Carbohydrate Research, 224 (1992) 29–40
Elsevier Science Publishers B.V., Amsterdam

4-Thiocellooligosaccharides. Their synthesis and use as ligands for the separation of cellobiohydrolases of *Trichoderma reesei* by affinity chromatography

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(Received July 9th, 1990; accepted in revised form March 12th, 1991)

ABSTRACT

4-Aminophenyl 1,4-dithio-β-cellobioside (**6**) was obtained by treatment of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-triflyl-α-D-galactopyranoside with the sodium salt of 1-thio-β-D-glucopyranose, followed by acetolysis and glycosylation of the corresponding bromide with 4-aminobenzenethiol and subsequent deacylation. A similar synthesis starting with the 1-thiolate of 1,4-dithio-β-cellobiose led to the trisaccharide 4-aminophenyl 1,4,4'-trithiocellotrioside (**16**). The 4-acetamidophenyl di- and tri-thiocellooligosaccharides were found to be excellent competitive inhibitors of the hydrolysis of 4-methylumbelliferyl β-lactoside with respective K_i values of 25 and 6.5mM. The two 4-aminophenyl oligosaccharides **6** and **16** were coupled to CH-Sepharose 4B, and the affinity gels were used for the purification of cellobiohydrolases from a crude commercial cellulolytic extract of *T. reesei*. Cellobiohydrolases I or II were selectively desorbed from gels bearing ligands **6** and **16**.

INTRODUCTION

The purification of cellulolytic enzymes requires usually complex and time-consuming techniques involving ion-exchange chromatography and gel-filtration, followed by isoelectrofocusing[1] or immunoadsorption[2]. An alternative approach based on the ability of cellulases to bind tightly to various cellulose matrices has been investigated for more than twenty years[3–6]. Difficulties in eluting adsorbed enzymes from polymers were however encountered[7]. Affinity chromatography using the well-defined low mol. wt. 4-aminophenyl 1-thio-β-cellobioside as ligand was recently introduced to solve this problem[8]. A limitation in the use of this ligand and higher oligosaccharides lies in their possible hydrolysis at interglycosidic bonds which necessitates the addition of appropriate inhibitors to prevent column deterioration.[8] For this reason, our laboratory has, over the course of several years, developed the synthesis of thio-linked di- and trisaccharides for use as analogs of substrate of glycanases[9], based upon the concept of

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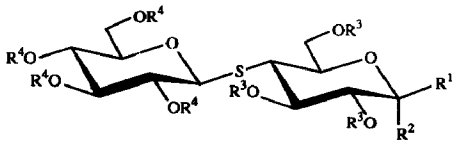
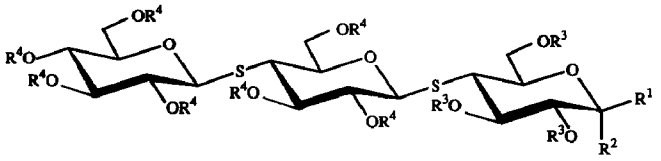
0008-6215/92/$ 05.00



1 R^1 = H, R^2 = OMe, R^3 = Bz, R^4 = Ac
2 R^1 = H, R^2 = OAc, R^3 = R^4 = Ac
3 R^1,R^2 = H, OH, R^3 = R^4 = H
4 R^1 = S$C_6H_4NH_2$(4), R^2 = H, R^3 = R^4 = Ac
5 R^1 = H, R^2 = Br, R^3 = R^4 = Ac
6 R^1 = S$C_6H_4NH_2$(4), R^2 = R^3 = R^4 = H
7 R^1 = SC_6H_4NHAc(4), R^2 = R^3 = R^4 = H
8 R^1 = SC_6H_4NHAc(4), R^2 = H, R^3 = R^4 = Ac
9 R^1 = 4-methylumbelliferyl O, R^2 = H, R^3 = R^4 = Ac
10 R^1 = 4-methylumbelliferyl O, R^2 = R^3 = R^4 = H
11 R^1 = SAc, R^2 = H, R^3 = R^4 = Ac



