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Specificity of amylases and cyclodextrin-glucanotransferase in reactions with 2-deoxy-maltooligosaccharides

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Abstract

2-Deoxy-maltooligosaccharides of different chain length were tested as substrates for exo- and endo-amylases. Cleavage occurred with β-amylase, yielding 2,2′-dideoxy-maltose, and with amyloglucosidase. With the α-amylase from *Thermomonospora curvata* tris-(2-deoxy)-maltotriose and the corresponding tetra- and pentasaccharides were formed. Porcine pancreatic α-amylase did not tolerate the deoxygenated substrate, nor were cyclization experiments with cyclodextrin-glucanotransferase (CGT) successful. In a coupling reaction with CGT, however, a series of transfer products to the acceptor 2-deoxyglucose were obtained. © 1997 Elsevier Science Ltd.

Keywords: Cyclodextrin; Exo- and endo-amylases; Glucanotransferase

1. Introduction

The substrate specificities of different amylases have been investigated with derivatives of amylose or oligosaccharides. In the past, various chemically modified polysaccharides were used especially to study the structural requirements of porcine pancreatic α-amylase [1,2]. Unfortunately, in most cases modifications were restricted to the primary hydroxy group and rather low degrees of substitution in the polysaccharide were obtained. Here we describe the action of endo- and exo-amylases on a maltooligosaccharide derivative with a long chain consisting exclusively of 2-deoxyglucose. This substrate was synthesized from D-glucal and maltotetraose with phosphorylase [3].

Amylases are classified according to their attack in the middle or at the non-reducing end of an amylose chain (endo- or exo-), and they are further divided into retaining (α-) and inverting (β-) enzymes. Recently, various exo-α-amylases were investigated which formed maltooligosaccharides of specific chain length [4,5]. The maltotetraose-forming amylase from *Pseudomonas stutzeri* as well as the maltotriose-forming enzyme from *Streptomyces griseus* have been applied in transglucosylation reactions with *p*-nitrophenyl glucosides [6,7]. The α-amylase from *Thermomonospora curvata* also catalyzed degrada-

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tion of starch into products from maltose to maltopentaose without any formation of glucose. Upon longer incubation maltose and maltotriose accumulated as final products [8]. The gene of this thermostable amylase has been characterized [9] and it was cloned and expressed in *Streptomyces lividans* [10]. In this work we investigated the reaction of the α-amylases (EC 3.2.1.1) from *T. curvata* and from porcine pancreas, of β-amylase (EC 3.2.1.2) and of amyloglucosidase (EC 3.2.1.3) with 2-deoxy-maltooligosaccharides as substrates.

Another enzyme acting on amylose is the cyclodextrin-α-1,4-glucanotransferase (CGT, EC 2.4.1.19). The CGT catalyzes three types of reactions:

1. cyclization:

$$G_n \rightarrow cG_x + G_{(n-x)}$$

where G_n is α-1,4-glucan; cG_x is cyclodextrin with $x = 6, 7, 8$.

2. coupling reaction:

$$cG_x + A \rightarrow G_x\text{–}A$$

where A is acceptor.

3. disproportionation:

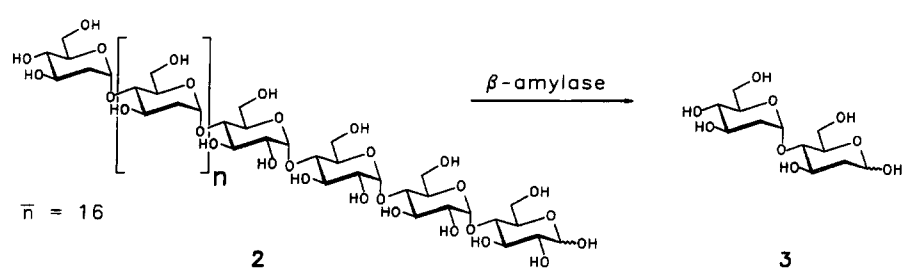
$$G_n + G_m \rightarrow G_{(n-x)} + G_{(m+x)}$$

Although it does not belong to the hydrolases, a hydrolytic activity of CGT is still discussed by some authors [11,12]. This enzyme was also incubated with the 2-deoxy-maltooligosaccharides and in a coupling reaction with 2-deoxyglucose as an acceptor.

