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Transfer reactions catalyzed by cyclodextrin glucosyltransferase using 4-thiomaltosyl and *C*-maltosyl fluorides as artificial donors [1]

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Abstract

Cyclodextrin glycosyltransferase enzyme from *Bacillus circulans* catalyzed the effective conversion of 4-thio-α-maltosyl fluoride into cyclo-α-(1 → 4²)-thiomalto -tetraoside, -pentaoside, -hexaoside and linear hemithiomaltooligosaccharides. However, under the same conditions, *C*-maltosyl fluoride afforded only linear modified maltotetraose, maltohexaose and maltooctaose in moderate yield. © 1997 Elsevier Science Ltd.

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1. Introduction

The understanding of substrate–enzyme interactions has been one of the most difficult tasks in glycobiology since recognition, binding and catalysis are all involved in the process. Their analysis can be conducted using either natural oligosaccharides and modified proteins inactivated by site-directed mutagenesis, or native proteins and non-natural substrate analogues. The latter approach in the glycosyl hydrolase field has been mainly developed by using enzyme-resistant substrates as competitive inhibitors. In these oligosaccharides, the oxygen(s) of the scissile bond(s) are replaced with methylene group(s), nitrogen or sulfur atom(s). Up until now, only chemical approaches have been reported for the access of *C*-oligosaccharides, whereas in the thio-series, both chemical and enzymatic methods have been devised [2,3].

We have also been involved in the synthesis and uses of substrate analogues of cyclodextrin glucosyltransferases to map their active sites and to develop a new methodology to obtain specifically modified cyclodextrins (CDs). Cyclodextrin glucosyl transferase (CGTase-enzymes catalyzed the cyclization reaction of starch and oligosaccharides into a mixture of cyclic oligosaccharides, referred to α-, β- or γ-cyclodextrins (consisting of six, seven or eight glucosyl units, respectively). We have shown that some maltosyl and maltotriosyl fluorides modified at C-6[2] or C-6[3] could easily afford modified CDs, which

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[1] For a preliminary description of this work, see Ref. [1].

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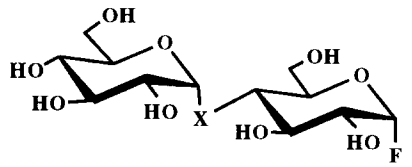


1 X = S
2 X = CH_2
3 X = O

Scheme 1.

indicated that these primary hydroxyl groups were not involved in direct contact with amino acids of subsite -2 (or -3) and +1 of the catalytic site [1]. This hypothesis was recently confirmed by the structures of CGTases complexed to various substrates or inhibitors determined by X-ray crystallography [4].

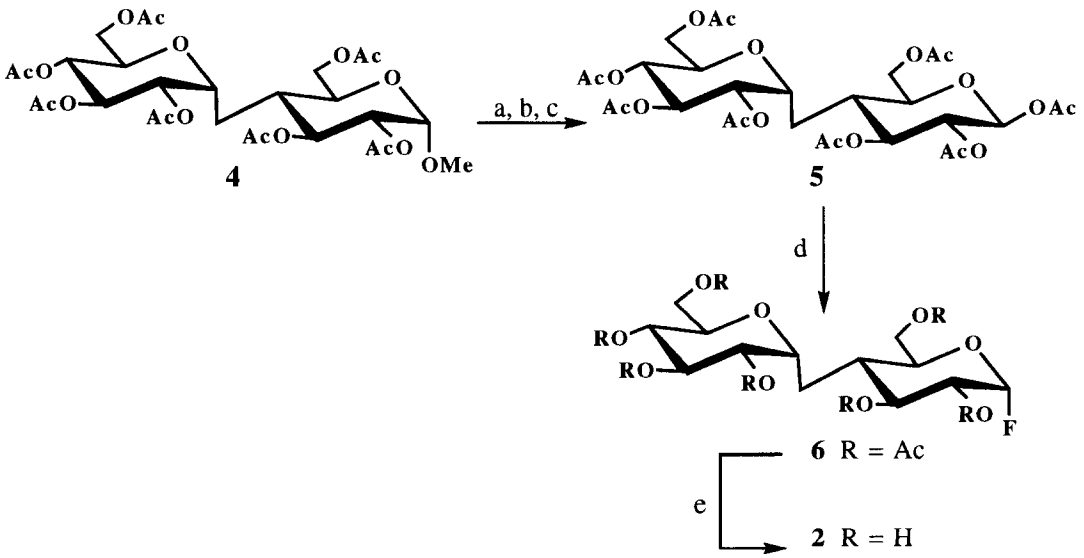
As an extension of this work, the present paper describes the effective conversion of 4-thio-α-maltosyl fluoride **1** into linear and cyclic hemithio-maltodextrins catalyzed by cyclodextrin glucosyltransferase enzyme (Scheme 1). We also present the first attempt for the enzymatic synthesis of *C*-maltooligosaccharides using the same approach. However starting from *C*-maltosyl fluoride **2**, only linear *C*-maltosyl dimer, trimer and tetramer were formed in appreciable yield.