12 R^1 = H, R^2 = OMe, R^3 = Bz, R^4 = Ac
13 R^1 = H, R^2 = OMe, R^3 = R^4 = Ac
14 R^1,R^2 = H, OAc, R^3 = R^4 = Ac
15 R^1 = S$C_6H_4NH_2$(4), R^2 = H, R^3 = R^4 = Ac
16 R^1 = S$C_6H_4NH_2$(4), R^2 = R^3 = R^4 = H
17 R^1 = SC_6H_4NHAc(4), R^2 = R^3 = R^4 = H
18 R^1 = SC_6H_4NHAc(4), R^2 = H, R^3 = R^4 = Ac

nucleophilic enhancement of a sulfide anion in a dipolar aprotic solvent such as *N'*, *N''*, *N'''*-hexamethylphosphoramide. We describe herein the synthesis of 4-thiocellobiose (**3**) and its glycosides **7** and **10**, and of the glycoside of 1,4,4'-trithiocellotrioside (**18**), and their inhibition of hydrolysis of methylumbelliferyl lactoside[8], a specific substrate of CBH I. In addition, the purification of cellobiohydrolases I and II (CBH I and CBH II) by affinity chromatography using 4-aminophenyl 1,4-dithio-*β*-cellobioside (**6**) and 1,4,4'-trithio-*β*-cellotrioside (**15**) as ligands is also discussed.

RESULTS AND DISCUSSION

Synthesis of aryl 4-thiocellobioside, aryl 1,4-dithio- and 1,4,4'-trithiocellotriosides. — A previous paper of this laboratory[10] described the preparation of 4-thiocellobiose as a gratuitous inducer of cellulase-degrading enzymes by reaction of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-α-D-galactopyranoside with the sodium salt of 1-thio-*β*-D-glucopyranose, followed by acetolysis of the resulting methyl 4-thiocellobioside **1**; experimental details are reported herein. An alternative approach

TABLE I

^{1}H-N.m.r. data for the ring protons of acylated thiooligosaccharides **1**, **2**, **4**, **8**, **11–15**, and **18** in $CDCl_3$[a]

Proton[b]	*Compound*									
	1	**2**	**4**	**8**	**11**	**12**	**13**	**14**	**15**	**18**
H-1	5.18	6.33	4.46	4.56	5.25	5.20	4.92	6.33	4.53	4.64
	d	d	d	d	d	d	d	d	d	d
($J_{1,2}$)	3.5	4.0	10.0	10.0	9.5	3.5	4.5	4.0	10.0	10.0
H-2	5.24	5.06	4.87	4.86	5.07	5.25	4.86	5.04	4.85	4.86
	dd	dd	t	t	dd	dd	dd	dd	dd	dd
($J_{2,3}$)	9.5	10.0	9.0	9.0	11.0	9.0	10.0	10.0	9	9.5
H-3	6.04	5.42	5.15	5.15	5.27	6.0	5.43	5.41	5.28	5.28
	dd	dd	dd	dd	t	dd	dd	dd	dd	dd
($J_{3,4}$)	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
H-4	3.29	3.04	2.91	2.89	3.2	3.29	2.92	3.00	2.88	2.89
	t	t	t	t	t	t	t	t	t	t
($J_{4,5}$)	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
H-5	4.44	4.28	3.8	3.82	4.03	4.50	4.20	4.31	4.06	3.96
	td	td	m	m	m	m	m	m	m	m
($J_{5,6a}$)	3.0	2.5								
H-6a			4.62	4.63					4.58	4.6
			dd	dd					dd	m
($J_{6a,6b}$)	4.8	4.47	12.0	12.0	4.48	4.8	4.5	4.47	12.0	
H-6b	m	m	4.37	4.37	m	m	m	m	4.38	4.37
			dd	dd					dd	dd
($J_{6b,5}$)			5	4.5					5.0	4.5
H-1′	4.97	4.74	4.73	4.72	4.82	5.0	4.81	4.78		
	d	d	d	d	d	d	d	d		
($J_{1',2'}$)	10.0	10.0	10.0	9.0	9.5	10.0	9.0	9.5	4.83	4.9
H-2′	4.90	4.94	4.92	4.90	4.94	4.86	4.84	4.87	m	m
	dd	t	t	t	dd	dd	dd	dd		
($J_{2',3'}$)	9.0	10.0	10.0	9.0	10.0	9.0	10.0	10.0		
H-3′	5.20	5.25	5.21	5.19	5.24	5.15	5.14	5.15	5.16	5.15
	t	t	t	t	t	dd	dd	dd	m	m
($J_{3',4'}$)	9.0	9.5	10.0	9.0	10.0	11.0	11.0	11.0		
H-4′	5.02	5.02	4.96	4.98	4.98	2.95	2.96	2.94	2.82	2.84
	t	t	t	t	t	t	t	t	t	t
($J_{4',5'}$)	9.0	10.0	10.0	9.5	10.0	10.0	11.0	11.0	11.0	11.0
H-5′	3.74	3.73	3.72	3.72	3.83	3.91	3.84	3.9	3.87	
	o	td			o			o		
($J_{5',6'a}$)	5.0	4.0	m	m	2.0	m	m	2.5	m	