In addition to a comparison of the specificities of different amylases the aim of this work was the synthesis of deoxygenated oligosaccharides. The smaller saccharides might be crystallized to study the hydrogen bonds in these compounds in which no interaction between OH-3 and OH-2′ can occur. Moreover, with new primers for phosphorylase, which must be at least a tetrasaccharide, homogenous oligosaccharides of a higher DP could be synthesized in the reaction with glucal.

2. Results and discussion

Action of exo-amylases on 2-deoxy-maltooligosaccharides.—The 2-deoxy-maltooligomers were synthesized by elongation of the primer maltotetraose with 2-deoxyglucosyl units in the reaction of phosphorylase with D-glucal [3]. Therefore, these compounds always carry four regular glucose units at the reducing end. However, this has no influence on the initial attack of the amylases since the degradation takes place from the non-reducing end. Two substrates have been employed in the experiments, a water-soluble fraction [13] of DP 12 (**1**) and a fraction with an average DP of 20 (**2**). The latter one is only soluble in alkali or in Me_2SO–water mixtures so that reaction conditions have to be adjusted for each enzyme. The activity of β-amylase from sweet potato was measured at different concentrations in Me_2SO. At 10% Me_2SO the activity was even higher than without Me_2SO. To keep the substrate **2** in solution at least 25% Me_2SO is necessary, and as observed β-amylase tolerated concentrations up to 40% of the solvent. Incubations were also performed with 35% Me_2SO in which still 70% of the activity in water was observed. In further assays the stability of the enzyme in this medium for at least 4 days was proven. The disaccharide 2,2′-dideoxymaltose (**3**) was isolated (Scheme 1) but no disaccharides containing glucose were formed. Apparently, the degradation of **2** was incomplete, and the yield did not exceed 32%. This was not due to the low solubility of the substrate because incubations with the water-soluble oligosaccharide **1** showed the same results. It may be assumed that 2,2′-dideoxymaltose (**3**) could act as an inhibitor for β-amylase.



Scheme 1.

For degradation experiments with amyloglucosidase only the soluble substrate **1** was used. This enzyme cleaved the oligosaccharide completely into 2-deoxyglucose and glucose in a ratio of 2:1, corresponding to the substrate $(2d\text{-}Glc)_8$–$(Glc)_4$. This finding is in accord with previous investigations by Bock et al. who synthesized a series of mono- and dideoxy-maltosides as substrates for amyloglucosidase and found that the 3-, 4′- and 6′-hydroxy groups were essential, whereas all the other hydroxy groups could be deleted without affecting the activity [14]. The application of a long chain of 2-deoxyglucosyl units as substrate revealed that for both exo-amylases OH-2 is not essential at any of the binding sites. 2,2′-Dideoxymaltose, the synthesis of which has not been described before, can be obtained now from glucal in two enzymatic steps.

Action of α-amylases on 2-deoxy-maltooligosaccharides.—Porcine pancreatic α-amylase also tolerates up to 30% Me_2SO in the buffer solution, and it is still active after 5 days at 35 °C in this medium. But even after 7 days incubation cleavage of **2** occurred only between glucosyl units in the maltotriose part of the molecule. Glucose, maltose and maltotriose were isolated besides a higher oligosaccharide fraction containing 2-deoxyglucose. No products with 2-deoxyglucose at the reducing end were detected which would be easily assigned by ^{1}H-NMR due to the additional H-2 signals.

Obviously, α-amylase is much more sensitive to modifications in the substrate than exo-amylases. Porcine pancreatic α-amylase has five subsites in the catalytic site, cleavage of the amylose chain takes place between subsites 2 and 3 (Fig. 1). Robyt et al. investigated the enzymatic cleavage products of 6-deoxy-amylose [2] and of hydroxyethylated amylose [1]. A 6-deoxy-glucosyl unit was found to bind at any of the subsites. On the contrary, a glucosyl unit with a hydroxyethyl substituent at C-6, was not recognized by subsites 3 and 4, and a hydroxyethyl substituent at C-2 was not compatible with subsites 2 and 3. It can be concluded that the subsites in the direct neighbourhood of the cleaving site are the most specific. Even though a deoxygenation cannot be compared with the hydroxyethyl substituent, neither sterically nor electronically, also these results with 2-deoxy-maltooligosaccharides demonstrate and ascertain that at subsites 2 and 3 the hydroxy group is really essential.

