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Note

Enzymatic synthesis of oligosaccharides from branched cyclodextrins

Christel T. Jørgensen, Allan Svendsen and Jesper Brask*

Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark

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Abstract—6″-α-Maltosyl-maltotriose and 6″-α-D-glucosyl-maltotriose were prepared from Novamyl™ degradation of 6-*O*-α-maltosyl-α-cyclodextrin and 6-*O*-α-D-glucosyl-α-cyclodextrin, respectively. NMR spectroscopy was used to elucidate their structural identities, in a combination of COSY experiments. Further, a mechanism for the degradation was proposed based on the Novamyl active site geometry.

Keywords: Novamyl; Cyclodextrins, branched; Oligosaccharides, NMR of; Biocatalysis; Chemoenzymatic synthesis

Oligosaccharides of high purity and defined composition are very valuable research tools. Some also possess important biological and pharmacological properties.[1] However, chemical synthesis of such compounds is still often laborious, going through multiple reaction steps.[2] This naturally causes limitations to the application and investigation of otherwise interesting oligosaccharides.

Novamyl™ is a 'maltogenic' α-amylase (EC 3.2.1.133) from *Bacillus stearothermophilus*, belonging to the glycoside hydrolase family 13.[3] Hence, Novamyl-catalyzed hydrolysis of starch results in maltose as the main product. Although Novamyl is primarily an exo-acting enzyme, under appropriate experimental conditions, it may also catalyze in an endo mechanism and thus, can degrade cyclodextrins (CDs).[4] The enzyme is used industrially to enhance the freshness of bread (anti-staling) and is produced on a large scale.

Using Novamyl to degrade commercially available branched CDs, we now show that defined oligosaccharides can be obtained from a single enzymatic reaction. As proof-of-concept, we have prepared 6″-α-maltosyl-maltotriose (G5, CAS 35175-17-8) from 6-*O*-α-maltosyl-αCD and 6″-α-D-glucosyl-maltotriose (G4, CAS 35175-16-7) from 6-*O*-α-D-glucosyl-αCD (Fig. 1).

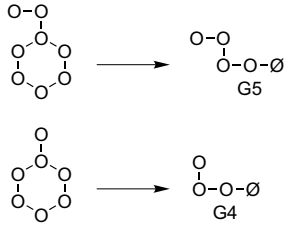

Figure 1. Schematic representation of the syntheses of G4 and G5 oligosaccharides from branched αCDs through Novamyl degradation (O = α-D-glucosyl; Ø = reducing end).

The G4 compound is commercially available, being prepared by hydrolysis of pullulan with pullulanase and β-amylase. The G5 oligosaccharide on the other hand has previously only been prepared in a small scale in mixtures. Iwaki and Fuwa reported that transglycosylation of a concentrated aqueous solution of maltose and maltotriose with pullulanase resulted in a mixture of pentaoses. Though the pentaoses were obtained in very poor yield, 6″-α-maltosyl-maltotriose was claimed to be one of the dominant isomers.[5] Also, Park and coworkers produced G5 in a mixture of branched oligosaccharides from the action of a maltogenic amylase of *Bacillus licheniformis* on liquefied starch. Two different pentaoses were produced in low yields, as verified by high-performance anion exchange chromatography (HPAEC) analysis. Further enzymatic degradation with

* Corresponding author. Tel.: +45 4443 6714; fax: +45 4442 7535; e-mail: jebk@novozymes.com

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pullulanase and glucoamylase led to the conclusion that the two structures would be 6″-α-maltosyl-maltotriose and 6″-α-maltotriosyl-maltose.[6]

