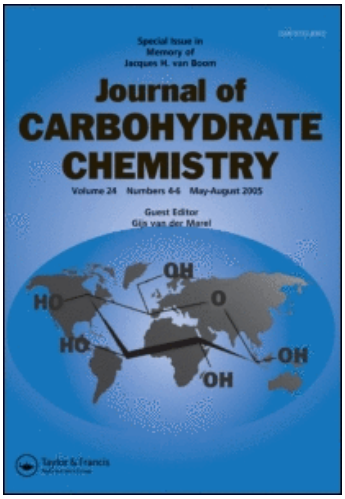

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4-Thiocellooligosaccharides : Their Synthesis and use as Inhibitors of Cellulases

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4-THIOCELLOOLIGOSACCHARIDES : THEIR SYNTHESIS AND USE AS INHIBITORS OF CELLULASES

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ABSTRACT

Methyl 4,4'-dithio-α-cellotrioside **1**, its tetra- and pentasaccharide homologues **8** and **14** were conveniently synthesized by treatment of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-triflyl-α-D-galactoside **6** with the respective peracetylated 1,4-dithio-, 1,4,4'-trithio- and 1,4,4',4"-tetrathiocellooligomers, in the presence of cysteamine and dithioerythritol in *N'*,*N"*,*N"'*-hexamethylphosphoramide. These methyl thiocellooligosaccharides were found to be excellent inhibitors of cellulases EGI and CBHII from *Humicola insolens*. The affinity increased strongly with an increasing number of glycosyl units in the thiooligosaccharides.

INTRODUCTION

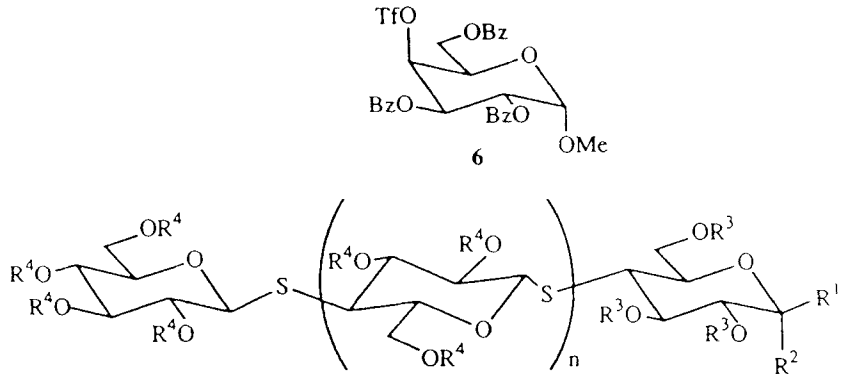
Cellulose, the major polysaccharide in plant cell walls, is hydrolyzed into glucose and cellobiose by several families of cellulases[1] displaying various types of synergy.[2] Because of the insolubility of the natural substrate, the precise mechanism of action of these enzymes may be more conveniently approached by the use of artificial and chromogenic substrates which should have at least a disaccharide structure.[3] However,

some of these enzymes require higher oligosaccharides for an efficient productive binding into their active site.[4] Since it has been shown that 1,4-dithio-β-cellobioside and 1,4,4'-trithio-β-cellotrioside were excellent competitive inhibitors of the hydrolysis of 4-methylumbelliferyl-β-lactoside by CBHI from *Trichoderma reesei*,[5] it will be of interest to synthesize higher oligomers in this series to map the active site of other cellulases.

This paper deals with the synthesis in the *S*-series of tri-, tetra- and pentasaccharides and their uses as inhibitors for endoglucanase I (EGI)[6] and cellobiohydrolase II (CBHII)[7] from *Humicola insolens*, which require, respectively, three and four unmodified β(1 → 4) glucosyl units to efficiently hydrolyze a glycosidic bond.[8]

RESULTS AND DISCUSSION

1. Synthesis of Methyl 4,4',4"-Trithio-α-Cellotetraoside **8** and Methyl 4,4',4",4"'-Tetrathio-α-Cellopentaoside **14**.