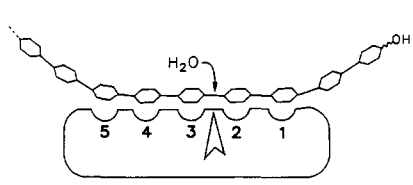
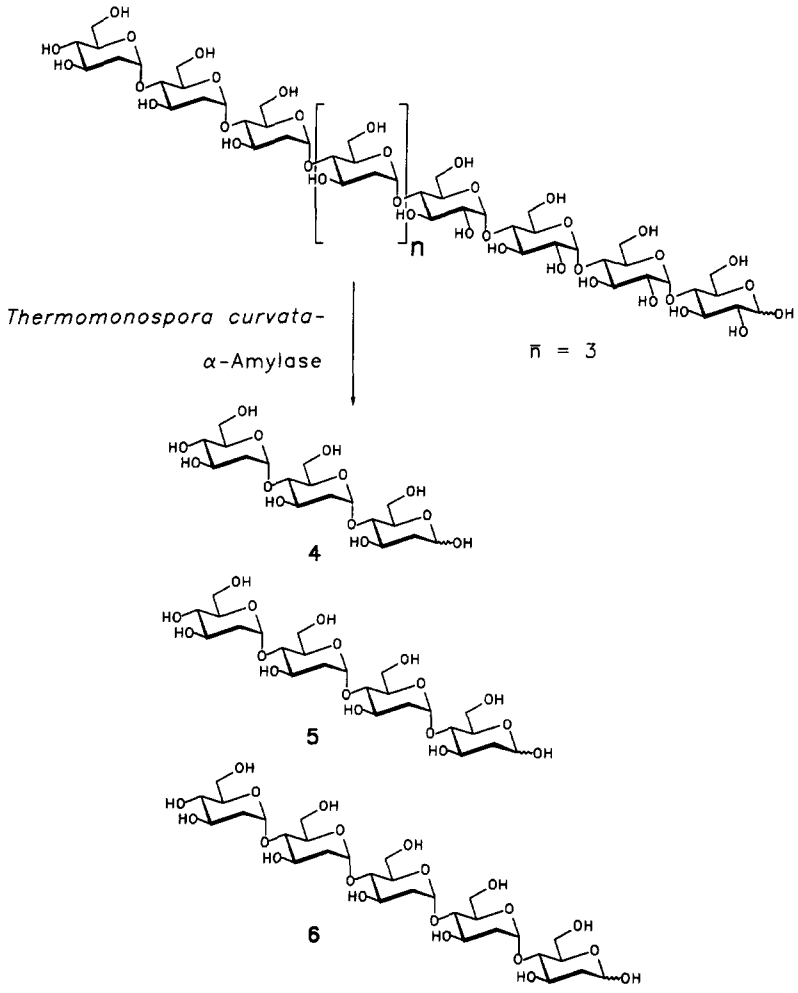


Fig. 1. Schematic representation of porcine pancreatic α-amylase with five binding sites. The substrate is cleaved between subsites 2 and 3.

Thermomonospora curvata, a facultatively thermophilic actinomycete, produces an extracellular α-amylase with an activity optimum at 65 °C and pH 6.0. This has been cloned and expressed in *Streptomyces lividans* so that sufficient amounts of the enzyme can be isolated [10]. Starch is degraded mainly into maltose and maltotriose and small amounts of the tetra- and pentamer are also formed [8]. Upon incubation of the *T. curvata* amylase with a water-soluble 2-deoxy-maltooligosaccharide fraction of DP 10, cleavage was observed between 2-deoxyglucosyl residues (Scheme 2). 2,2′,2″-Trideoxy-maltotriose (**4**) and the corresponding per-deoxy-maltotetraose **5** and -pentaose **6** were isolated besides a fraction of partially degraded oligosaccharides. Compounds **4**, **5** and **6** were obtained in 13, 27 and 16% yield, respectively. So far, these new 2-deoxy-oligosaccharides were synthesized only on a milligram scale. Nevertheless, the soluble substrate is available by direct synthesis with phosphorylase in a simple way, and thus the experiments with the *T. curvata* amylase could be run on a larger scale. Compounds **5** and **6** can serve as a primer for the phosphorylase reaction to determine the kinetic parameters of a per-2-deoxy-maltooligosaccharide. Previously, only a primer with a single 2-deoxyglucosyl residue was available for such investigations [15]. The formation of a trideoxy-maltotriose proved that 2-deoxy-glucosyl units were tolerated at any subsite of the bacterial α-amylase. This enzyme is apparently less specific than the amylase from porcine pancreas and its behaviour resembles that of exo-amylases. The action of the *T. curvata* amylase however is not only due to a hydrolytic mechanism. Transglycosylation and condensation reactions were also assumed to play a role in the degradation of maltooligosaccharides, explaining the absence of glucose in the reaction mixtures [8].