Branched or modified CDs have previously been subjected to enzymatic degradation with the aim of preparing valuable oligosaccharides and/or to study enzyme specificity. In an early report by Melton and Slessor, 6′-substituted maltoses were prepared from corresponding monosubstituted αCDs (azido-, tosyl-, or halogen-) by *Aspergillus oryzae* amylase (Taka-amylase) degradation.[7] Fujita and coworkers have further investigated the 'Taka amylolysis' of various substituted and modified CDs. In one publication, 3″,6″-anhydromaltotetraose was prepared from degradation of an anhydro-CD.[8] Also, Simiand et al. used an anhydro-CD as a substrate for cyclodextrin glucosyltransferase (CGTase) catalyzed ring-opening and condensation with α-D-glucosides.[9] Uchida and coworkers used CGTase and human salivary α-amylase (HSA) to prepare 6″-modified maltopentaoses and -tetraoses from transglycosylation of mono-6-*O*-*p*-toluenesulfonyl-αCD with maltose.[10] Finally, related to the present work, Okada et al. studied the degradation of di-*O*-glycosylated γCDs with CGTase and bacterial saccharifying α-amylase (BSA). The two enzymes showed the same hydrolysis pattern, to provide a series of branched oligosaccharides, which were analyzed by LC-MS, but not isolated.[11] In a follow-up on this, Okada et al. studied the BSA-degradation of di-*N*-acetylglucosaminyl-βCDs.[12]

The present investigation focused on finding an easy, robust, and preparative-scale synthesis of branched oligosaccharides. Enzyme (Novamyl) and substrates were chosen so that only one oligosaccharide was obtained, to increase yield and facilitate separation. Hence, 6″-α-maltosyl-maltotriose (G5) was prepared in 63% yield from degradation of 6-*O*-α-maltosyl-αCD, followed by purification by size-exclusion chromatography (SEC). Reaction products could also be worked-up by a per-*O*-acetylation/flash chromatography/Zemplen deacetylation sequence. In this way, the G5 oligosaccharide was obtained in 25% overall yield, whereas 6″-α-D-glucosyl-maltotriose (G4) was obtained in modest 16% yield from 6-*O*-α-D-glucosyl-αCD. Enzyme dosages were optimized for the reactions to finish within a reasonable timeframe. However, this was not a crucial parameter, as over-dosage (with product degradation) did not pose a problem. TLC and HPAEC analysis showed that the enzymatic reactions were essentially quantitative and that product formation was accompanied by release of one G1 (glucose) and one G2 (maltose) unit in both reactions.

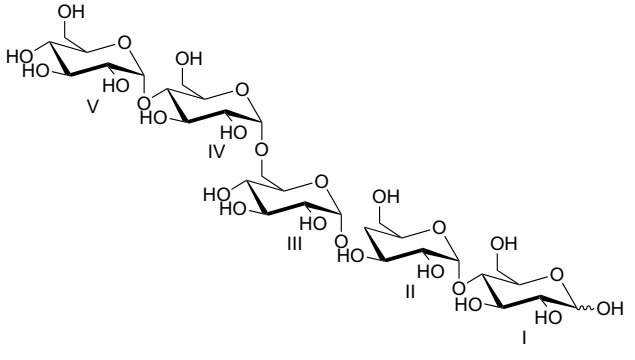

Figure 2. Structure of 6″-α-maltosyl-maltotriose (G5) with indication of glucose units I–V. The 6″-α-D-glucosyl-maltotriose (G4) structure is that of units I–IV.

The structures of G5 and G4 oligosaccharides were elucidated by ^{1}H NMR, using data and guidelines published by Bock.[13] In the present case, it was not found necessary to form the β-methyl glycosides for structural determination, even though this is easily done from the per-*O*-acetylated structures. The G4 structure could be established simply by a comparison with commercially available 6″-α-D-glucosyl-maltotriose. Elucidation of the G5 structure started out from the 1D proton spectrum (Fig. 3), where signals from anomeric protons showed one reducing end (α + β form), one α1,6-branch, and three internal α1,4-linkages. From COSY (Fig. 4, left) and relayed COSY (Fig. 4, right) experiments, H-2 and H-3 on the 1,6-bound glycosyl unit IV could easily be assigned (Fig. 2). H-3 was found at 4.02 ppm, indicating that this unit has a glycosidic bond in the 4-position.[13] For comparison, in the G4 structure, where IV is a terminal unit, H-3 was found at 3.74 ppm. Now knowing that G5 is a 6-α-maltosyl-maltotriose, the only remaining question was if the maltosyl branch was placed on the I, II, or III ring. In a COSY experiment, couplings from H-4 in the two non-reducing ends (V and III) were studied. One of the protons was found to couple to signals at 3.94 ppm, assigned to H-3/5 in a 6-substituted glycosyl unit (III).[13] Also in G4, a coupling to 3.94 ppm was observed. On the other hand, for both CD substrates, where the non-reducing ends (glucosyl and maltosyl) clearly are not 6-substituted, the H-4 signals couple to protons in the region 3.67–3.77 ppm (6-*O*-α-maltosyl-αCD) or 3.76–3.80 (6-*O*-α-D-glucosyl-αCD). This is all consistent with the G5 structure as illustrated in Figure 2.

