Chemoenzymatic Synthesis of Branched Oligo- and Polysaccharides as Potential Substrates for Starch Active Enzymes

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Oligo- and polysaccharides embodying the α-maltotriosyl-6^{II}-maltotetraosyl structure were readily synthesized by transglycosylation of maltosyl fluoride onto panose and pullulan catalysed by the bacterial transglycosylase cyclodextrin glycosyltransferase (CGTase). The two products obtained proved useful for increasing the knowledge of substrate binding and processing at the active site of barley limit dextrinase that is involved in the metabolism of amylopectin by acting upon its branch points.

Introduction

Increased understanding of starch metabolism is of primary importance, since the two constituents, amylose and amylopectin, are the most widely used polysaccharides in food and nonfood industries.[1] As a consequence of the structural complexity of amylopectin, the difficulties associated with the isolation of pure oligomers from natural sources and the complexity of the chemical syntheses of branched oligosaccharides representing the branch point of amylopectin,[2–4] most steps in the biosynthesis of amylopectin have been elucidated by using a genetic approach involving specific gene deletions.[5] However, insight into the mechanisms by which enzymes specifically act on and recognize amylopectin is lagging behind and this motivated the recent development of a new chemoenzymatic procedure for the preparation of substrate analogue inhibitors of α-1,6-degrading enzymes.[6] Here, we report the use of the same methodology to obtain new oligosaccharides embodying the natural α-maltotriosyl-6^{II}-maltotetraosyl structure as substrate for enzymes involved in the degradation, biosynthesis and conversion of amylopectin.[1]

Results and Discussion

Chemical synthesis of substrates 4 and 8

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19, GH family 13) has previously been employed in the preparation of branched *gluco*-oligosaccharides by coupling cyclohexaamylose and panose (**1**).[7, 8] The reported results, however, were unclear because mixtures of branched oligosaccharides terminated by panose were obtained and no experimental conditions were given. In contrast, Vetter et al. found that under kinetic control, the elongation reaction occurred only at the 4^{II}-OH of panose.[8] Summers and French described a different strategy for the synthesis of this type of branched oligosaccharide by using pullulan and dextran as acceptors.[9] In the present work, the substrate specificity of the active site of CGTase was reinvestigated by using 4^{II}-*O*-tetrahydropyranyl-maltosyl fluoride (**2**) as donor, a methodology we developed previously.[6] The 4^{II}-OH site of this derivative was temporarily blocked by a tetrahydropyranyl (THP) group to prevent self-condensation of the donor and multiple elongation of the reaction product. Enzymatic condensation onto panose (**1**) gave, after a series of protection/deprotection steps, the acetylated α-maltotriosyl-6^{II}-maltotetraose (**3**) in 80% overall yield (Scheme 1). The free heptasaccharide **4** was subsequently obtained in 96% yield by catalytic transesterification. These compounds were characterized by HRMS and partial assignment of their NMR spectra and compared with literature data for analogues in this series.[2, 10] The ultimate proof of the presence of the branch point, however, was achieved by MS/MS analysis of the reduced derivative **5**. By following the systematic nomenclature proposed by Domon and Costello to design fragments of branched oligosaccharides,[11] the negative electrospray MS spectrum of **5** had two highly abundant secondary fragments: $^{0,4}A_{3\beta}$ at $m/z = 545$ and $^{2,4}A_{3\alpha}$ at $m/z = 383$. The MS/MS experiment showed that the two ions were independent, which confirmed the structure of **5** and consequently that of **4**.