Substrate specificity of cyclodextrin-α-1,4-glucanotransferase.—The enzymatic synthesis of modified cyclodextrins has been accomplished with maltosyl fluoride derivatives, but the position of the modification was confined to C-6′, yielding 6^A,6^C,6^E-O-substituted α-cyclodextrins. Driguez et

al. applied 6′-O-methyl- or 6′-O-acetyl-maltosyl fluorides successfully in cyclization reactions [16]. No substituents were tolerated at C-6, the glucosyl unit binding at subsite 1, next to the cleaving site (Fig. 1). In the present experiments with 2-deoxy-maltooligosaccharides as substrates for CGT the specificity of the enzyme was confirmed. After incubations of **1** or **2** in water or in Me_2SO–water with CGT at different temperatures, concentrations, or reaction times no cyclization products were found. Three CGTs from *Bacillus macerans, Klebsiella oxytoca*, and an alcalophilic bacillus species, the activity of which was confirmed in the presence of Me_2SO, were tested. Cleavage of the oligosaccharide chain oc-



Scheme 2.

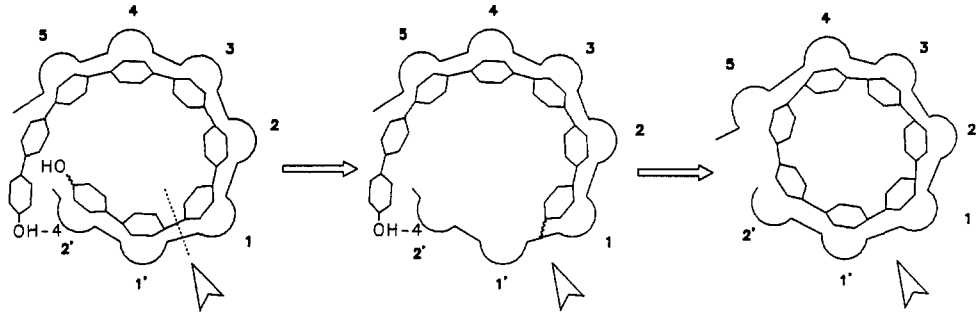


Fig. 2. Cyclization mechanism of CGT from Bacillus species with seven subsites in the active center, according to Klein et al. [18].

curred only between glucosyl units, formation of products with 2-deoxyglucose at the reducing end was not observed, implying that subsite 1 is particularly specific for glucose. These results are similar to those with porcine pancreatic α-amylase. Sequence similarities have been found between CGT and α-amylases with four conserved regions constituting the active center. Therefore, a similar reaction mechanism of both enzymes was proposed [17].

The active center of CGT from *Bacillus* species consists of seven subsites (Fig. 2) [18]. After cleavage of an amylose chain one fragment remains bound to the enzyme with the reducing end at subsite 1. This is subsequently transferred to a new oligosaccharide binding at subsites 1′ and 2′, the acceptor binding sites, or, in the case of cyclization, to the non-reducing end of the first fragment. Subsite 1′ is more flexible with regard to the substrate structure. Various glucose derivatives such as xylose [19], sucrose, D-glucono-1,5-lactone [20], inositol [21] or the plant glycoside rutin [22] have been used as acceptors in the coupling reaction with cyclodextrins or with starch. According to Kitahata et al. 2-deoxyglucose is a poor acceptor for the *Bacillus macerans* and *B. megaterium* enzymes [23]. In addition to the transfer products this study has shown the formation of a series of natural maltooligosaccharides upon incubation with starch and 2-deoxyglucose. Vetter et al. reported that 2-deoxyglucose did not act as an acceptor at all [20]. In contrast, in our findings a series of mono-deoxy-maltooligosaccharides could be isolated exclusively as transfer products of a coupling reaction with cyclodextrin. Upon incubation of the CGT from *B. macerans* with α-cyclodextrin and an excess of 2-deoxyglucose, 2-deoxy-maltose **7**, 2-deoxy-maltotriose **8** and 2-deoxy-maltotetraose **9** were obtained in 19, 17, and 13% yield, respectively. Higher analogues that eluted together with α-cyclodextrin, could not be separated but were observed in the nmr spectrum. The primary step in the coupling reaction is the transfer of a maltohexaosyl unit from α-cyclodextrin. Products of different chain length are formed in the course of the incubation due to disproportionation. The natural maltoligosaccharides found by Kitahata et al. in addition to the transfer products might be a result of the use of soluble starch as donor, and since their formation was not observed with other acceptors this can be ascribed to the poor acceptor properties of 2-deoxyglucose with the enzyme tested [23]. In our investigations, however, formation of glucose and higher oligosaccharides was not observed.