A crystal structure of Novamyl with an acarbose-derived hexasaccharide in the active site has been published (1QHO.pdb).[3] The hexamer, located in subsites −3 to +3, is believed to be the transglycosylation product of maltose and acarbose. Looking at O-6 coordinates in this hexasaccharide relative the solvent accessible surface in the active site, possible locations of a 1,6-branch was estimated. Whereas branches in subsites −1 and −2 could be ruled out as impossible, a 1,6-branch in +3 is definitely possible. The remaining positions, subsites +2, +1, and −3 looked intermediate, that is, a branch might be possible, but appears tight. However, knowing the products of the two investigated reactions (glucose, maltose, G4/G5), it seemed that the most likely scenario is that initial

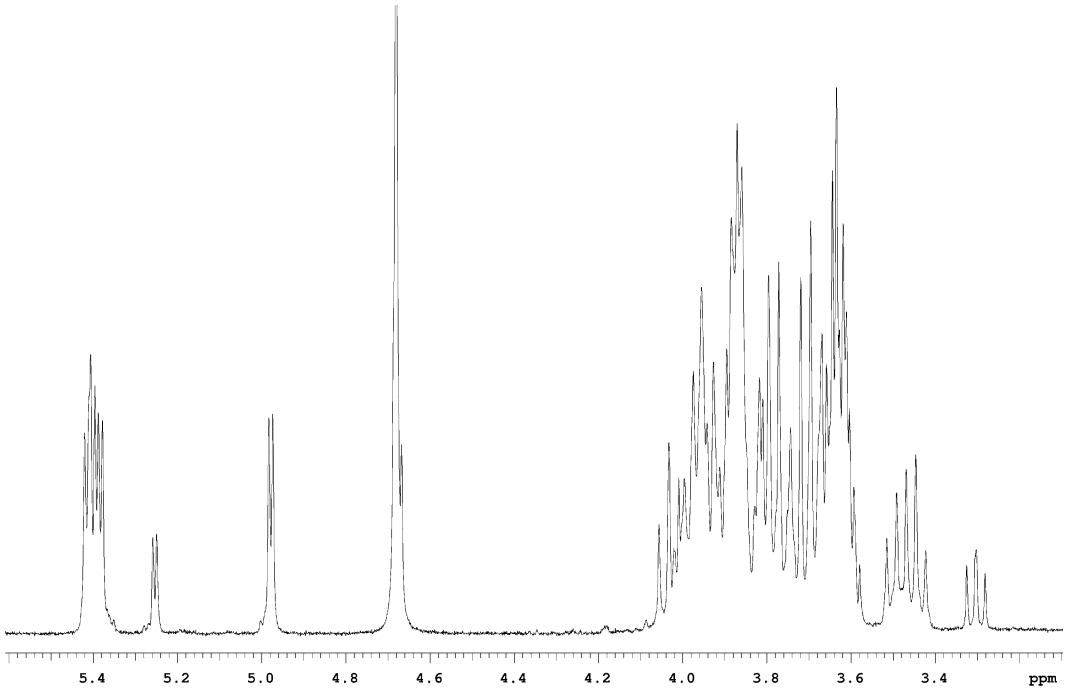

Figure 3. ^{1}H NMR spectrum of 6″-α-maltosyl-maltotriose (G5).