1 n = 1	R^1 = H, R^2 = OMe, R^3 = R^4 = H	**8** n = 2	R^1 = H, R^2 = OMe, R^3 = R^4 = H
2 n = 1	R^1 = H, R^2 = OMe, R^3 = R^4 = Ac	**9** n = 2	R^1 = H, R^2 = OMe, R^3 = R^4 = Ac
3 n = 1	R^1, R^2 = OAc,H, R^3 = R^4 = Ac	**10** n = 2	R^1, R^2 = OAc,H, R^3 = R^4 = Ac
4 n = 1	R^1 = H, R^2 = Br, R^3 = R^4 = Ac	**11** n = 2	R^1 = H, R^2 = Br, R^3 = R^4 = Ac
5 n = 1	R^1 = SAc, R^2 = H, R^3 = R^4 = Ac	**12** n = 2	R^1 = SAc, R^2 = H, R^3 = Ac, R^4 = Ac
7 n = 2	R^1 = H, R^2 = OMe, R^3 = Bz, R^4 = Ac	**13** n = 3	R^1 = H, R^2 = OMe, R^3 = Bz, R^4 = Ac
		14 n = 3	R^1 = H, R^2 = OMe, R^3 = R^4 = H

Figure 1 : Chemical structure of compounds **1** - **14**

The synthesis of peracetylated 4,4'-dithiocellotriose has been carried out previously[5] by reaction of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-triflyl-α-D-galactopyranoside[9] (**6**) with the sodium salt of 1,4-dithiocellobiose, followed by acetolysis of the resulting methyl 4,4'-dithiocellotrioside. A new method initially developed for thiomaltodextrins,[10] is presently described. It couples a peracetylated 1-thiocellooligosaccharide with a triflate in a one-pot reaction by using cysteamine which *S*-deacetylates and activates the C-1 by forming a sulfide anion. The presence of dithioerythritol helps maintaining the sulfide anion in the reduced form and the dipolar aprotic solvent *N',N'',N'''*-hexamethylphosphoramide (HMPA) enhancing the nucleophilicity of this sulfide anion. By this method, trisaccharide **2** was synthesized, and its properties were identical to the compound obtained by the previous synthetic route.[5] Methyl 4,4'-dithio-α-cellotrioside **1** was obtained by *O*-deacetylation of the derivative **2**. The key compound in the synthesis of the tetrasaccharide **7** was the peracetylated 1,4,4'-trithiocellotriose **5** which was obtained by treatment of the bromide **4** with tetrabutylammonium thioacetate in toluene as already described for the synthesis of the trisaccharide.[5] The strategy elaborated for the synthesis of the 4,4'-dithiocellotriose series was used again: coupling **5** with an excess of triflate **6** in the presence of cysteamine and dithioerythritol in HMPA gave tetrasaccharide **7** in 95% yield. After de-*O*-acylation, the methyl benzoate formed was removed by extraction of the aqueous solution with ether affording the pure methyl 4,4',4"-trithio-α-cellotetraoside **8**. Acetylation, acetolysis, bromide formation and treatment of the bromide with tetrabutylammonium thioacetate gave the acetylated 1,4,4',4"-tetrathio-β-cellotetraose **12** in 63% yield from **8**. Finally, methyl 4,4',4",4"'-tetrathio-α-cellopentaoside **14** was obtained after a condensation between the tetrasaccharide **12** and the galactoside **6** followed by deacylation as already described for the synthesis of **8**.

2. Compounds **1**, **8** and **14** as Inhibitors of EGI and CBHII Activities.

An efficient method has been developed for the determination of kinetic constants of cellulases using a second enzyme, cellobiose oxidase (CBO) from *Humicola insolens*.[11] CBO oxidizes cellodextrins at their reducing end in the presence of the electron acceptor 2,6-dichlorophenol-indophenol, DCPIP.[12,13] By using a non-reducing substrate such as a reduced cellodextrin, only the reducing ends formed by the cellulase can be oxidized by CBO. The concomitant reduction of the coloured acceptor is monitored continuously (600 nm) and allows the enzymatic reaction rates to be determined.

The kinetic constants of EGI have been measured by this method using reduced cellotetraose[14] as the substrate giving cellobiose and reduced cellobiose as the hydrolysis products.[8] The enzyme activities were determined with different concentrations of inhibitor and the kinetic constants were calculated using Lineweaver-Burk plots. Methyl

TABLE 1. Inhibition Constants (K_i) for *H. insolens* EGI and CBHII with the 4-Thiocellooligosaccharides 1, 8 and 14.