TABLE I (*continued*)

^{1}H-N.m.r. data for the ring protons of acylated thiooligosaccharides **1**, **2**, **4**, **8**, **11–15**, and **18** in $CDCl_3$[a]

Proton[b]	*Compound*									
	1	**2**	**4**	**8**	**11**	**12**	**13**	**14**	**15**	**18**
H-6'a			4.04		4.23	4.7	4.66	4.68	4.65	4.65
			dd		dd	dd	dd	m	dd	m
($J_{6'a,6'b}$)	4.1	4.17	12.0	4.10	12.0	12.0	12.0		12.0	
H-6'b	m	m	4.14	m	4.07	4.33	4.32	4.26	4.05	4.10
			dd		dd	dd	dd	m	dd	dd
($J_{6'b,5'}$)			3.0		7.0	4.5	4.5		4.5	4.5
H-1″						4.93	4.77	4.74	4.80	4.81
						d	d	d	d	d
($J_{1'',2''}$)						10.0	9.5	9.5	10.0	10.0
H-2″						4.80	4.94	4.92	4.92	4.93
						m	t	t	dd	dd
($J_{2'',3''}$)							9.5	9.5	9.0	9.0
H-3″							5.22	5.22	5.22	5.22
							t	t	t	t
($J_{3'',4''}$)							10.0	9.5	9.0	9.0
H-4″						5.06	5.05		5.10	5.10
						t	t		t	t
($J_{4'',5''}$)						9.5	10.0		9.0	9.0
H-5″						3.8	3.75	3.76	3.77	3.78
						o	m	m	o	m
($J_{5'',6''a}$)						2.5			2.5	
H-6″a						4.26	3.78	4.23	4.46	4.44
						dd	m	m	dd	dd
						12.0			12.0	12.0
H-6″b						4.18	4.24	4.23	4.08	4.09
						dd	m	dm	dd	dd
						5.0			4.5	4.5

[a] δ values, J in Hz. Abbreviations: d, doublet; dd, doublet of doublets; m. multiplet; o, octet; t, triplet. [b] The primed numbers refer to the protons of the central residue for trisaccharides or the terminal nonreducing group for disaccharides; double-primed for the protons of the terminal nonreducing group of trisaccharides.