2. Results and discussion

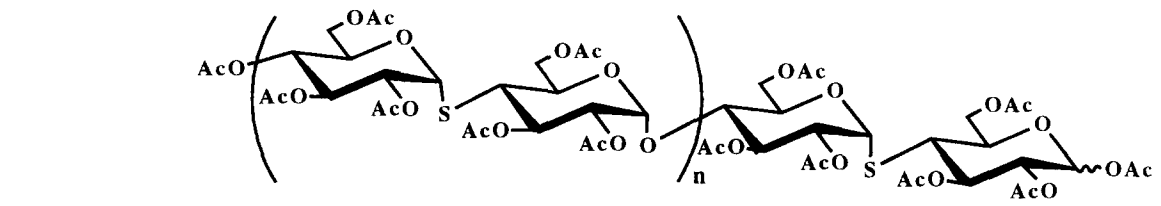
Maltosyl fluorides **1–3** were easily obtained as already described [5] or by conventional procedure from the known methyl α-*C*-maltoside **4** (Scheme 2) [6]. Acetolysis afforded an anomeric mixture of acetylated *C*-maltose which was converted into pure β-anomer by a two-step procedure (69% from **4**). The maltose derivative **5** was treated by a commercial mixture of hydrogen fluoride–pyridine at 0 °C and the fluoride **6** was isolated in 96% yield. It is worthy to note that fluoride **2** was more prone to spontaneous hydrolysis than its analogues **1** and **3**.

Following the methodology which we developed previously for modified maltosyl and maltotriosyl fluorides using commercially available CGTase [5], we decided to incubate compounds **1–3** under autocondensation conditions using pure CGTase from *Bacillus circulans* strain 251 [7]. The TLC patterns and HPLC profiles of the reaction mixtures revealed the formation of oligosaccharides.

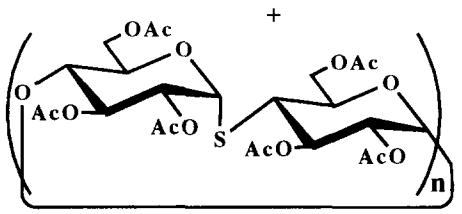
After incubation of 4-thiomaltosyl fluoride **1** with CGTase enzyme for 24 h, the reaction mixture was freed of enzyme by heating at 100 °C and spinning at 10,000 rpm. The supernatant was freeze-dried and submitted to two different treatments (Scheme 3). In the first set of experiments, the residue was acetylated and after the usual work-up, was purified by chromatography using an open-column. Acetylated 4-thiomaltose **7**, linear hemithiomaltodextrins **8** and **9** were isolated in 42, 4 and 4% yield, respectively. Besides these linear compounds, cyclothiomaltins were also obtained and characterized. The acetylated cyclo-α-(1 → 4^2)-4-thio-maltotetraoside **10**, -maltopentaoside **11** and -maltohexaaoside **12** were isolated in 13, 16 and 7% yield respectively.



Scheme 2. (a) Ac_2O, CH_3COOH, H_2SO_4. (b) CH_2Cl_2, hydrogen bromide/CH_3COOH (33%). (c) Ac_2O, CH_3COOH, silver acetate. (d) Hydrogen fluoride/pyridine. (e) MeOH, MeO^-Na^+.