From this experiment, we concluded that the 4^{II}-OH and 4^{III}-OH sites of **1** were glycosylated with the same efficiency, which provided new information on the topology of the active site and the broad acceptor – sugar specificity of the CGTase. In fact, from examination of the X-ray structure of CGTase complexed with

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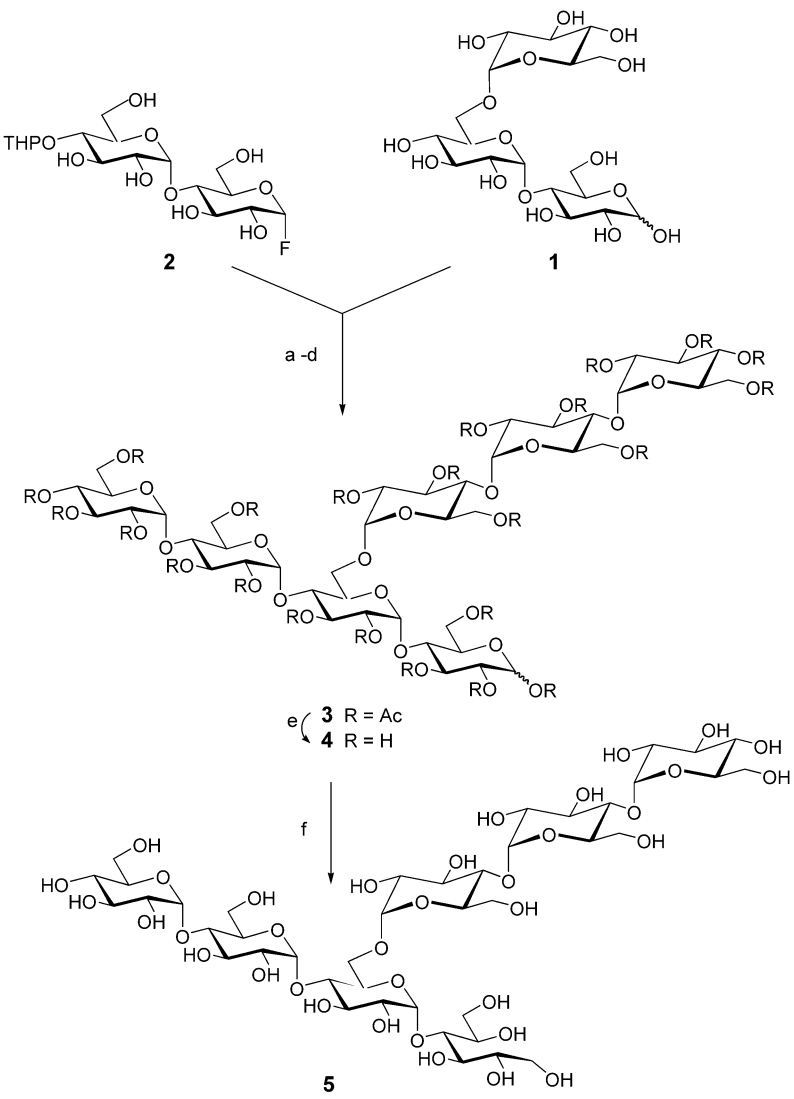
 DOI: 10.1002/cbic.200300692

various substrate analogues, it was quite unexpected that both 4-OH groups in the two glucosyl residues that constitute the nonreducing-end isomaltose unit of panose could be activated by the same general acid/base residue.[12–14] Thus, the acceptor subsites +1 and perhaps +2 of the active site can accommodate the isomaltosyl structure. We then exploited this broad specificity for the de novo preparation of a new polysaccharide from pullulan (**6**). Pullulan is a soluble linear polymer formed by maltotriosyl repeating units linked through α-(1,6) bonds and isolated from bacterial sources.[15] CGTase-catalysed transglycosylation of **6** with 1.5 equivalents of the fluoride donor **2** gave a new polymer **7**, as shown by ^{1}H NMR spectroscopy. Mild acid hydrolysis of **7** led to the removal of *O*-THP groups to give **8** (Scheme 2). Analysis of characteristic protons in the spectra of compounds **6**–**8** revealed that 30–40% of the 4^{III}-OH groups of the maltotriosyl residues of **6** were substituted by maltosyl units. This substitution level was confirmed by methylation, acid hydrolysis, $NaBH_4$ reduction, acetylation and gas chromatography analysis of **8**.[16] The results showed the presence of the 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol and of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol which correspond to the unmodified and modified repeating units of the polysaccharide **8**, respectively. The molecular ratio of these two partially methylated hexitols permitted the estimation of the substitution to be 36%.