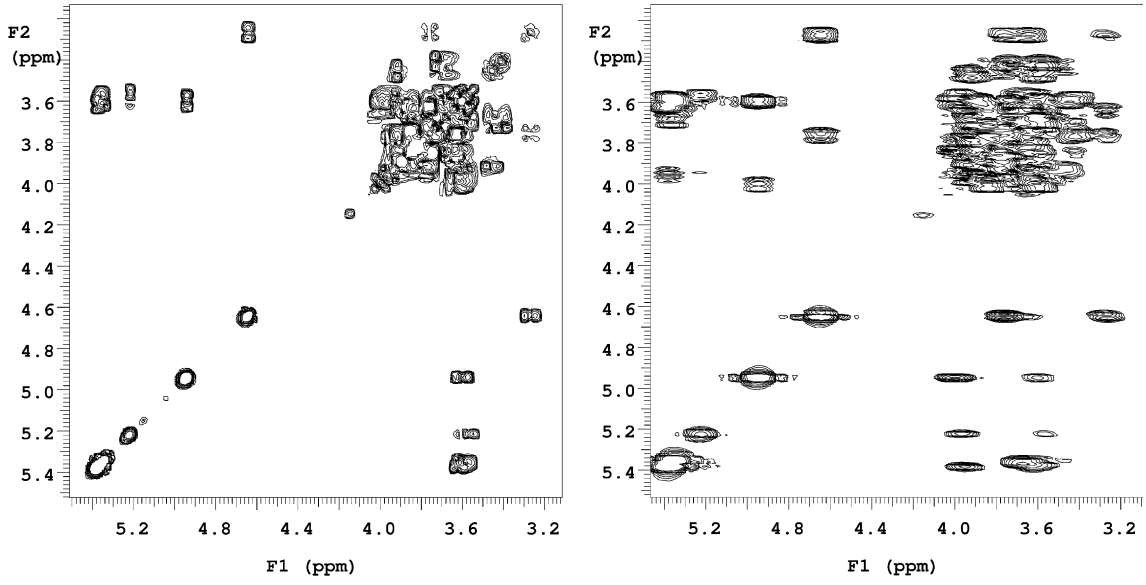

Figure 4. COSY (left) and relayed COSY (right) spectra of G5. In the COSY spectrum couplings from anomeric protons to H-2 protons are easily identified, whereas in the relayed spectrum, also couplings to H-3 protons are seen.

ring-opening of the CDs is followed by cleavage of maltose from the non-reducing end of the linear, branched oligosaccharides, in which the 1,6-branch is placed in the +1 subsite. Hence, we propose that the glucose unit is cleaved off the reducing end. This speculative reaction pathway is illustrated in Figure 5.

In conclusion, we found that Novamyl degrades 6-*O*-α-maltosyl-αCD and 6-*O*-α-D-glucosyl-αCD to D-glucose, maltose, and 6″-α-maltosyl-maltotriose (G5) or 6″-α-D-glucosyl-maltotriose (G4), respectively. The high-value oligosaccharides can be isolated directly by SEC or by flash chromatography after acetylation and deacetylation. SEC resulted in 63% recovery, whereas purification by flash chromatography lowered the yields to 16–25%. The structural identities of the products were determined by ^{1}H NMR spectroscopy, solely using chemical shifts of a few key protons. From knowing what reaction products are formed, as well as from structural information about the Novamyl active site geometry, it is proposed that after initial ring-opening,

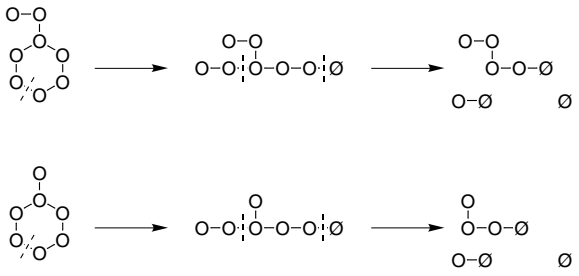

Figure 5. Schematic representation of the proposed mechanism for formation of G5 and G4 oligosaccharides (dashed line represent bond cleavage). After initial ring-opening, the 1,6-branch is placed in the +1 subsite to liberate a maltose unit from the non-reducing end.

a maltose unit is cleaved from the oligosaccharide non-reducing end by placing the 1,6-branch in the +1 subsite.