was later described for the synthesis of the acylated compound **1**, which involved phase-transfer catalysis for the coupling reaction[11]. The preparation of aromatic glycosides of **3** was, then, readily achieved from its acetylated precursor **2** by use of the glycosyl bromide intermediate **5**. Reaction of either 4-aminobenzenethiol or 4-methylumbelliferone with **5**, in acetone containing potassium carbonate, afforded the corresponding 1,2-*trans*-aryl glycosides **4** and **9** in a good yield. The assignment of the

anomeric configuration is clearly inferred from the large coupling constants for H-1–H-2 in the ^{1}H-n.m.r. spectra (Table I). A further confirmation of the β-D-anomeric configuration for **9** was obtained from an examination of its ^{13}C-n.m.r. spectrum, which showed for C-1 the expected chemical displacement at δ 104 for β-D-glucosides. Deacetylation of **4** and **9** gave 4-aminophenyl 1,4-dithio-β-cellobioside (**6**) and 4-methylumbelliferyl 4-thio-β-cellobioside (**10**), respectively. Further *N*-acetylation of the aminophenyl derivative **4** with acetic anhydride–pyridine led to the *N*-acetyl derivative **8** and *O*-deacetylation afforded **7**.

The key compound in the synthesis of the trisaccharide analogs of **6** and **7**, namely, 4-aminophenyl (**16**) and 4-acetamidophenyl 1,4,4′-trithiocellotriosides (**18**) was 2,3,6-tri-*O*-acetyl-1-*S*-acetyl-4-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-1,4-dithio-β-D-glucopyranose (**11**). This compound was obtained in 63% yield by treatment of bromide **5** with tetrabutylammonium thioacetate in toluene.

At this stage, the sequence of reactions already developed for the synthesis of 4-thiocellobiose was used again, *i.e.*, reaction of deacetylated **11** with methyl 2,3,6-tri-*O*-benzoyl-4-*O*-triflyl-α-D-galactopyranoside gave, after acetylation, the trisaccharide derivative **12** in 58% yield. Subsequent *O*-deacylation and acetylation afforded **13** in crystalline form in 60% yield. The configuration of the newly formed thioglycosidic bond was expected to be 1,2-*trans* since mutarotation of a thiolate anion has never been observed under the conditions used[9,12,13]. This conclusion was again supported by the ^{1}H-n.m.r. spectra of **12** and **13** (Table I). Acetolysis of the methyl group of **13** led to the fully acetylated 4,4′-dithiocellotriose **14**. The transformation of **14** into 4-aminophenyl (**16**) and 4-acetamidophenyl 1,4,4′-trithiocellotriosides (**18**) was performed as described for the synthesis of **6** and **7**.

Thiocellobiosides **7** *and* **10**, *and 1,4,4′-trithiocellotrioside* **18** *as inhibitors of cellobiohydrolase I.* — It has been known for several years that alkyl and aryl thioglycosides are inhibitors of glycosidases and can be used as ligands for the purification of these enzymes by affinity chromatography[8–10,14,15]. We anticipated that 1,2-*trans*-*S*-interglycosidically-linked D-glucooligosaccharides might provide useful tools for cellulase purification if the enzymic affinity for such a substrate analog is sufficient.

The enzymic hydrolysis of 4-methylumbelliferyl β-lactoside by CBH I was studied in the absence (K_m 80μM) or in the presence of compounds **7**, **10**, and **18** at various concentrations (see Experimental section). The value reported herein is almost identical to that reported by Van Tilbeurg *et al.*[16] (K_m 52μM). Lineweaver–Burk double-reciprocal plots (Figs. 1A and 1B) indicated that **7** and **18** are competitive inhibitors with dissociation constants of 24 and 6.5μM, respectively. These results were promising for a biospecific adsorption of CBH I on a matrix bearing compound **6** or **16**. An unusual behavior was observed with the methylumbelliferyl derivative **10** (Fig. 1C), which was expected to be a specific substrate for CBH-I. In contrast to the corresponding cellobioside or lactoside, which are hydrolyzed at both C-1 and C-1′ bonds or only at the C-1 bond, respectively, the thio derivative **10** is a competitive inhibitor (K_i 67μM) like 4-nitrobenzyl 1-thio-β-D-lactoside (K_i 29μM)[16]. These results strongly suggested that the sulfur exocyclic atom induces a preferential, nonproductive binding in the active site of CBH I.