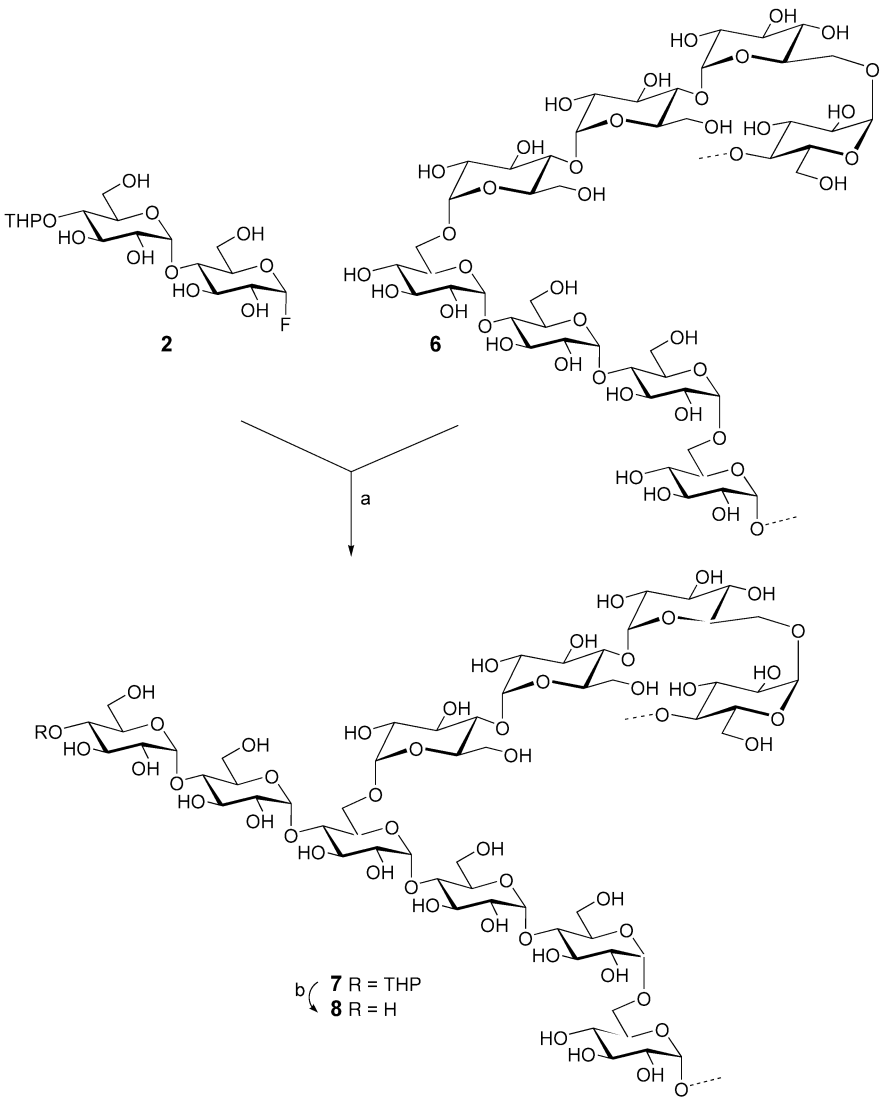
Scheme 1. *Syntheses of the target molecules **4** and **5**. Experimental conditions: a) CGTase, phosphate buffer, 40 °C, 2 h; b) Ac_2O, pyridine, 70 °C, 12 h; c) TFA, CH_2Cl_2, 30 min, RT; d) Ac_2O, pyridine, overnight, RT; e) MeONa, MeOH, overnight, RT; f) $NaBH_4$, water.*

