[M + Na]^+$: 689.2116; Found: *m*/*z*, 689.2123; δ_C ([$^2H_6$]DMSO;

D_2O; 303 K) 61.2 (C-6β, -6[II]), 61.3 (C-6α), 61.8 (C-6[IV]), 61.9 (C-6[III]), 69.4 (C-4[III]), 71.0–77.4 (C-2–C-5), 80.1 (C-4[II]), 80.3 (C-4β), 80.5 (C-4α), 86.1 (C-3[III]), 93.1 (C-1α), 97.2 (C-1β), 103.5 (C-1[II]), 103.7 (C-1[III]) and 104.3 (C-1[IV]).

Compound 7. (78 mg, 0.094 mmol, 33% from **1**). Analytical data: $[a]_D$ +16.00 (*c* 2.75, DMSO); HR-MS, Calc. for $C_{30}H_{52}O_{26}$ [M + Na]$^+$: 851.2645; Found: *m*/*z*, 851.2601; δ_C ([$^2H_6$]DMSO; D_2O; 303 K) 61.0 (C-6β), 61.1 (C-6[II]), 61.2 (C-6α), 61.8 (C-6[V]), 62.0 (C-6[III,IV]), 68.8 (C-4[V]), 69.3–80.1 (C-2–C-5), 80.3 (C-4β), 80.5 (C-4α), 86.3 (C-3[IV]), 93.0 (C-1α), 97.1 (C-1β), 103.4 (C-1[II]), 103.6 (C-1[III,IV]) and 104.2 (C-1[V]).

Compound 8. (77 mg, 0.078 mmol, 27% from **1**). Analytical data: $[a]_D$ +14.39 (*c* 2.78, DMSO); HR-MS, Calc. for $C_{36}H_{62}O_{31}$ [M + Na]$^+$: 1013.3173; Found: *m*/*z*, 1013.3142; δ_C ([$^2H_6$]-DMSO; D_2O; 303 K) 61.1 (C-6β, -6[II–IV]), 61.8 (C-6α), 62.0 (C-6[V]), 69.3–80.6 (C-2–C-5), 86.3 (C-3[V]), 93.0 (C-1α), 97.2 (C-1β), 103.5 (C-1[II]), 103.7 (C-1[III–V]) and 104.2 (C-1[VI]).

Compound 9. (49 mg, 0.042 mmol, 15% from **1**). Analytical data: $[a]_D$ +7.33 (*c* 3.00, DMSO); HR-MS, Calc. for $C_{42}H_{72}O_{36}$ [M + Na]$^+$: 1175.3701; Found: *m*/*z*, 1175.3697; δ_C ([$^2H_6$]-DMSO; D_2O; 303 K) 60.1–60.9 (C-6[II–IV,VI,VII]), 61.4 (C-6[V], -6β), 61.6 (C-6α), 69.0–80.8 (C-2–C-5), 87.0 (C-3[VI]), 92.8 (C-1α), 97.0 (C-1β), 103.1 (C-1[II]), 103.3 (C-1[III–VI]) and 104.2 (C-1[VII]).

Compound Glcβ3Glcβ4Glc 11

Barley (1→3,1→4)-β-D-glucan (60 mg) was dissolved in 12 ml of 50 mmol l^{-1} sodium acetate buffer, pH 5.0. Finizyme enzyme preparation (600 µl in the same buffer) was added and the mixture was incubated at 37 °C. After 1, 2, 3, 5 and 22 h, 2 ml aliquots were removed, placed in a boiling water-bath for 5 min, precipitated in 10 ml of ice-cold acetone and filtered through Celite. After *ca.* 18 h the filtrates were concentrated by evaporation, desalted with Amberlite 77(H^+), and filtered through 0.45 µm Millipore filters. The formation of compound **11** in the reaction mixtures was detected by HPLC, which showed that after 3 h of incubation, optimal yields of trisaccharide **11** were obtained.

For preparative-scale syntheses of compound **11**, the reaction mixture was scaled up 34-fold. Barley (1→3,1→4)-β-D-glucan (2 g) was hydrolysed for 3 h. The compound was isolated by flash chromatography (gradient 3:1 ⟶ 1:1.5 light petroleum–EtOAc) after the mixture had been fully *O*-acetylated as described.[20] Eluted per-*O*-acetylated compound **11** (2.4 g) was de-*O*-acetylated by the Zemplen transesterification procedure, as described above. The de-*O*-acetylation afforded 1.1 g (54% yield) of compound **11**.