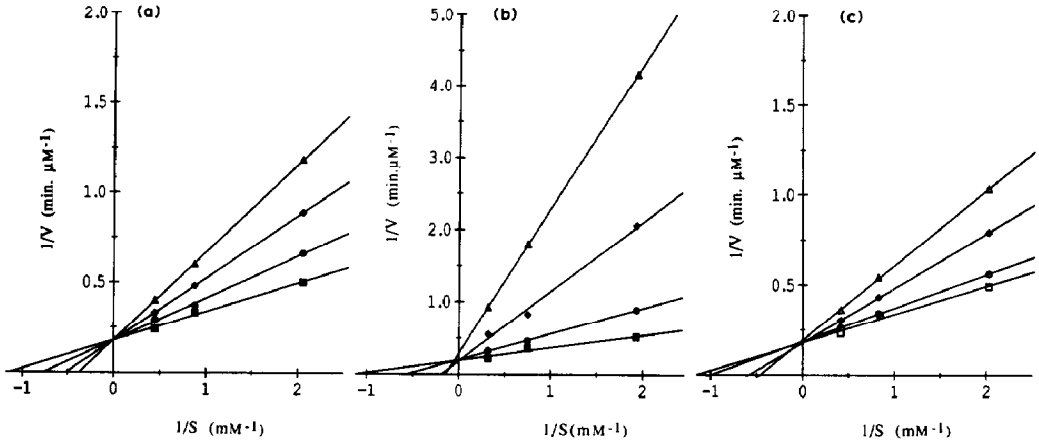


Fig. 1. Lineweaver–Burk plots for the hydrolysis of methylumbelliferyl lactoside catalyzed by CBH I in the presence or absence of inhibitors; the plotted values were the means of three different experiments: (A) (■) Without inhibitor; with (●) 7.5, (◆) 30, and (▲) 75μM of inhibitor **7**. (B) (■) Without inhibitor; with (●) 7.5, (◆) 30, and (▲) 75μM of inhibitor **18**. (C) (□) Without inhibitor; with (○) 12 (□) 60, and (△) 120μM of inhibitor **10**.

Efficiency of 4-aminophenyl 1,4-dithiocellobioside (**6**) *and 1,4,4'-trithiocellotrioside* (**16**) *as ligands for affinity chromatography.* — In a primary test, pure CBH I from *T. reesei* was applied to three differently substituted CH-Sepharose 4B gels. Aniline and 4-aminophenyl derivatives **6** and **16** were coupled to CH-Sepharose 4B as described in the Experimental section. Aniline was chosen as a nonbiospecific ligand for a "mock affinity gel". The possible adsorption attributable to nonbiospecific interactions with the hydrophobic spacer arm was first studied by loading pure CBH I (4 mg) onto the three gels (1 mL). CBH I was completely retained on these gels. However CBH I could be eluted only from Gel 1 by increasing the ionic strength of the buffer (sodium chloride 2M), and these results suggested that biospecific interactions are the main factors for the adsorption of this protein by Gels 2 and 3. Desorption from these gels was achieved by applying either 0.1M lactose or M urea in sodium acetate buffer containing M sodium chloride. Attempts to elute CBH I with D-glucose, lactose, cellobiose, or methylumbelliferyl lactoside in sodium acetate buffer without sodium chloride were unsuccessful. Desorbing agents could be removed either by precipitation of the protein with acetone or by postchromatography on Sepharose G-25 fine column. The recovery of proteins and of the enzyme activity was essentially total under these conditions.