Specificity of the limit dextrinase from barley

Compounds **4** and **8** were tested as potential substrates for the barley limit dextrinase (EC 3.2.1.142, GH family 13). As expected from earlier results,[6] these two compounds were not recognized and hydrolyzed with the same efficiency by the enzyme. Relative to pullulan (**6**), the polymer **8** was hydrolyzed at a higher rate, but with somewhat reduced affinity (Table 1). The heptasaccharide **4**, however, was hydrolyzed with the highest k_{cat} value and a smaller K_m value than polymer **8**. Three situations can be considered for the attack on the modified pullulan **8**: the enzyme may have comparable affinity for the linear α-1,6-regions naturally present in pullulan (**6**) and the truly branched regions of polymer **8**, which are produced by the glycosylation of the free 4^{III}-OH present in the maltotriosyl repeating unit of the linear polymer; it may also be that the enzyme prefers either the former or the latter α-1,6-linkage, which is introduced chemoenzymatically. The lower K_m value for pullulan (**6**) relative to **8** suggests that the affinity is higher for the linear regions (Table 1). By considering the degree of substitution of 36% estimated for **8**, this means that on a molar basis the same amount of polymer by weight will contain 24% fewer α-1,6-linkages than **6**. This small difference, however, does not change the important conclusion that the affinity is decreased for the branched polymer **8**. The simple branched maltoheptaose **4** had the highest k_{cat} value and also reasonable affinity, which was lower than for pullulan but higher than for compound **8** (Table 1), indicating that the accommodation of the branched pullulan derivative may be slightly impeded compared to the simple branched maltoheptaose (Table 1). Transition state stabilization or the catalytic efficiency which is indicated by the k_{cat}/K_m value was superior for the branched maltoheptaose. The data thus suggest that barley limit dextrinase has a preference for truly branched oligosaccharides over both the natural linear and the transglycosylated branched derivative of pullulan and this specificity differs from bacterial pullulanases and isoamylases.[7]

Experimental Section

General procedures: Roman numerals in ascending order are given to the residues from the reducing end to the terminal unit of the



Scheme 2. *Synthesis of the target molecule* ***8****. Experimental conditions: a) CGTase, phosphate buffer, 40 °C, 2 h; b) HCl, 2 h, RT.*

Table 1. *Kinetics for barley limit dextrinase.*

Substrate	k_{cat} [s^{-1}]	K_m [mg mL^{-1}]	k_{cat}/K_m [mL mg^{-1} s^{-1}]
Pullulan (**6**)	60.0	0.44	140
8	165	2.2	75
4	215	0.81 (0.78 mM)	280 (273 s^{-1} mM^{-1})

branch. NMR spectra were recorded at 303 K on a Bruker AC 300, Bruker Avance 400 or Varian Unity 500. Proton chemical shifts (δ) are reported in ppm downfield from TMS. Coupling constants (J) are in hertz (Hz) and multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), triplet (t) or multiplet (m). Carbon chemical shifts (δ) are reported in ppm and are internally referenced to the solvent.

High-resolution mass spectra (HRMS) were recorded on VG ZAB and low-resolution mass spectra (MS) were recorded on a Nermag R-1010C spectrometer. MS/MS experiments were carried out in negative mode by using a Micromass Qualtroll spectrometer. Progress of synthesis was monitored by analytical thin-layer chromatography using silica gel 60 F254 precoated plates (E. Merck, Darmstadt). The enzyme CGTase from *Bacillus sp.* was a gift from Wacker Industrie SA. (Lyon, France)

All reactions in organic media were carried out with freshly distilled solvents. After work-up, organic phases were dried over anhydrous Na_2SO_4.

GC conditions were as follows: Hewlett–Packard 5890 gas chromatographer with FID; capillary column SP-2380 (Supelco) 30 m × 0.53-mm I.D.; column temperature program, a 2.5 °C min^{-1} gradient from 165 to 225 °C and two isocratic periods of 3 min at 165 and 225 °C; injector temperature 260 °C; detector temperature 280 °C. Nitrogen was used as the vector gas with a flow rate of 4 mL min^{-1}. The ratio between the different modified hexitols was obtained by signal integration using an integrator HP 3395.