3. Experimental

Methods.—Substrates **1** and **2** were synthesized by elongation of maltotetraose with 2-deoxyglucosyl residues with phosphorylase [3,13]. Enzyme was removed from the reaction mixtures by ultrafiltration in a stirred ultrafiltration cell (Amicon 8050) with a membrane with 10 kDa cutoff (Diaflo YM-10). For removal of buffer salts the solution was subjected to an anion exchanger (DEAE-Fractogel 650-S, 3*35 cm) loaded with OH^- and the eluate was neutralized with Dowex HCR-W2 (H^+ form). Oligosaccharides were separated by size exclusion chromatography on a column (Fractogel TSK HW-40S, 2*90 cm) equipped with peristaltic pump and R_I-detector and product fractions were freeze dried. All reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck) with detection by spraying with 10% H_2SO_4 in EtOH and charring. Oligosaccharides were analysed with the solvents CH_3CN/ethyl acetate/propanol/water 85:20:50:50 or with CH_2Cl_2/MeOH/EtOH/H_2O 12:5:1:0.5. ^{1}H-NMR spectra were recorded on a Bruker AMX-400 spectrometer.

Enzymes.—Porcine pancreatic α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) from sweet potato and amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* were purchased from Sigma. *T. curvata* α-amylase was expressed in a *S. lividans* TK 24 strain and isolated from the culture medium [9]. CGT (1,4-α-D-glucan 4-α-D-glucanotransferase, EC 2.4.1.19) from *B. macerans* was obtained from Amano Pharmaceuticals, Nagoya, Japan. CGT from *K. oxytoca* and from alcalophilic bacillus sp. were from Wacker-Chemie, Germany.

Amylase assay.—Amylase activity was determined by measuring the reducing sugar formed by amylolytic degradation of starch. 3,5-Dinitro-salicylic acid was reduced to nitroaminosalicylic acid and determined photometrically according to Rick et al. [24].

Degradation with β-amylase.—2-deoxy-maltooligosaccharide **2** (DP 20, 30 mg, 10 μmol) was dissolved in Me_2SO (7 mL), diluted with buffer solution (10 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.02% NaN_3, pH 6.0) to a final Me_2SO concentration of 35% and incubated with β-amylase (500 U) for 2 days at 30 °C. In addition to a fraction of oligosaccharides degraded to an average DP of 11, 2,2′-dideoxymaltose **3** was isolated (8.2 mg, 26.5 μmol, 33%). $[\alpha]_D^{20}$ +126.6° (H_2O, *c* 0.47); ^{1}H NMR (400 MHz, D_2O): δ 5.48 (m, 1 H, H-1′), 5.33 (m, 0.5 H, α-H-1), 4.88 (dd, 0.5 H, β-H-1), 4.04 (ddd, 0.5 H, α-H-3), 3.66–3.88 (m, 6 H), 3.61 (m, 1 H, H-5′), 3.53 (dd̨t, 0.5 H, α-H-4), 3.45 (dd̨t, 0.5 H, β-H-4),

3.34 (ddd, 0.5 H, β-H-5), 3.32 (dt, 1 H, H-4′), 2.15–2.24 (m, 1.5 H, H-2′eq + β-H-2eq), 2.05 (ddd, 0.5 H, α-H-2eq), 1.64–1.75 (m, 1.5 H, H-2′ax + α-H-2ax), 1.50 (dt, 0.5 H, β-H-2ax); α-anomer: $J_{1,2ax}$ 2.6, $J_{1,2eq}$ 1.6, $J_{2ax,2eq}$ 13.7, $J_{2ax,3}$ 12.2, $J_{2eq,3}$ 5.1, $J_{3,4}$ 8.7, $J_{4,5}$ 9.5 Hz; β-anomer: $J_{1,2ax}$ 9.6, $J_{1,2eq}$ 2.0, $J_{2ax,2eq}$ 12.2, $J_{2ax,3}$ 12.2, $J_{3,4}$ 9.7, $J_{4,5}$ 9.7, $J_{5,6a}$ 5.6, $J_{5,6b}$ 2.0 Hz; $J_{3',4'}$ 9.7, $J_{4',5'}$ 9.7, $J_{5',6a'}$ 5.1 Hz.