Enzyme	Constants	1	8	14
EGI	K_i (μM)	330 ± 1.6	73 ± 1.6	35 ± 2.7
EGI	K_{is}(μM)	2,000 ± 11	1,000 ± 100	
CBHII	K_i (μM)	1,400 ± 250	270 ± 2.3	15 ± 0.9

thiocellotrioside **1** and methyl thiocellotetraoside **8** displayed a mixed-type inhibition mechanism[15] (Table 1) implying two binding sites for the inhibitor. The inhibition has a predominant competitive character as $K_{is} >> K_i$. Methyl thiocellopentaoside **14** was a competitive inhibitor of EGI. When reduced cellopentaose[14] was used as the substrate for CBHII cellobiose and reduced cellotriose were reaction products[8] with this enzyme. The mode of inhibition was competitive for all 3 compounds (Table 1). For both enzymes, a strong decrease in K_i was observed upon increasing the number of glycosyl units of the inhibitors.

EXPERIMENTAL

General Methods. NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C. The mass spectra were recorded on a Nermag R-1010C spectrometer. For the FAB mode a 0.1 M HCl-glycerol matrix (1:4 v/v) was used. Optical rotations were measured at 20 °C on a Perkin Elmer 241 polarimeter. All solvents were evaporated at reduced pressure (40 °C). When dichloromethane solutions were extracted with aqueous solutions the water phases were back-extracted with dichloromethane and the collected organic phase was dried on sodium sulfate. For flash chromatography Merck Silica gel 60 230-400 mesh was used.

Methyl *S*-β-D-Glucopyranosyl-(1→4)-*S*-4-thio-β-D-glucopyranosyl-(1→4)-4-thio-α-D-glucopyranoside (1). Compound **1** was prepared from the acetylated methyl 4,4'-dithio-α-cellotrioside[5] (**2**, 100 mg, 0.10 mmol) by deacetylation

with sodium methoxide (1 M in methanol, 0.2 mL) in methanol (10 mL). The mixture was stirred overnight at room temperature, neutralized with Amberlite resin (H^+ form), filtered and concentrated to give pure **1** (55 mg, 100 %): mp 171-173 °C (dec.) ; $[\alpha]_D$ -8.8° (*c* 0.08, water); MS (FAB$^+$) m/z 573 $[M+Na]^+$, 551 $[M+H]^+$; ^{13}C NMR (D_2O/H_2O) δ(ppm) 100.9 (C-1), 85.4 and 85.0 (C'-1 and C''-1), 81.9, 81.5, 78.9, 75.9, 75.3, 74.1 (2C), 73.5, 71.3 and 71.2 (3xC-2, 3xC-3, 3xC-5 and C''-4), 63.3, 63.1 and 62.6 (3xC-6), 56.7 (OCH_3), 48.6 (2C) (C-4 and C'-4); ^1H NMR (D_2O/H_2O) δ (ppm) 2.77 (t, 1 H, H-4, $J_{3,4} = J_{4,5}$= 11 Hz), 2.72 (t, 1 H, H'-4, $J_{3',4'} = J_{4',5'}$= 11 Hz).

Anal. Calcd for $C_{19}H_{34}O_{14}S_2.3H_2O$: C, 37.74; H, 6.67; O, 44.98; S, 10.61. Found: C, 38.22; H, 6.60; O, 45.38; S, 9.80.