GC/MS conditions were as follows: a gas chromatographer Delsi Di 700 was coupled with the mass spectrometer Nermag R10.10C (France); system quadrupole with electronic impact ionisation; ionisation voltage 70 eV. The chromatographic conditions were the same as previously described.

Enzyme assays: Barley limit dextrinase was purified from green malt as described elsewhere.[17] Compound **4** was hydrolysed in sodium acetate (10 mM, pH 5.0) at 40 °C and limit dextrinase (2 nM). The reaction was stopped by the addition of NaOH and the mixture was neutralised by HCl prior to injection onto a PA100 column (HPAEC). A linear gradient over 20 min from buffer A (100 mM NaOH, 50 mM sodium acetate) to B (100 mM NaOH, 250 mM sodium acetate) was used. The activity was calculated from the PAD detector output using both of the products maltotriose and maltotetraose as standards. Compound **8** and pullulan were hydrolysed in 50 mM sodium acetate (pH 5.0) at 40 °C. Twelve substrate concentrations in the range of 0.11–4 mM (for compound **4**) and 0.06–10 mg mL^{-1} (for compounds **8** and **6**) were used. The enzyme concentration was 1.0 nM in all experiments on polymeric substrate. The activity was calculated from the release of reducing sugar measured by the copper-bicinconinate method[18] using maltose as standard. The kinetic constants, k_{cat} and K_m, were calculated from the initial rates of hydrolysis.

Compound 3: CGTase (207 μL) was added to a solution of fluoride donor **2** (171 mg, 0.4 mmol) and commercially available panose (**1**; 80 mg, 0.158 mmol) in sodium phosphate buffer (8 mL, 0.1 M, pH 7.0). The reaction mixture was gently shaken in an oven at 40 °C for 2 h. The mixture was then boiled for 5 min and filtered through a cotton plug, freeze-dried and acetylated (acetic anhydride/pyridine, 1:1 *v/v*, 25 mL) in the presence of a trace of dimethylaminopyridine. After 12 h at 70 °C, the reaction mixture was cooled to 0 °C, quenched by adding MeOH (10 mL) and concentrated in vacuo. The residue was

dissolved in CH_2Cl_2 and washed with water and saturated aqueous $NaHCO_3$. The organic layers were concentrated and coevaporated with toluene. The residue was dissolved in CH_2Cl_2 (10 mL) and trifluoroacetic acid was added (10 mL). After 30 min at room temperature, the reaction mixture was evaporated and acetylated (acetic anhydride/pyridine, 1:1.5 *v/v*, 3.5 mL). After 14 h at room temperature, the reaction mixture was cooled to 0 °C, quenched by adding MeOH (1 mL) and concentrated in vacuo. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:1 *v/v*) to generate compound **3** (230 mg, 80%). HRMS (ES^+): *m/z* calcd. for $C_{88}H_{118}O_{59}$ $[M+Na]^+$: 2141.6131; found: 2141.6145; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 6.23$ (d, 0.5 H, $J_{1,2} = 3.7$ Hz; H-$1^{I\alpha}$), 5.74 (d, 0.5 H, $J_{1,2} = 8.0$ Hz; H-$1^{I\beta}$), 5.54 – 5.16 (m; 13 H, H-$1^{II,III,IV,V,VI,VII}$, H-$3^{I,II,III,IV,V,VI,VII}$), 5.04 (apparent t, 2 H, $J = 9.8$ Hz; H-$4^{IV,VII}$), 4.96 (apparent t, 0.5 H, $J_{3,4} = 9.5$ Hz; H-$2^{I\beta}$), 4.84 – 4.60 (m; 6.5 H, H-$2^{II,III,IV,V,VI,VII}$), 4.59 – 3.76 (m; 26 H, H-$4^{I,II,III,V,VI}$, H-$5^{I,II,III,IV,V,VI,VII}$, H-6a^{I,II,III,IV,V,VI,VII}, H-6b^{I,II,III,IV,V,VI,VII}), 2.20 – 1.96 (m, 69 H, OCOC*H*$_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.37 - 169.56$ (O*C*OCH$_3$), 96.15, 95.61 (C-$1^{II,III,IV,V,VI,VII}$), 91.33 (C-$1^{I\beta}$), 88.76 (C-$1^{I\alpha}$), 75.31, 75.07, 73.98, 73.64, 73.38, 73.20, 72.80, 72.58, 72.49, 72.28, 71.96, 71.64, 71.43, 71.20, 70.89, 70.77, 70.51, 70.33, 70.18, 69.97, 69.67, 69.49, 69.36, 68.95, 68.42, 68.15, 67.99, 65.95 (C-$2^{I,II,III,IV,V,VI,VII}$, C-$3^{I,II,III,IV,V,VI,VII}$, C-$4^{I,II,III,IV,V,VI,VII}$, C$5^{I,II,III,IV,V,VI,VII}$), 65.95, 65.51 (C-$6^{II\alpha,II\beta}$), 62.69, 62.27, 62.17, 61.39 (C-$6^{I,III,IV,V,VI,VII}$), 20.79 – 20.51 (OCO*C*H$_3$) ppm.