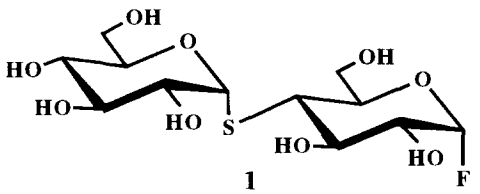
7 **n** =0
8 **n** = 1
9 **n** = 2



10 **n** = 4
11 **n** = 5
12 **n** = 6

pathway 1

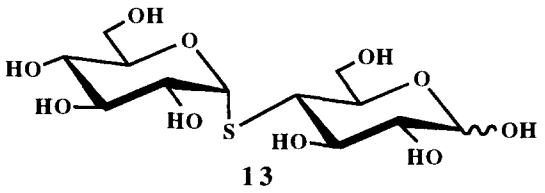
a, b



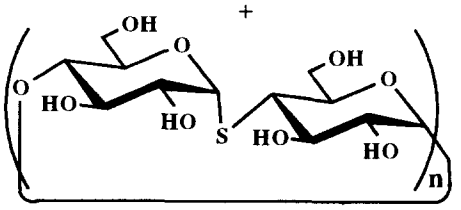
1

a, c

pathway 2



13



14 **n** = 4
15 **n** = 5
16 **n** = 6

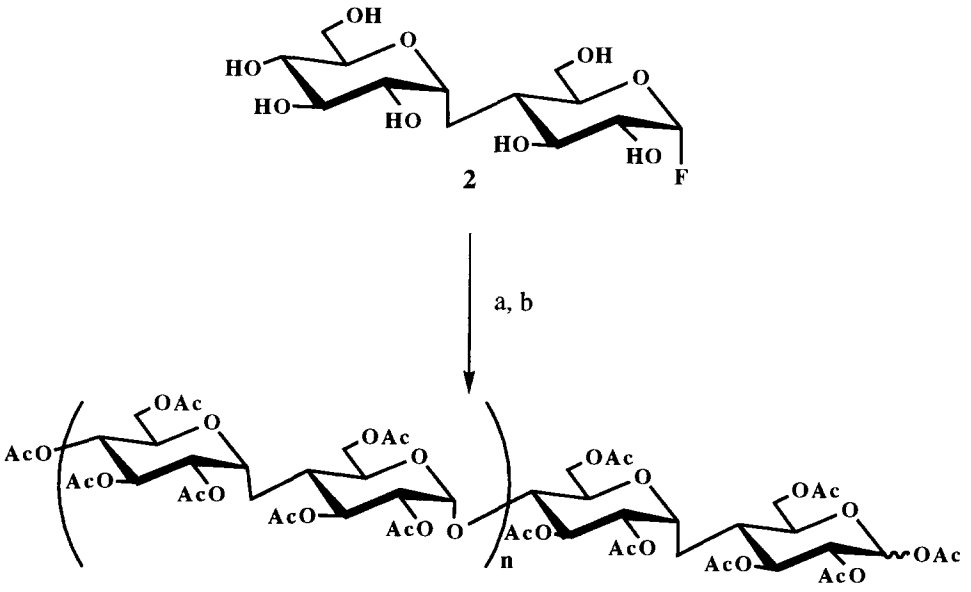
Scheme 3. (a) Phosphate buffer 0.2 M, pH 6.5, CGTase, 24 h, 40 °C. (b) Ac_2O, Pyridine, DMAP, 8 h, 60 °C. (c) Phosphate buffer 0.2 M, pH 6.5, β-Amylase, 24 h, 40 °C.

For ease of the purification, an alternative method was developed. A subsequent enzymatic treatment of the freeze-dried compounds with β-amylase, an *exo*-enzyme which hydrolyses the penultimate bond at the non-reducing end of maltooligosaccharides and liberates β-maltose unit [8], afforded only 4-thiomaltose **13** and cyclothiomaltins **14**, **15** and **16**. These compounds were isolated by preparative HPLC using μ-Bondapak NH_2 column in 45, 16, 14 and 7% yield.

At this stage, we turned our attention to increase the production of linear hemithiomaltodextrins which could act as potent inhibitors of glucoamylases, since this type of *exo*-enzyme catalyzes the hydrolytic release of β-D-glucose from non reducing ends of starch and related oligo- and poly-saccharides [9]. The stepwise addition of the fluoride **1** (0.1 equiv.) over 4 days (total 3 equiv.) to a solution of CGTase and 4-thiomaltose **13** (1 equiv.), which acts as an acceptor in the condensation reaction, gave after acetylation of the reaction mixture, acetylated hemithio-maltotetraose **8** and -maltohexaose **9** in 18 and 14% yield, respectively (these yields were based on the donor molecule **1**).

The CGTase enzyme from *B. circulans* strain 251 is known to be a β-CGTase; that means β-CD is the major product of the cyclisation reaction but α- and γ-CDs are also produced [7]. It will thus be of interest to find out (1) why cyclo-α(1 → 4²)-4-thiomaltotrioside was not isolated, and (2) the origin of the shift in the reaction products towards larger CD ring sizes. Under the same experimental conditions, α-maltosyl fluoride **3** was assayed and within 1 h it was consumed. We therefore followed the time-course of the transformation of compounds **1** and **3** into cyclic molecules by HPLC using analytical μ-Bondapak NH_2 column. After 1 h, natural α-, β- and γ-CDs were synthesized in 20, 47 and 7% yield (Fig. 1A) together with glucose and maltose (not shown). Then α-CD–β-CD interconversion occurred and after 24 h approximately half of the initial β-CDs were found in the mixture. The kinetics of transformation of **1** was different. A lag phase of 3 h was necessary for the formation of cyclothiomaltins, but linear hemithiomaltodextrins were also produced and β-amylase treatment was used to estimate the cyclic molecule yields. After 9 h the reaction plateaued without interconversion (Fig. 1B), suggesting possible resistance of cyclothiomaltins to enzymatic hydrolysis and a preferential binding in the active site with thiomaltosyl unit spanning the catalytic site. From these results, a possible explanation could be that cyclic hexamers are the thermodynamic products and for that reason they were not found in the cyclothiomaltin series.

Since the spontaneous hydrolysis of the *C*-maltosyl fluoride **2** was rapid (half-life ~ 2 h), we decided to perform the enzymatic condensation in a 1:1 mix-



Scheme 4. (a) Phosphate buffer/CH_3CN, (1:1), CGTase, 24 h, 40 °C. (b) Pyridine, Ac_2O, 24 h, 20 °C.