Degradation with amyloglucosidase.—2-deoxy-maltooligosaccharide **1** (DP 12, 15 mg, 8 μmol) was incubated in buffer (3 mL, 0.2 M NaOAc, 0.2 M Na_2HPO_4, 0.02% NaN_3, pH 4.5) with amyloglucosidase (5 U) for 3 days at 40 °C. **1** was then completely degraded into glucose and 2-deoxyglucose in a ratio of 2:1. A control sample without enzyme showed no decomposition under the same conditions.

Incubations with α-amylases.—Porcine pancreatic α-amylase (500 U) was incubated for 2 days at 35 °C with **2** (DP 20, 20 mg, 7 μmol) in buffer solution (30 mL, 10 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.02% NaN_3, pH 6.0) containing 20% Me_2SO. Alternatively, **1** (DP 12, 10 mg, 5 μmol) was applied without Me_2SO. No cleavage was observed between 2-deoxyglucosyl residues.

For degradation with *T. curvata* α-amylase 2-deoxy-maltooligosaccharide (DP 10, 40 mg, 26 μmol) was dissolved in buffer (12 mL, 0.2 M NaOAc, 0.2 M Na_2HPO_4, 0.02% NaN_3, pH 6.1) and incubated with amylase (200 U) at 50 °C for 2 days. After chromatographic separation tris-(2-deoxy)-maltotriose **4** (3.0 mg, 13%), tetrakis-(2-deoxy)-maltotetraose **5** (4.3 mg, 27%), and pentakis-(2-deoxy)-maltopentaose **6** (3.2 mg, 17%) were isolated. The yield of **4** was calculated assuming that two moles can be formed per mole substrate.

Compound **4**: $[\alpha]_D^{20}$ +122° (H_2O, *c* 0.24); ^1H NMR (400 MHz, D_2O): δ 5.44 (m, 2 H, H-1′, H-1″), 5.30 (m, 0.6 H, α-H-1), 4.86 (dd, 0.4 H, β-H-1), 4.00 (m, 1.6 H, α-H-3, H-3′), 3.32 (dq̇t, 1 H, H-4″), 2.12–2.24 (m, 2.4 H, β-H-2eq, H-2′eq, H-2″eq), 2.04 (ddd, 0.6 H, α-H-2eq), 1.61–1.80 (m, 2.6 H, α-H-2ax, H-2′ax, H-2″ax), 1.50 (ddq̇dt, 0.4 H, β-H-2ax); α-anomer: $J_{1,2eq} < 1.0$, $J_{2ax,2eq}$ 11.8, $J_{2eq,3}$ 5.6 Hz; β-anomer: $J_{1,2ax}$ 10.2, $J_{1,2eq}$ 2.0, $J_{2ax,eq}$ 11.7, $J_{2ax,3}$ 10.2 Hz; $J_{3'',4''}$ 9.7, $J_{4'',5''}$ 9.7 Hz.

Compound **5**: $[\alpha]_D^{20}$ +123° (H_2O, *c* 0.40); ^1H NMR (400 MHz, D_2O): δ 5.44 (m, 3 H, H-1^{2-4}), 5.30 (m, 0.6 H, α-H-1), 4.86 (dd, 0.4 H, β-H-1), 4.00 (m, 2.6 H, α-H-3, H-3^2, H-3^3), 3.34 (dq̇t, 1 H, H-4^4), 2.12–2.24 (m, 3.4 H, β-H-2eq, H-2eq$^{2-4}$), 2.04 (dd, 0.6 H, α-H-2eq), 1.61–1.80 (m, 3.6 H, α-H-2ax, H-2ax$^{2-4}$), 1.50 (ddq̇dt, 0.4 H, β-H-2ax).