Compound 4: Sodium methoxide in methanol (1 M, 500 μL) was added to a solution of **3** (100 mg, 0.546 mmol) in methanol (30 mL). The mixture was stirred overnight, neutralized with Amberlite IRN 120(H^+) resin, concentrated, then diluted with water and freeze-dried to give compound **4** (61 mg, 96%). HRMS (ES^+): *m/z* calcd. for $C_{88}H_{118}O_{59}$ $[M+Na]^+$: 1175.3701; found: 1175.3701 and *m/z* calcd. for $[M+K]^+$: 1191.3440; found: 1191,3441; ^1H NMR (400 MHz, D_2O): $\delta =$ 5.02 – 5.07 (m, 5 H; H-$1^{II,III,IV,VI,VII}$), 5.07 (d, 0.5 H, $J_{1,2} = 3.6$ Hz; H-$1^{I\alpha}$), 4.81 (d, 1 H, $J_{1,2} = 3.6$ Hz; H-1^{V}), 4.49 (d, 0.5 H, 1 H, $J_{1,2} = 8.0$ Hz; H-$1^{I\beta}$), 3.9 – 3.4 (m; 39.5 H, H-$2^{I\alpha,II,III,IV,V,VI,VII}$, H-$3^{I,II,III,IV,V,VI,VII}$, H-$4^{I,II,III,V,VI}$, H-$5^{I,II,III,IV,V,VI,VII}$, H-6a^{I,II,III,IV,V,VI,VII}, H-6b^{I,II,III,IV,V,VI,VII}), 3.25 (apparent t, 2 H, 0.5 H, $J = 9.6$ Hz; H-$4^{IV,VII}$), 3.11 (apparent t, 0.5 H, $J_{2,3} = 9.6$ Hz; H-$2^{I\beta}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 100.37 - 100.14$ (C-$1^{II,III,IV,VI,VII}$), 98.93 (C-1^{V}), 96.14 (C-$1^{I\beta}$), 92.24 (C-$1^{I\alpha}$), 79.16, 78.39, 78.20, 78.01, 77.90, 77.31, 77.19, 76.43, 75.01, 74.30, 73.70, 73.48, 73.48, 73.32, 73.23, 73.07, 72.10, 72.01, 71.67, 71.59, 70.71, 70.55, 70.43, 69.69 (C-$2^{I,II,III,IV,V,VI,VII}$, C-$3^{I,II,III,IV,V,VI,VII}$, C-$4^{I,II,III,IV,V,VI,VII}$, C-$5^{I,II,III,IV,V,VI,VII}$), 67.86, 67.70 (C-$6^{II\alpha,II\beta}$), 61.21 – 60.86 (C-$6^{I,III,IV,V,VI,VII}$) ppm.