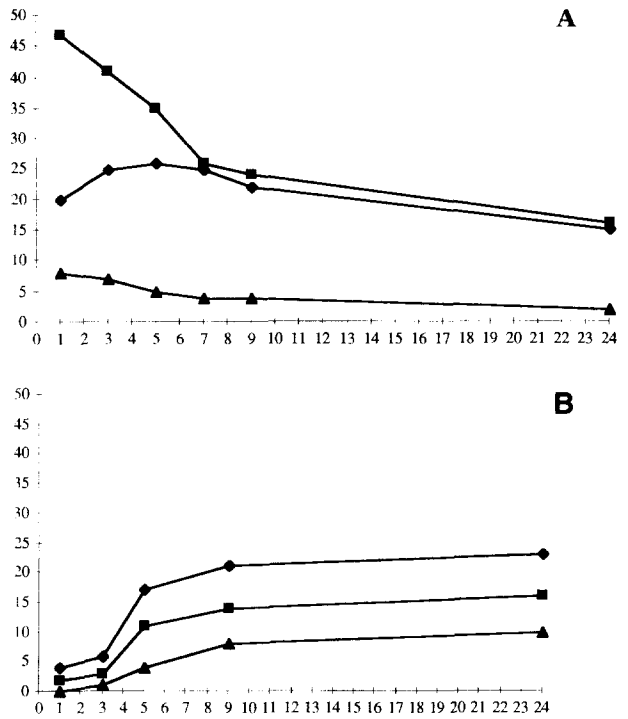


Fig. 1. Time course transformation of 4-thiomaltosyl fluoride **1** and maltosyl fluoride **3**. (A) Compound **3** (-◆-) α-CD, (-■-) β-CD, (-▲-) γ-CD. (B) Compound **1** (-◆-) 14, (-■-) 5, (-▲-) 16.

ture of phosphate buffer–acetonitrile (Scheme 4), conditions already used successfully for enzymatic synthesis of cellooligosaccharides [10]. After incubation of **2** with CGTase for 24 h, and elimination of the enzyme by heating and spinning, the reaction mixture was acetylated. Acetylated *C*-maltose **5**, tetra-**17**, hexa-**18** and octa-mers **19** were isolated in 50, 18, 11 and 5% yield respectively.

These results led to the hypothesis that in the active site, alternate *C*-maltooligosaccharides could adopt a different preferred conformation than their *O*- or *S*-analogues and that prevent the formation of cyclic molecules, even if in the free state *C*-oligosaccharides may exhibit much higher flexibility as suggested by recent studies on the conformation and the internal dynamics of *C*-disaccharides and related derivatives [11–13].

3. Experimental

General.—As already described in [10].

*1,2,3,6-Tetra-*O*-acetyl-4-deoxy-4-*C*-(2,6-anhydro-3,4,5,7-tetra-*O*-acetyl-1-deoxy-*D*-glycero-*D*-ido-heptityl)-β-D-glucopyranose* (**5**).—The acetylated methyl *C*-maltoside **4** (120 mg, 0.19 mmol) was dissolved in a mixture of CH_3COOH, Ac_2O, H_2SO_4 (3:7:0.07 v/v, 10 mL) and stirred for 18 h at room temperature. The reaction mixture was neutralized with anhydrous NaOAc (500 mg) and the solvents were evaporated and coevaporated with toluene (3 × 20 mL). A CH_2Cl_2 solution of the residue was washed with saturated $NaHCO_3$ solution (2 ×) and ice-cold water. After concentration of the residue, flash-column chromatography (1:1 EtOAc–light petroleum) gave a mixture of **5** and its α-anomer (100 mg, 80%, α:β ratio 2:8 estimated by ^1H NMR). This anomeric mixture was dissolved in dry CH_2Cl_2 (4 mL), cooled to 0 °C and hydrogen bromide in CH_3COOH (33%, 2 mL) was added. After being stirred at 0 °C for 30 min, the reaction mixture was kept at room temperature for 1.5 h, diluted with CH_2Cl_2 and washed with ice-cold water, and then ice-cold saturated $NaHCO_3$ solution (3 ×). The organic phase was concentrated to dryness and the crude bromide was used directly in the next reaction. To a solution of this bromide in Ac_2O–CH_3COOH (1:1, 5 mL) silver acetate (375 mg) was added. The reaction mixture was stirred in the dark for 15 h at room temperature, then filtered on Celite. The solution was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$ solution (×3) and concentrated. After purification by flash chromatography (4.5:6.5 EtOAc–light petroleum) the expected **5** was crystallized (70 mg, 69%): mp 86 °C (from Et_2O–hexane); $[\alpha]_D^{20}$ +44° (*c* 0.58, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.65 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 5.13 (m, 2 H, H-3, H-4^2), 4.91 (m, 3 H, H-2, H-3^2, H-5^2), 4.33 (dd, 1 H, $J_{5,6}$ 2.8, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.24 (dd, 1 H, $J_{6^2,7^2}$ 5.3, $J_{7a^2,7b^2}$ 12.6 Hz, H-7a^2), 4.23 (m, 1 H, H-2^2), 4.12 (dd, 1 H, $J_{5,6b}$ 5.4 Hz, H-6b), 4.05 (dd, 1 H, $J_{7b^2,5^2}$ 2.6 Hz, H-7b^2)), 3.79 (m, 1 H, H-6^2), 3.69 (m, 1 H, H-5), 2.19 (m, 1 H, H-4), 2.01 (m, 21 H, $COCH_3$), 1.83, 1.55 (m, 2 H, H-1^2); ^{13}C NMR ($CDCl_3$): δ 170.5, 170.4, 169.7, 169.4, 169.3, 168.8 (*C*OCH_3), 91.5 (C-1), 75.2, 74.3, 71.5, 70.6, 69.8, 69.5, 69.4, 68.2 (C-2^2, C-2, C-3^2, C-3, C-4^2, C-5^2, C-5, C-6^2), 63.8, 61.9 (C-6, C-7^2), 37.9 (C-4), 26.8 (C-1^2), 20.7, 20.6, 20.5, 20.4 (CO*C*H_3). FABMS, Anal. Calcd for $C_{29}H_{40}O_{18}$ [M + Li] 683.2374. Found: 683.2321.