***S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1,4-dithio-β-D-glucopyranose (5).** The thiocellotriosyl bromide **4** was prepared from the peracetylated thiocellotriose[5] (**3**, 1.50 g, 1.50 mmol) as described[5] and was used without further purification. Simultaneously, tetrabutylammonium thioacetate was prepared from tetrabutylammonium hydroxide (25 % v/v in methanol, 7 mL, 5.5 mmol) and thioacetic acid (0.4 mL, 5.5 mmol) in toluene/methanol (9:1, 40 mL). The solvents were evaporated and coevaporated several times with toluene. The tetrabutylammonium salt was finally dissolved in toluene (40 mL) and added to the bromide **4**. The reaction mixture was stirred overnight at room temperature, concentrated and dissolved in dichloromethane. The organic phase was washed twice with ice-cold saturated sodium hydrogen carbonate and with water and concentrated. The dark brown residue was then treated with charcoal in hot methanol and filtered on celite for colour removal. After concentration the thioacetate **5** was purified by flash chromatography (ethyl acetate/light petroleum, 2:3) and crystallized from ether (0.87 g, 57 %): mp 125-128 °C; $[\alpha]_D$ -41.3° (*c* 0.15, chloroform); (FAB$^+$) MS m/z 1037 $[M+Na]^+$, 1015 $[M+H]^+$; ^{13}C NMR ($CDCl_3$) δ (ppm) 191.7 (S-C=O), 170.7 (2C), 170.4, 170.4, 170.0, 169.9, 169.7, 169.3 (2C) and 169.1 (10xO-C=O), 81.2, 80.3 and 79.8 (C-1, C'-1 and C''-1), 78.0, 77.7, 75.8, 73.8, 71.4, 70.9, 70.7, 70.4, 68.0 and 65.8 (3xC-2, 3xC-3, 3xC-5 and C''-4), 64.0, 63.7 and 61.2 (3xC-6), 45.0 and 45.9 (C-4 and C'-4), 30.7 (CH_3-CO-S), 20.7, 20.6 and 20.5 (10 x CH_3-CO-O); ^1H NMR ($CDCl_3$) δ (ppm) 2.91 (t, 1 H, H-4, $J_{3,4} = J_{4,5}$ = 11 Hz), 2.79 (t,1 H, H'-4, $J_{3',4'} = J_{4',5'}$ = 11 Hz).

Anal. Calcd for $C_{40}H_{54}O_{24}S_3.H_2O$: C, 46.51; H, 5.46; O, 38.72; S, 9.31. Found: C, 46.91; H, 5.43; O, 38.80; S, 8.86.

Methyl *S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-

thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside (7). To a solution of **5** (0.87 g, 0.86 mmol), freshly prepared methyl 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-α-D-galactopyranoside[10] (**6**, 1.3 g, 2.0 mmol) and dithioerythritol (160 mg, 1.0 mmol) in HMPA (6 mL) cysteamine (100 mg, 1.3 mmol) were added. The reaction mixture was stirred for 1 h and more cysteamine (50 mg, 0.65 mmol) was added. After stirring at room temperature overnight, the reaction mixture was poured onto ice water and a light yellow precipitate was formed. The precipitate was filtered on celite and washed with water. The precipitate was then dissolved in dichloromethane and the organic phase was washed with water and concentrated. After purification by flash chromatography (ethyl acetate/hexane,1:1.5) **7** crystallized from ethanol (1.2 g, 95 %): mp 131-134 °C; $[\alpha]_D$ -8.7° (*c* 0.46, chloroform); MS (FAB$^+$) m/z 1483 $[M+Na]^+$; ^{13}C NMR ($CDCl_3$) δ (ppm) 169.1-170.7 (10xCO-CH_3), 166.1, 165.7 and 165.5 (3xCO-C_6H_5), 128.3-133.3 (C_6H_5), 97.2 (C-1), 81.4, 80.8 and 80.5 (C'-1, C''-1 and C'''-1), 77.4, 77.0, 75.8, 73.8, 73.4, 71.3, 71.2, 71.0, 70.7, 70.3, 69.2, 68.0 and 67.5 (4xC-2, 4xC-3, 4xC-5 and C'''-4), 63.9, 63.6, 63.1 and 61.0 (4xC-6), 55.6 (OCH_3), 46.2, 46.1 and 45.8 (C-4, C'-4 and C''-4), 19.8-20.8 (10xCO-CH_3). ^1H NMR ($CDCl_3$) δ (ppm) 3.22 (t, 1 H, H-4, $J_{3,4} = J_{4,5}$ =11 Hz), 2.94 (t, 1 H, H'-4, $J_{3',4'} = J_{4',5'}$ = 11 Hz), 2.84 (t, 1H, H''-4, $J_{3'',4''} = J_{4'',5''}$ = 11 Hz).