Compound 5: Compound **4** (5 mg, 0.43 μmol) was dissolved in water (1 mL) and this solution was made slightly alkaline by adding ammonia solution (4%, 1 drop). Sodium borohydride was then added (1 mg, 0.26 mmol) and the mixture was stirred for 12 h at room temperature. The reaction mixture was then acidified with acetic acid solution (50%). The mixture was evaporated and then coevaporated with methanol. The residue was dissolved in water, desalted on MB3 Amberlite resin and freeze-dried to give alditol **5** in quantitative yield.

Modified pullulan (7): CGTase (150 μL) was added to a solution of fluoride donor **2** (63.4 mg, 0.15 mmol) and commercially available pullulan (**6**, 50 mg) in sodium phosphate buffer (5 mL, 0.1 M, pH 7.0). The reaction mixture was gently shaken at room temperature for 2 h, after which TLC (CH_3CN/H_2O, 4:1 v/v) indicated complete conversion of the fluoride **2**. The mixture was boiled for 5 min and then filtered through a cotton plug. The filtrate was precipitated by addition of ethanol (200 mL). After centrifugation, the polysaccharide **7** was dried overnight under reduced pressure (65 mg). ^1H NMR (300 MHz, D_2O): $\delta = 5.26 - 5.24$ (m, 2.6 H; H-1 of the glucosidic bond α-1,4), 4.83 (m, 1 H; H-1 of the glucosidic bond α-1,6), 3.9 – 3.4 (m, H-2, H-3, H-4, H-5, H-6a, H-6b), 1.71 – 1.42 (m, 1.7 H; C*H*$_2$ of the THP group) ppm.

Modified pullulan (8): The modified polysaccharide **7** (21 mg) was dissolved in HCl solution (10^{-2} M, 2 mL), stirred for 2 h at room temperature and then neutralized with ammonia. The mixture was precipitated in ethanol and dried overnight under reduced pressure to give the compound **8** (16 mg). ^1H NMR (300 MHz, D_2O): $\delta = 5.26 -$ 5.24 (m, 2.7 H; H-1 of the glucosidic bond α-1,4), 4.83 (m, 1 H; H-1 of the glucosidic bond α-1,6), 3.9 – 3.4 (m, H-2, H-3, H-4, H-5, H-6a, H-6b) ppm.

The structural analysis of **8** was performed by standard methylation analysis of polysaccharides.[16] Sodium hydride (2.5 g, 1 mmol) was dissolved in dry DMSO (25 mL). The mixture was warmed to 60 °C for 2 h and then cooled to room temperature. The resultant dimsyl anion can be stored for several months at −18 °C. The polymer **8** was dissolved in dry DMSO (800 μL) and dimsyl anion (500 μL) was added. The mixture was stirred overnight at room temperature under nitrogen and then methyl iodide was added (3 × 170 μL). After 1 h at room temperature, the mixture was dialyzed against water and freeze-dried. This procedure was repeated twice. The solid was dissolved in formic acid and heated at 100 °C for 1 h. The mixture was evaporated and trifluoroacetic acid (2 M, 500 μL) was added. The mixture was stirred 3 h at 100 °C, evaporated, coevaporated with water until neutrality and then freeze-dried. The residue was dissolved in water and this solution was made slightly alkaline by adding ammonia solution (4%, 1 drop). Sodium borohydride (1 mg) was added and the mixture was stirred for 12 h at room temperature and then acidified with acetic acid solution (50%). The mixture was evaporated and then coevaporated with water and finally with a hydrogen chloride solution in methanol (1%, 3 ×). The residue was acetylated (acetic anhydride/pyridine, 1:1 v/v, 1 mL). After 1 h at 100 °C, the reaction was quenched by adding water (1 mL) at 0 °C, evaporated, coevaporated with water and finally with methanol. The mixture of alditols was analysed by gas chromatography and characterized by GC-MS.

Acknowledgements

This work was supported by CNRS and the European Union 4th Framework Program on Biotechnology (BIO4-CT98-0022).

Keywords: active site mapping · carbohydrates · enzyme catalysis · glycosyltransferases · oligosaccharides

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Received: June 20, 2003 [F692]