*2,3,6-Tri-*O*-acetyl-4-deoxy-4-*C*-(2,6 wfhydro-3,4,5,7-tetra-*O*-acetyl-1-deoxy-*D*-glycero-*D*-ido-heptityl)-α-*D*-glucopyranosyl fluoride* (**6**).—In a plastic vial a hydrogen fluoride–pyridine solution (7:3 v/v, 2 mL) of **5** (70 mg, 0.104 mmol) was stirred at 0 °C for 30 min, diluted with $CHCl_3$ (20 mL) and neutralized (pH paper ~ 6) with 3 M ammonium hydroxide. The organic phase was decanted, washed twice with cold

saturated $NaHCO_3$ solution and then water. After solvent removal, the residue was purified by column chromatography (1:1 EtOAc–hexane) and the expected **6** was obtained (64 mg, 96%): mp 69 °C (from Et_2O–hexane) $[\alpha]_D^{20}$ +71° (*c* 0.41, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.68 (dd, 1 H, $J_{1,2}$ 2.5, $J_{1,F}$ = 53.5 Hz, H-1), 5.37 (dd, 1 H, $J_{3,4}$ 9.0, $J_{3,2}$ 9.3 Hz, H-3), 5.16 (dd, 1 H, $J_{4^2,3^2} = J_{4^2,5^2}$ 9.0 Hz, H-4^2), 4.96–4.91 (m, 2 H, H-3^2, H-5^2), 4.79 (dddd, 1 H, $J_{2,3}$ 10.0, $J_{2,F}$ 23.9 Hz, H-2), 4.38 (dd, 1 H, $J_{7a^2,6^2}$ 2.5, $J_{7a^2,7b^2}$ 12.4 Hz, 7a^2), 4.26–4.20 (m, 2 H, H-7b^2, H-6^2), 4.11 (dd, 1 H, $J_{6a,5}$ 5.0, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.07–3.99 (m, 2 H, H-6b, H-2^2), 3.77 (m, 1 H, H-5), 2.26 (m, 1 H, H-4), 2.02 (m, $COCH_3$), 1.81–1.66 (m, 2 H, H-1^2); ^{13}C NMR ($CDCl_3$): δ 170.5, 170.4, 170.1, 169.8, 169.4 ($COCH_3$), 104.3 (C-1, $J_{1,F}$ 226 Hz), 71.9, 71.6, 71.5, 71.1, 71.0, 69.9, 69.5, 68.3 (C-2^2, C-2, C-3^2, C-3, C-4^2, C-5^2, C-5, C-6^2), 63.6, 62.0 (C-6, C-7^2), 37.6 (C-4), 25.1 (C-1^2), 20.8, 20.6, 20.5 ($COCH_3$). FABMS, Anal. Calcd for $C_{27}H_{37}FO_{16}$ [M + Li] 643.2225. Found: 643.2389.

*4-Deoxy-4-*C*-(2,6-anhydro-1-deoxy-*D*-glycero-*D*-ido-heptityl)-α-D-glucopyranosyl fluoride* (**2**).—Peracetylated fluoride **6** (55 mg, 0.086 mol) was treated in MeOH (5 mL) with NaOMe (1 N, 50 μL). After 2 h at room temperature, neutralization with Amberlite IRN 77 (H^+), filtration, evaporation to dryness and freeze-drying led to **2** (27 mg, 91%). This compound was pure by HPLC and was immediately used in enzymatic condensation.

Enzymatic condensation of 4-thio-α-maltosyl fluoride (**1**)—*Pathway 1.*—CGTase (325 U · mL^{-1}, 440 μL) was added to a solution of **1** (400 mg, 1.12 mmol) in phosphate buffer (0.2 M, pH 6.5, 36 mL). The mixture was incubated at 40 °C for 24 h and boiled for 10 min. Denaturated proteins were eliminated by spinning (14,000 rpm, 10 min), the supernatant was freeze-dried and acetylated (1:2 Ac_2O-pyridine, 60 mL) in the presence of 4-dimethylaminopyridine (50 mg). After being kept for 8 h at 60 °C, the mixture was poured onto ice-water and the precipitate collected, washed with water, dissolved in CH_2Cl_2 and dried. Open column chromatography (3.5:5 acetone–cyclohexane) was used to solve the mixture of compounds. Pure oligomers were eluted in the following order.