Anal. Calcd for $C_{66}H_{76}O_{31}S_3$: C, 54.24; H, 5.24; O, 33.94; S, 6.58. Found: C, 53.82; H, 5.25; O, 34.45; S, 6.48.

Methyl *S*-β-D-Glucopyranosyl-(1→4)-*S*-4-thio-β-D-glucopyranosyl-(1→4)-4-thio-β-D-glucopyranosyl-(1→4)-4-thio-α-D-glucopyranoside (8). Compound **7** (1.1 g, 0.75 mmol) was stirred overnight with methanol (50 mL) and sodium methoxide (1 M in methanol, 1 mL). After neutralization with Amberlite resin (H^+ form) and filtration the methanol was evaporated. The residue was dissolved in water and extracted with ether in order to remove the methyl benzoate. The water phase was then freeze dried to give the pure methyl thiocellotetraoside (**8**, 500 mg, 91%): mp 176-180 °C (dec.); $[\alpha]_D$ -32.8° (*c* 0.54, water); MS (FAB$^+$) m/z 751 $[M+Na]^+$, 729 $[M+H]^+$; (FAB$^-$) m/z 727 $[M-H]^-$; ^{13}C NMR (D_2O/H_2O) δ (ppm) 100 9 (C-1), 85.4, 85.1 and 84.9 (C'-1, C''-1 and C'''-1), 81.9 (2C), 81.4, 78.8, 75.8 (2C), 75.2 (2C), 74.1 (2C), 73.5, 71.3 and 71.1 (4xC-2, 4xC-3, 4xC-5 and C'''-4), 63.3, 63.2, 63.1 and 62.6 (4xC-6), 56.7 (OCH_3), 48.6 (2C) and 48.5 (C-4, C'-4 and C'''-4) ; ^1H NMR (D_2O/H_2O) δ (ppm) 2.79 (t, 2 H, H-4 and H-4', $J_{3,4} = J_{4,5}$ = 11 Hz), 2.73 (t, 1 H, H''-4, $J_{3'',4''} = J_{4'',5''}$ = 11 Hz).

Anal. Calcd for $C_{25}H_{44}O_{18}S_3.2H_2O$: C, 39.26; H, 6.33; O, 41.84; S, 12.58. Found: C, 39.60; H, 6.30; O, 43.44; S, 10.66.

Methyl *S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-4-thio-α-D-glucopyranoside (9). For the preparation of **9**, methyl thiocellotetraoside (**8**, 500 mg, 0.69 mmol) was dissolved in pyridine (20 mL) and acetic anhydride (10 mL) and stirred overnight at room temperature. The reaction mixture was cooled to 0 °C and methanol was added. The solvents were evaporated and coevaporated with toluene and the residue was dissolved in dichloromethane and washed consecutively with ice cold potassium hydrogen sulfate solutions (10%) in water, saturated sodium hydrogen carbonate and ice cold water. After concentration in water and purification by flash chromatography (ethyl acetate/light petroleum, 1:1), **9** was obtained by crystallisation from ether (790 mg, 90 %): mp 136-139 °C; $[\alpha]_D$ -21.9° (*c* 0.31, chloroform); (FAB^+) m/z 1297 MS $[M+Na]^+$; (FAB^-) NBA matrix m/z 1273 $[M-H]^-$, 1320 $[M+NO_2]^-$; ^{13}C NMR ($CDCl_3$) δ (ppm) 169.1-170.8 (13xCO-CH_3), 96.9 (C-1), 81.1, 80.6 and 80.5 (C'-1, C''-1 and C'''-1), 77.3, 77.1, 75.7, 73.8, 72.3, 71.5, 71.3, 70.8, 70.5, 70.3, 68.6, 67.9 and 67.3 (4xC-2, 4xC-3, 4xC-5 and C'''-4), 63.6, 63.4, 62.9 and 60.5 (4xC-6), 55.4 (OCH_3), 45.9 (2C) and 45.5 (C-4, C'-4 and C''-4), 20.4, 20.5 and 20.6 (13xCO-CH_3); ^1H NMR ($CDCl_3$) δ (ppm) 2.93 (t, 1 H, H-4, $J_{3,4} = J_{4,5} = 11$ Hz), 2.87 (t, 1 H, H'-4, $J_{3',4'} = J_{4',5'} = 11$ Hz), 2.84 (t, 1 H, H''-4, $J_{3'',4''} = J_{4'',5''} = 11$ Hz).