Compound **6**: $[\alpha]_D^{20}$ +115° (H_2O, *c* 0.30); ^1H NMR (400 MHz, D_2O): δ 5.44 (m, 4 H, H-1^{2-5}), 5.30 (m, 0.6 H, α-H-1), 4.86 (dd, 0.4 H, β-H-1), 4.00 (m, 3.6 H, α-H-3, H-3^{2-4}), 3.34 (dq̇t, 1 H, H-4^5), 2.12–2.24 (m, 4.4 H, β-H-2eq, H-2eq$^{2-5}$), 2.04 (dd, 0.6 H, α-H-2eq), 1.61–1.80 (m, 4.6 H, α-H-2ax, H-2ax$^{2-5}$), 1.50 (m, 0.4 H, β-H-2ax).

Incubations with CGT.—For cyclization experiments compound **2** (DP 20, 50 mg, 16 μmol) was dissolved in Me_2SO (10 mL) and diluted with buffer (20 mL, 5 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3) or 20 mg of the soluble substrate compound **1** were dissolved in buffer (30 mL) with the following conditions: pH 6.0/60° for *B. macerans* CGT; pH 6.5/40 °C for *K. oxatoca* CGT; and pH 8.0/50 °C for alcalophilic bacillus sp. CGT. After incubation times of 1 to 6 days with CGT (30 U) at 60 °C only glucose, maltose and maltotriose were isolated besides a fraction of 2-deoxy-maltooligosaccharides that still carried at least one glucosyl residue at the reducing end.

In the coupling reaction 2-deoxyglucose (180 mg, 1.10 mmol) and α-cyclodextrin (130 mg, 0.13 mmol) in buffer (10 mL, 5 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, pH 6.0) were shaken gently with CGT (25 U) at 40 °C for 2.5 h. Upon usual work-up the transfer products 2-deoxy-maltose **7** (8 mg, 19%), 2-deoxy-maltotriose **8** (11 mg, 17%), and 2-deoxy-maltotetraose **9** (11 mg, 13%) were isolated. Yields are referred to cyclodextrin.

Compound **7**: $[\alpha]_D^{20}$ +126° (H_2O, *c* 0.14); NMR ^1H (400 MHz, D_2O): δ 5.32–5.38 (m, 1.5 H, α-H-1, H-1′), 4.90 (dd, 0.5 H, β-H-1), 4.15 (m, 0.5 H, α-H-3), 3.38 (dq̇t, 1 H, H-4′), 2.22 (ddd, 0.5 H, β-H-2eq), 2.09 (ddd, 0.5 H, α-H-2eq), 1.72 (ddd, 0.5 H, α-H-2ax), 1.51 (ddq̇dt, 0.5 H, β-H-2ax); α-anomer: $J_{1,2ax}$ 3.6, $J_{1,2eq}$ 1.0, $J_{2ax,2eq}$ 13.5, $J_{2ax,3}$ 11.7, $J_{2eq,3}$ 5.1 Hz; β-anomer: $J_{1,2ax}$ 9.6, $J_{1,2eq}$ 2.0, $J_{2ax,2eq}$ 12.2, $J_{2ax,3}$ 12.2, $J_{2eq,3}$ 4.9 Hz; $J_{3',4'}$ 9.5, $J_{4',5'}$ 9.5 Hz.

Compound **8**: $[\alpha]_D^{20}$ +191° (H_2O, *c* 0.27); ^1H NMR (400 MHz, D_2O): δ 5.30–5.37 (m, 2.5 H, α-H-1, H-1′, H-1″), 4.90 (dd, 0.5 H, β-H-1), 4.15 (m, 0.5 H, α-H-3), 3.38 (dq̇t, 1 H, H-4″), 2.22 (ddd, 0.5 H, β-H-2eq), 2.08 (ddd, 0.5 H, α-H-2eq), 1.71 (ddd, 0.5 H, α-H-2ax), 1.50 (ddq̇dt, 0.5 H, β-H-2ax).

Compound **9**: $[\alpha]_D^{20}$ +138° (H_2O, *c* 0.12); ^1H NMR (400 MHz, D_2O): δ 5.35 (m, 3.5 H, α-H-1, H-1^{2-4}), 4.90 (d, 0.5 H, β-H-1), 4.15 (m, 0.5 H, α-H-3), 3.38 (dq̇t, 1 H, H-4^4), 2.22 (m, 0.5 H, β-H-2eq), 2.09 m, 0.5 H, α-H-2eq), 1.72 (ddq̇t, 0.5 H, α-H-2ax), 1.51 (m, 0.5 H, β-H-2ax).

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