*Octa-*O*-acetyl-4-thio-α-maltose* (**7**). (316 mg, 42%) identified by comparison to an authentic sample [14].

*Hepta-*O*-acetyl-4^2-*O*-(hepta-*O*-acetyl-4-thio-α-maltosyl)-4-thiomaltose* (**8**). (29 mg, 4%); ^{13}C NMR ($CDCl_3$): δ 170.5, 170.0, 169.7, 169.5 ($COCH_3$), 96.1 (C-1^3), 91.3 (C-1β), 88.6 (C-1α), 82.4 (C-1^2, -1^4), 74.7, 73.5, 71.6, 70.4, 69.8, 69.3, 68.7, 67.9 (C-2-C-2^4, C-3, C-3^4, C-4^2, C-5-C-5^4), 63.0, 62.9, 61.5 (C-6, C-6^4), 43.5, 43.4 (C-4, -4^3), 20.8, 20.7, 20.6 ($COCH_3$). FABMS, Anal. calcd for $C_{52}H_{70}O_{33}S_2$ $[M + Na]^+$ 1309.3138. Found: 1309.3234.

Acetylated hemithiomaltohexaose (**9**). (28 mg, 4%); ^{13}C NMR ($CDCl_3$): δ 170.4, 170.2, 170.0, 169.7, 169.3, 169.2 ($COCH_3$), 97.0, 96.4 (C-1^3, C-1^5), 91.0 (C-1β), 88.7 (C-1α), 82.5, 82.2 (C-1^2, C-1^4, C-1^6), 75.0, 74.7, 73.3, 73.1, 72.7, 72.0, 71.6, 70.7, 70.6, 70.2, 69.3, 69.1, 68.7 (C-2–C-2^6, C-3–C-3^6, C-4^2, C-4^4, C-4^6, C-5–C-5^6), 63.8, 62.4, 62.3, 62.0 (C-6, C-6^6), 44.0, 43.8 (C-4, C-4^3, C-4^5), 20.9, 20.7, 20.5, 20.4, 20.3, 20.1 ($COCH_3$). FABMS, Anal. Calcd for $C_{76}H_{102}O_{48}S_3$ $[M + Na]^+$ 1901.4600 Found: 1901.4757.

*Per-*O*-acetylated cyclo-α-(1 → 4^2)-4-thiomaltotetraoside* (**10**). (84 mg), 13%); $[\alpha]_D^{20}$ +101° (*c* 0.33, $CHCl_3$); ^1H NMR (C_6D_6, 345 K)): δ 6.01 (d, 1 H, *J*12,22 5.8 Hz, H-1^2), 5.76 (dd, 1 H, $J_{3,2} = J_{3,4}$ 10.2 Hz, H-3), 5.58 (dd, 1 H, *J*32,22 10.0, *J*32,42 8.4 Hz, H-3^2), 5.48 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1), 5.10–4.80 (m, 5 H, H-2, H-2^2, H-5, H-6a, H-6a^2), 4.71 (m, 1 H $J_{5^2,6b^2} \sim 2$, $J_{5^2,6a^2}$ 4.3, $J_{5^2,4^2}$ 9.8 Hz, H-5^2), 4.57 (m, 2 H, H-6b, H6b^2), 4.03 (dd, 1 H, $J_{4^2,3^2}$ 8.6 Hz, H-4^2), 3.21 (dd, 1 H, $J_{4,5}$ 10.8 Hz, H-4); ^{13}C NMR (C_6D_6, 345 K) δ: 170.7, 170.5, 170.3, 169.7, 169.1 ($COCH_3$), 97.2 (C-1), 81.8 (C-1^2), 73.7, 73.1, 72.5, 72.0, 71.7, 70.5, 68.9 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 63.3 (C-6, C-6^2), 45.6 (C-4), 20.8, 20.5, 20.2, 20.1 ($COCH_3$). FABMS: *m/z* 2376.9 $[M + Li]^+$.

*Per-*O*-acetylated cyclo-α-(1 → 4^2)-4-thiomaltopentaoside* (**11**). (104 mg, 16%); $[\alpha]_D^{20}$ +102° (*c* 0.36, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.66 (d, 1 H, $J_{12,22}$ 5.7 Hz, H-1^2), 5.32 (dd, 1 H, $J_{3,2}$ 10.4, $J_{3,4}$ 10.9 Hz, H-3), 5.25 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 5.17 (dd, 1 H, $J_{3^2,2^2}$ 9.2 $J_{3^2,4^2}$ 8.4 Hz, H-3^2), 4.83 (dd, 1 H, H-2^2), 4.70 (dd, 1 H, H-2), 4.54 (bd, 1 H, $J_{6a,6b}$ 10.8 Hz, H-6a), 4.43 (bd, 1 H, $J_{6a^2,6b^2}$ 11.5 Hz, H-6a^2), 4.28 (dd, 1 H, $J_{6b^2,5^2}$ 3.5 Hz, H-6b^2), 4.12 (m, 3 H, H-5, H-5^2, H-6b), 3.76 (m, 1 H, H-4^2), 2.99 (dd, 1 H, H-4); ^{13}C NMR ($CDCl_3$) δ: 170.5, 170.3, 170.0, 169.4 ($COCH_3$), 96.2 (C-1), 81.6 (C-1^2), 73.5, 71.9, 71.4, 71.0, 70.4, 69.7, 68.0 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 62.8, 62.3 (C-6, C-6^2), 44.4 (C-4). FABMS: *m/z* 2969.5 $[M + Li]^+$.