Anal. Calcd for $C_{51}H_{70}O_{31}S_3$: C, 48.03; H, 5.53; O, 38.89; S,7.54. Found: C, 47.75; H, 5.60; O, 39.44; S, 7.21.

S-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1,4-dithio-β-D-glucopyranose (12). The acetylated methyl thiocellotetraoside **9** (690 mg, 0.54 mmol) was dissolved in a mixture of acetic acid, acetic anhydride and sulfuric acid (3:7:0.07 v/v, 30 mL) and stirred overnight at room temperature. The reaction mixture was neutralized with sodium acetate (1.0 g) and the solvents were evaporated and coevaporated with toluene. The residue was dissolved in dichloromethane and washed consecutively with ice cold aqueous solutions (10%) of potassium hydrogen sulfate, saturated sodium hydrogen carbonate and ice cold water. After concentration to dryness, product **10** was used without any further purification. Crude **10** was dissolved in dry dichloromethane (7 mL), cooled to 0 °C and HBr in acetic acid (33 %, 1.5 mL) was added. After being stirred at 0 °C for 15 min, the reaction mixture was kept at 5 °C overnight. The reaction mixture was then diluted with dichloromethane and washed twice with ice cold saturated sodium hydrogen carbonate and with water. The organic phase was concentrated to dryness to

give the crude bromide **11** which was used directly in the next reaction. Compound **11** was treated with tetrabutylammonium thioacetate which was prepared from tetrabutylammonium hydroxide (2.5 mL, 2 mmol), thioacetic acid (0.15 mL, 2 mmol) and toluene/methanol (95:5, 15 mL) as described for the preparation of **5**. Reaction time and work-up procedure were the same as described for **5**. Product **12** was purified by flash chromatography (ethyl acetate/light petroleum, 1:1) and crystallized from ether (500 mg, 70 % on the basis of **9**); mp 131-134 °C; $[\alpha]_D$ -40.4° (*c* 0.23, chloroform); MS (FAB^+) m/z 1341 $[M+Na]^+$; (FAB^-) NBA matrix m/z 1471 [M+NBA]; ^{13}C NMR ($CDCl_3$) δ (ppm) 169.1-170.7 (13xCO-CH_3), 81.5, 80.2, 79.9 and 79.7 (C-1, C'-1, C''-1, C'''-1), 78.0, 77.8, 77.4, 75.7, 73.8, 72.0, 71.5, 71.1, 70.8 (2C), 70.5, 70.2 and 67.9 (4xC-2, 4xC-3, 4xC-5 and C'''-4), 64.2, 63.8, 62.1 and 61.2 (4xC-6), 46.3, 45.8 and 45.7 (C-4, C'-4 and C''-4), 30.8 (CH_3-CO-S), 20.4-20.8 (13xCH_3-CO-O).

Anal. Calcd for $C_{52}H_{70}O_{31}S_4$: C, 47.34; H, 5.35; O, 37.59; S, 9.72. Found: C, 46.84; H, 5.43; O, 38.43; S, 9.30.