Analytical data: $[a]_D$ +16.12 (*c* 2.73, DMSO); HR-MS: Calc. [M + Na]$^+$ $C_{24}H_{42}O_{21}$, 527.1588; Found: *m*/*z*, 527.1600; δ_C (D_2O; 303 K) 60.7 (C-6α), 60.9 (C-6β), 61.4 (C-6[II]), 61.5 (C-6[III]), 66.8–76.8 (C-2–C-5), 79.4 (C-4β), 79.6 (C-4α), 84.9 (C-3[II]), 92.6 (C-1α), 96.5 (C-1β), 103.1 (C-1[II]) and 103.6 (C-1[III]).

Preliminary chemoenzymic syntheses of (1→3,1→4)-β-D-gluco-oligosaccharides 12–14

Cellodextrin phosphorylase (EC 2.4.1.49) (0.4 U), glucose oxidase (EC 1.1.3.4) (35 U), catalase (EC 1.11.1.6) (735 U) and then 304 mg of G-1P (corresponding to a 10-molar excess relative to Glcβ3Glcβ4Glc **11**) were added to a solution containing compound **11** (50 mg, 0.1 mmol) in 1.8 ml of 100 mmol l^{-1} 3-morpholinopropanesulfonic acid (MOPS) buffer, pH 7.0 [containing 4 mmol l^{-1} sodium ethylenediaminetetraacetate and 2 mmol l^{-1} dithiothreitol (DTT)], to make up a final reaction volume of 2.2 ml. The mixture was vigorously stirred at 25 °C and after 8, 22 and 46 h, 0.7 ml aliquots were removed. Enzymes were inactivated in a boiling water-bath for 3 min, the aliquots were desalted with mixed-bed resin MB-3 and filtered through 0.45 µm Millipore filters, and the simultaneous presence of (1→3,1→4)-β-D-glucooligosaccharides transglucosylation products **12**–**14** was determined by HPLC using an isocratic elution with MeCN–water (65:35, v/v).

Semi-preparative syntheses of Glcβ4Glcβ3Glcβ4Glc 12; Glcβ4Glcβ4Glcβ3Glcβ4Glc 13; and Glcβ4Glcβ4Glcβ4Glcβ3Glcβ4Glc 14

The syntheses of the (1→3,1→4)-β-D-glucooligosaccharides **12**–**14** were performed as described above, except that reaction mixtures were scaled up 8-fold and the reaction time was 22 h.

Compound 12. (76 mg, 0.114 mmol, 14% from **11**). Analytical data: $[a]_D$ +13.19 (*c* 2.73, DMSO); HR-MS, Calc. for $C_{24}H_{42}O_{21}$ [M + Na]$^+$: 689.2116; Found: *m*/*z*, 689.2068; δ_C ([$^2H_6$]DMSO; D_2O; 303 K) 61.4 (C-6[I,III]), 62.1 (C-6[II,IV]), 69.5 (C-4[II]), 71.0-77.5 (C-2–C-5), 79.9 (C-4β, -4[III]), 80.0 (C-4α), 85.6 (C-3[II]), 93.3 (C-1α), 97.2 (C-1β), 103.8 (C-1[II,IV]) and 104.1 (C-1[III]).

Compound 13. (45 mg, 0.078 mmol, 10% from **11**). Analytical data: $[a]_D$ +8.06 (*c* 2.73, DMSO); HR-MS, Calc. for $C_{30}H_{52}O_{26}$ [M + Na]$^+$: 851.2645; Found: *m*/*z*, 851.2651; δ_C ([$^2H_6$]DMSO; 333 K) 60.3–61.0 (C-6[I–VI]), 70.0 (C-4[II]), 73.0–80.4 (C-2–C-5), 87.7 (C-3[II]), 91.9 (C-1α), 96.6 (C-1β) and 102.8–103.7 (C-1[II–VI]).

Compound 14. (37 mg, 0.037 mmol, 5% from **11**). Analytical data: $[a]_D$ +4.40 (*c* 2.73, DMSO); HR-MS, Calc. for $C_{36}H_{62}O_{31}$ [M + Na]$^+$: 1013.3173; Found: *m*/*z*, 1013.3147; δ_C ([$^2H_6$]-DMSO; 353 K) 60.2–60.8 (C-6[I–VI]), 69.9 (C-4[II]), 72.1–79.7 (C-2–C-5), 86.9 (C-3[II]), 91.7 (C-1α), 96.5 (C-1β) and 102.4–103.3 (C-1[II–VI]).

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