*Per-*O*-acetylated cyclo-α-(1 → 4^2)-4-thiomaltohexaoside* (**12**). (55 mg, 7%); $[\alpha]_D^{20}$ +95° (*c* 0.4, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.63 (d, 1 H, $J_{1^2,2^2}$ 5.6 Hz, H-1^2), 5.31 (m, 2 H, H-3^2, H-1), 5.15

(dd, 1 H, $J_{3^2,2^2}$ 8.9, $J_{3^2,4^2}$ 7.3 Hz, H-3^2), 4.90 (dd, 1 H, H-2^2), 4.77 (dd, 1 H, $J_{2,1}$ 4.0, $J_{2,3}$ 10.0 Hz, H-2), 4.46 (bd, H-6a, H-$6a^2$), 4.32 (dd, 1 H, $J_{6b^2,5b^2}$ 7.0 Hz, H-4^2), 3.02 (dd, 1 H, $J_{4,5}$ 11.0 Hz, H-4). ^{13}C NMR ($CDCl_3$) δ: 170.6, 170.5, 170.4, 170.0, 169.5, 169.4 ($COCH_3$), 96.7 (C-1), 82.2 (C-1^2), 74.3, 71.2, 70.8, 70.2, 69.9, 69.5 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 63.1, 62.7 (C-6, C-6^2), 45.0 (C-4), 20.8, 20.6, 20.5, 19.7 ($COCH_3$). FABMS: *m/z* 3561.9 $[M + Li]^+$.

Pathway 2.—CGTase (19.5 U · mL^{-1}, 60 μL) was added to a solution of fluoride **1** (55 mg, 0.15 mmol) in phosphate buffer (0.2 M, pH 6.5, 5 mL). The mixture was incubated at 40 °C for 10 h and the enzymatic reaction was stopped by boiling for 10 min. After spinning, the supernatant was freeze-dried and treated with β-amylase (20 U · mL^{-1}, 1 μL) in the same phosphate buffer (1.25 mL) at 40 °C for 24 h. After boiling and spinning, the solution was treated with TMD-8 mixed bed resin and freeze-dried. The compounds were purified on HPLC (μ-Bondapak NH_2 column using 60:40 acetonitrile–water as eluent).

4-Thiomaltose (**13**). Compound **13** (24 mg, 45%) was isolated and identified with an authentic sample [12].

Cyclo-α-(1 → 4²)-4-thiomaltotetraoside (**14**). (8 mg, 16%); $[\alpha]_D^{20}$ +198° (*c* 0.3, H_2O); ^{1}H NMR (D_2O): δ 5.46 (d, 1 H, $J_{1^2,2^2}$ 5.1 Hz, H-1^2), 5.35 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 3.75 (m, H-2, H-2^2, H-3, H-3^2, H-4^2, H-5, H-5^2, H-6a, H-$6a^2$, H-6^2, H-$6b^2$), 2.71 (dd, 1 H, $J_{4,3} = J_{4,5}$10.7 Hz, H-4).^{13}C NMR (D_2O): δ 102.2 (C-1), 89.0 (C-1^2), 76.6, 76.5, 75.4, 74.5, 74.3, 73.5 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 64.1, 62.3 (C-6, C-6^2), 51.0 (C-4). FABMS, Anal. Calcd for $C_{48}H_{80}O_{36}S_4$ $[M + Na]^+$ 1383.3209. Found: 1383.3258.

Cyclo-α-(1 → 4²)-4-thiomaltopentaoside (**15**). (7 mg, 14%); $[\alpha]_D^{20}$ +209° (*c* 0.75, H_2O); ^{1}H NMR (D_2O): δ 5.53 (d, 1 H, $J_{1^2,2^2}$ 4.4 Hz, H-1^2), 5.36 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.72 (m, H-2, H-2^2, H-3, H-3^2, H-4^2, H-5, H-5^2, H-6a, H-$6a^2$, H-6b, H-$6b^2$), 2.71 (dd, 1 H, $J_{4,3} = J_{4,5}$ 10.3 Hz, H-4). ^{13}C NMR (D_2O): δ 101.2 (C-1), 87.1 (C-1^2), 77.9, 75.4, 74.4, 73.6, 73.2, 72.4 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 63.0, 61.9 (C-6, C-6^2), 48.6 (C-4). FABMS, Anal. Calcd for $C_{60}H_{100}O_{45}S_5$ $[M + Na]^+$ 1723.4038. Found: 1723.4067.