1. Experimental

1.1. General

Cyclodextrins were purchased from Wako Pure Chemical Industries Ltd., 6″-α-D-glucosyl-maltotriose was from Megazyme International Ireland Ltd., and Novamyl was from Novozymes A/S. Size-exclusion chromatography (SEC) was performed on a K26/100 column with Bio-Gel P-2 from Bio-Rad Laboratories operated at 60 °C, and equipped with RI detector. Flash chromatography was performed with pre-packed 4×15 cm columns on a Flash+™ instrument from Biotage, Inc. High-performance anion exchange chromatography (HPAEC) was performed on a Dionex DX-500 system with PAD detector. The system was equipped with a CarboPac PA-100 4×250 mm column and run with a gradient of 0–0.6 M NaOAc in 0.15 M NaOH. TLC was performed on silica gel 60 F_{254} precoated aluminum sheets from Merck. Compounds were visualized by staining with 10% H_2SO_4 in EtOH, followed by heating. NMR was performed on a Varian Mercury-VX instrument with a 4-nucleus auto-switchable probe. Spectra were acquired in D_2O at 37 °C and 399.99 MHz (^1H) or 100.58 MHz (^{13}C) with chemical shifts being referenced to internal acetone (2.22 ppm, for ^1H) or dioxane (67.4 ppm, for ^{13}C). Relayed COSY experiments were performed with a propagation time of 50 ms ($\tau = 0.05$). Elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden.

1.2. 6″-α-Maltosyl-maltotriose (G5), SEC purification

6-*O*-α-Maltosyl-αCD (153 mg) was dissolved in 10 mM acetate buffer pH 5.0 (9.83 mL) and purified Novamyl enzyme (3.47 mg/mL, 170 μL) was added. The solution was incubated at 60 °C for 24 h and then quenched by the addition of H_2O (18.8 mL) and 0.1 M NaOH (1.5 mL). Next, the reaction mixture was concentrated to approx. 4 mL and purified by SEC, eluting with H_2O. The product containing fractions were pooled, concentrated, and freeze dried to yield the pure title compound as a white powder (61 mg, 63%). ^1H NMR (assigned signals in ppm): 5.39–5.35 (m, H-$1^{II+III+V}$), 5.22 (d, 3.8 Hz, H-$1\alpha^{I}$), 4.95 (d, 3.8 Hz, H-1^{IV}), 4.64 (d, overlap with solvent signal, H-$1\beta^{I}$), 3.46 (t, 9.4 Hz, H-4^{III}), 3.41 (t, 9.4 Hz, H-4^{V}), 3.27 (t, 8.4 Hz, H-$2\beta^{I}$). ^{13}C NMR (assigned signals in ppm): 100.9 (C-$1^{III/V}$), 100.7 (C-$1^{III/V}$), 100.4 (C-$1\alpha/\beta^{II}$), 100.3 (C-$1\alpha/\beta^{II}$), 98.8 (C-1^{IV}), 96.6 (C-$1\beta^{I}$), 92.7 (C-$1\alpha^{I}$), 77.0 (C-3^{I}), 75.4 (C-5^{I}), 66.9 (C-6^{III}), 61.6 (C-$6^{I/II/IV/V}$), 61.4 (C-$6^{I/II/IV/V}$), 61.3 (C-$6^{I/II/IV/V}$). MS (MALDI-TOF, DHB matrix), calculated mass 828.71 Da, found *m*/*z*: 851.68 $[M+Na]^+$, 867.78 $[M+K]^+$. Anal. Calcd for $C_{30}H_{52}O_{26}$: C, 43.48; H, 6.32. Found: C, 43.3; H, 6.6.