Methyl *S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*S*-(2,3, 6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-*S*-(2,3,6-tri-*O*-acetyl-4-thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside (13). Compound **13** was prepared from **12** (480 mg, 0.36 mmol), **6** (485 mg, 0.76 mmol), dithioerythritol (75 mg, 0,47 mmol) and cysteamine (50 mg, 0.65 mmol and 25 mg, 0.33 mmol) in HMPA (4 mL) as described for **7**. After purification by flash chromatography (ethyl acetate/light petroleum, 1:1.5), **13** crystallized from ether (350 mg, 55 %);. mp 134-137 °C; $[\alpha]_D$ -18.3° (*c* 0.35, chloroform); MS (FAB^+) m/z 1787 $[M+Na]^+$; ^{13}C NMR ($CDCl_3$) δ (ppm) 169.2-170.6 (13xCO-CH_3)166.0, 165.7 and 165.5 (3xCO-C_6H_5), 128.4-133.3 (C_6H_5), 97.2 (C-1), 81.4, 80.5 (2C) and 80.3 (C'-1, C''-1, C'''-1 and C''''-1), 77.9, 77.2, 76.6, 75.7, 73.8, 73.5, 71.9, 71.5, 71.2, 71.0, 70.7, 70.6, 70.2, 69.2, 67.8 and 67.6 (5xC-2, 5xC-3, 5xC-5 and C''''-4), 63.9, 63.8, 63.3, 62.0 and 61.3 (5xC-6), 55.6 (OCH_3), 46.2 (2C), 45.9 and 45.4 (C-4, C'-4, C''-4 and C'''-4), 19.8-20.9 (CO-CH_3). ^1H NMR ($CDCl_3$) δ (ppm) 3.23 (t, 1 H, H-4, $J_{3,4} = J_{4,5} = 11$ Hz), 2.98 (t, 1 H, H'-4, $J_{3',4'} = J_{4',5'} = 11$ Hz), 2.93 (t, 1 H, H''-4, $J_{3'',4''} = J_{4'',5''} = 11$ Hz), 2.82 (t, 1 H, H'''-4, $J_{3''',4'''} = J_{4''',5'''} = 11$ Hz).

Anal. Calcd for $C_{78}H_{92}O_{38}S_4$: C, 53.05; H, 5.25; O, 34.43; S, 7.26. Found: C, 52.76; H, 5.47; O, 34.78; S, 6.99.

Methyl *S*-β-D-Glucopyranosyl-(1→4)-*S*-4-thio-β-D-glucopyranosyl-(1→4)-*S*-4-thio-β-D-glucopyranosyl-(1→4)-*S*-4-thio-D-glucopyranosyl-(1→4)-4-thio-α-D-glucopyranoside (14). Methyl thiocellopentaoside **14** was

prepared from **13** (100 mg, 0.057 mg) as described for the preparation of **8**. The expected compound was obtained (50 mg, 97 %): mp 195-200 °C (dec.); $[\alpha]_D$ -47.0° (*c* 0.50, water); MS (FAB$^+$) m/z 929 $[M+Na]^+$, 907 $[M+H]^+$; ^{13}C NMR (D_2O/H_2O) δ (ppm) 100.9 (C-1), 85.4, 85.1 (2C) and (C'-1, C''-1, C'''-1 and C''''-1), 81.9 (2C), 81.8, 81.4, 78.8, 75.7 (2C), 75.2 (4C), 74.1 (2C), 73.5, 71.3 and 71.1 (5xC-2, 5xC-3, 5xC-5 and C''''-4), 63.3 (3C), 63.1 and 62.6 (5xC-6), 56.7 (OCH_3), 48.6 (2C) and 48.5 (2C) (C'-4, C''-4, C'''-4 and C''''-4); ^1H NMR (D_2O/H_2O) δ (ppm) 2.79 (t, 3 H, H-4, H-4' and H-4'', $J_{3,4} = J_{4,5} = 11$ Hz), 2.74 (t, 1 H, H'''-4, $J_{3''',4'''} = J_{4''',5'''} = 11$ Hz).

Anal. Calcd for $C_{31}H_{54}O_{22}S_4.2H_2O$: C, 39.48; H, 6.19; O, 40.72; S, 13.60. Found: C, 39.70; H, 6.12; O, 42.31; S, 11.87.

Enzyme assay: The enzymes were obtained from Novo Nordisk A/S where they have been cloned and characterized as described.[6,7] For the enzyme assay, enzymes and substrates were mixed to a final volume of 550 µL with the concentrations of 12 to 200 µM reduced cellodextrin,[14] 90 µM DCPIP, 1.7 nM EG I or 650 nM CBH II and 1200 nMCBO. The inhibitor concentrations were the following: EG I: **1**: 0.5 and 1.0 mM, **8**: 0.25 and 0.5 mM, **14**: 0.1 and 0.2 mM; CBH II: **1**: 1.0 and 2.0 mM, **8**: 1.0 mM, **14**: 0.1 mM. The steady state kinetics was performed on a HP 8452A spectrophotometer and followed at 600 nm for 8 min. The data were the result of dual determinations.

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