Cyclo-α-(1 → 4²)-4-thiomaltohexaoside (**16**).—(4 mg, 7%); $[\alpha]_D^{20}$ +195° (*c* 0.2, H_2O); ^{1}H NMR (D_2O): δ 5.58 (d, 1 H, $J_{1^2,2^2}$ 5.0 Hz, H-1^2), 5.32 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.73 (m, H-2, H-2^2, H-3, H-3^2, H-4^2, H-5, H-5^2, H-6a, H-$6a^2$, H-6b, H-$6b^2$), 2.75 (dd, 1 H, $J_{4,3} = J_{4,5}$ 10.4 Hz, H-4). ^{13}C NMR (D_2O): δ 101.7 (C-1), 87.0 (C-1^2), 79.1, 75.3, 74.4, 73.8, 73.2, 73.0, 72.3 (C-2, C-2^2, C-3, C-3^2, C-4^2, C-5, C-5^2), 63.0, 62.0 (C-6, C-6^2), 48.3 (C-4). FABMS, Anal. Calcd for $C_{72}H_{120}O_{54}S_6$ $[M + Na]^+$ 2063.4866. Found: 2063.4805.

Enzymatic coupling of 4-thio-α-maltosyl fluoride (1) to 4-thio-maltose (**13**).—CGTase (325 U · mL^{-1}, 36 μL) was added to a solution of 4-thiomaltose (50 mg, 0.14 mmol) in phosphate buffer (0.2 M, pH 6.5, 9 mL) followed by a stepwise addition of **1** (150 mg, 0.4 mmol, 3 equiv.) over 4 days time. After the usual work-up and acetylation as described above, the products were purified by flash chromatography on silica gel (3:7 acetone–hexane). Compounds **7** (157 mg, 41%), **8** (94 mg, 18%) and **9** (52 mg, 14%) were obtained.

Enzymatic condensation of C-maltosyl fluoride (**2**).—CGTase (325 U · mL^{-1}, 18 μL) was added to a solution of compound **2** (23 mg, 0.036 mmol) in phosphate buffer (0.2 M pH 6.5, 750 μL) and acetonitrile (750 μL). The solution was kept 24 h at 40 °C, then heated for 10 min at 100 °C to deactivate the enzyme. After spinning, freeze-drying of the supernatant and acetylation of the products with 1:1 Ac_2O–pyridine (4 mL) in the presence of *N-N*-dimethyl-4-aminopyridine, flash chromatography (3:7, 4:6 and 5:5 acetone–cyclohexane) afforded octa-*O*-acetyl *C*-maltose (**5**) (12 mg, 50%).

Acetylated hemi-C-maltotetraose (**17**).—(5 mg, 18%). ^{13}C NMR ($CDCl_3$): δ 170.6, 170.5, 170.2, 169.9, 168.1 ($COCH_3$), 97.9 (C-1^3), 91.5 (C-1β), 89.0 (C-1α), 75.1, 72.7, 72.2, 71.9, 71.5, 70.4, 70.2, 69.7, 69.3, 68.4 (C-2–C2^4, C-3–C-3^4, C-4^2, C-4^4, C-5–C-5^4, C-6^2, C-6^4), 63.9, 62.1 (C-6, C-6^3, C-7^2, C-7^4), 39.1, 38.3 (C-4, C-4^3), 26.8 (C-1^2, C-1^4) 20.8, 20.6, 19.7 ($COCH_3$). FABMS, Anal. Calcd for $C_{54}H_{74}O_{33}$ $[M + Na]^+$ 1273.4010. Found: 1273.4042.

Acetylated hemi C-maltohexaose (**18**).—(3 mg, 11%). ^{13}C NMR ($CDCl_3$): δ 170.7, 170.4, 170.1, 169.8, 169.3, 169.1, 168.9 ($COCH_3$), 97.7 (C-1^3, C-1^5), 91.5 (C-1β), 89.0 (C-1α), 75.5, 75.2, 73.8, 73.0, 72.3, 71.6, 70.6, 70.0, 69.7, 69.3, 68.4 (C-2–C-2^6, C-3–C-3^6, C-4^2, C-4^4, C-4^6, C-5–C-5^6, C-6^2, C-6^4, C-6^6), 64.3, 63.0, 62.7 (C-6, C-6^3, C-6^5, C-7^2, C-7^4, C-7^6), 38.3, 37.8 (C-4, C-4^3, C-4^5), 27.0, 26.8 (C-1^2, C-1^4, C-1^6), 20.8, 20.7, 20.5, 20.1 ($COCH_3$). FABMS, Anal. Calcd for $C_{79}H_{108}O_{48}$ $[M + Na]^+$ 1847.5791. Found: 1847.5907.

Acetylated hemi C-maltooctaose (**19**).—(1.1 mg, 5%). FABMS, Anal. Calcd for $C_{104}H_{142}O_{63}$ $(M + Na)^+$ 2421.7805. Found: 2421.7893.

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