1.3. 6″-α-Maltosyl-maltotriose (G5), flash chromatography purification

6-*O*-α-Maltosyl-αCD (311 mg) was dissolved in 10 mM NaOAc-buffer, pH 5.0 (20 mL). Novamyl L (4000 MANU/g, 1.25 g/mL, 50 μL) was added and the reaction mixture was stirred overnight at 60 °C. TLC (MeCN/EtOAc/*n*-PrOH/H_2O, 85:20:50:50) revealed three products, which were identified as G1 (glucose), G2 (maltose), and G5 (product) by HPAEC analysis. The solution was concentrated to absolute dryness before acetylation. Pyridine (3 mL) was added and the solution cooled on ice. Next, Ac_2O (4 mL) and DMAP (12 mg) was added and the mixture allowed to warm to room temperature while stirring over weekend. The reaction was then quenched with ice (50 mL) and transferred to a separation funnel with CH_2Cl_2 (100 mL). The organic phase was washed with 0.1 M HCl (3×50 mL), 5% $NaHCO_3$ (3×50 mL), and water (3×50 mL), dried (Na_2SO_4) and concentrated. Acetylated G5 product was purified by flash chromatography with EtOAc/heptane (4:2), followed by EtOAc/heptane (5:1). The late eluting product was identified by TLC (EtOAc/heptane, 5:1), having $R_f = 0.31$, and pooled to yield 189 mg, 51%. Finally, deacetylation was performed by dissolving the acetylated G5 (189 mg) in MeOH (8 mL), followed by the addition of NaOMe (30% solution in MeOH, 240 μL). The solution turned milky-white and was stirred overnight at room temperature. The reaction was neutralized by the addition of Dowex 50WX8 (H^+), followed by filtration and addition of Amberlite IRA68 (OH^-) to give pH approx. 5. After filtration the clear solution was concentrated, redissolved in water, and freeze dried to yield the title compound as a white powder (50 mg, 25% overall yield).

1.4. 6″-α-D-Glucosyl-maltotriose (G4)

6-*O*-α-D-Glucosyl-αCD (300 mg) was dissolved in 10 mM NaOAc buffer, pH 5.0 (20 mL). Novamyl L

(100 μL) was added and the reaction mixture was stirred overnight at 60 °C. TLC (MeCN/EtOAc/*n*-PrOH/H_2O, 85:20:50:50) revealed three products, which were identified as G1 (glucose), G2 (maltose), and G4 (product) by HPAEC analysis. Acetylation and subsequent workup was performed as described for the G5 product. Yield 155 mg, 48%, R_f = 0.34 (EtOAc/heptane, 5:1). Deacetylation was likewise performed as for the G5 product, but using 250 μL (35 equiv) NaOMe solution, to yield the title compound as a white powder after freeze drying (28 mg, 16% overall). ^{1}H NMR (assigned signals in ppm): 5.38 (d, 4.7 Hz, H-1$^{II/III}$), 5.37 (d, 4.4 Hz, H-1$^{II/III}$), 5.22 (d, 3.9 Hz, H-1α^{I}), 4.95 (d, 3.6 Hz, H-1^{IV}), 4.64 (d, overlap with solvent signal, H-1β^{I}), 3.49 (t, 9.4 Hz, H-4^{III}), 3.42 (t, 9.4 Hz, H-4^{IV}), 3.27 (dd, 8.3 Hz, 9.4 Hz, H-2β^{I}). ^{13}C NMR (assigned signals in ppm): 100.8 (C-1^{III}), 100.4 (C-1α/β^{II}), 100.3 (C-1α/β^{II}), 98.9 (C-1^{IV}), 96.6 (C-1β^{I}), 92.7 (C-1α^{I}), 77.0 (C-3^{I}), 75.4 (C-5^{I}), 66.9 (C-6^{III}) 61.6 (C-6$^{I/II/IV}$), 61.4 (C-6$^{I/II/IV}$), 61.3 (C-6$^{I/II/IV}$). MS (MALDI-TOF, DHB matrix), calculated mass 666.57 Da, found *m*/*z*: 690.07 $[M+Na]^+$, 705.73 $[M+K]^+$. Anal. Calcd for $C_{24}H_{42}O_{21}$: C, 43.25; H, 6.35. Calcd for $C_{24}H_{42}O_{21}\cdot H_2O$: C, 42.11; H, 6.48. Found: C, 42.1; H, 6.5.

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