A sample of the crude, commercially available enzyme, Celluclast™, was applied to a column of Gel 2 equilibrated in sodium acetate buffer; the elution profile is shown in Fig. 2A. After washing and desorption of nonspecifically bound enzymes by sodium acetate buffer (F1) and 2M sodium chloride in sodium acetate buffer (F2), a fraction was obtained by applying a sodium acetate buffer containing M sodium chloride and 0.1M lactose (F3). This fraction was submitted to SDS–poly(acrylamide) gel electrophoresis, and the protein was shown to correspond to one of the major proteins of the crude enzyme solution (67 kDa). However, minor impurities could be detected after increasing the amount of protein (13 to 67 μg) submitted to electrophoresis.

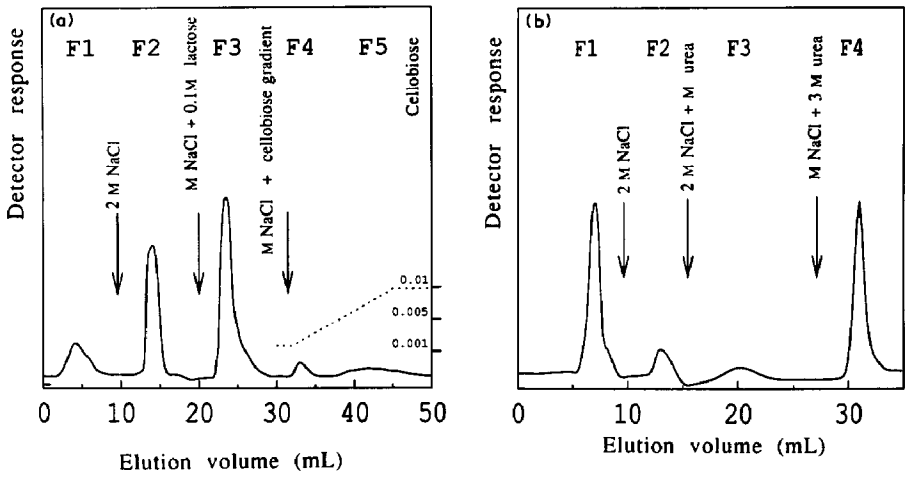


Fig. 2. Gel affinity chromatography: (A) Elution profile of Celluclast ™ obtained on Gel 2. (B) Resolution of the cellobiose fraction obtained from Gel 2 on Gel 3 (see Experimental section).

Another almost homogeneous protein (~ 47 kDa) was eluted when a sodium acetate buffer containing M sodium chloride and a gradient of cellobiose (1–10mM) was applied (F4 and F5). The activity tested on methylumbelliferyl lactoside[8] was mainly associated with the lactose fraction F3, whereas activity on bacterial microcrystalline cellulose[17] was found in both lactose and cellobiose fractions. From these results, it could be concluded that these fractions are mainly constituted of CBH I and CBH II, respectively. However, these proteins still possess residual cellulase activity on carboxymethylcellulose, indicative of incomplete purification (not shown). The lactose fraction (F3) was again applied onto the same column and eluted as described above. After this step of purification, the properties of CBH I were identical to those of an authentic sample of CBH I (no activity on carboxymethylcellulose, same activities on 4-methylumbelliferyl lactoside and bacterial microcrystalline cellulose[17]).

The final purification of CBH II was best achieved by chromatography on Gel 3 (Fig. 2B). A CBH II sample was applied to the trisaccharide column. Some proteins were eluted with the sodium acetate buffer containing 2M sodium chloride and 2M sodium chloride–M urea, whereas the sodium acetate buffer with M sodium chloride and 3M urea could desorb pure CBH II (F4). The specificities of this protein coincide with those reported earlier[17]. The usefulness of standard cellulase assays to monitor purification of this enzyme is limited, since its specific activity decreases during its purification; this may result from the synergistic action of cellulolytic enzymes[18]. We have already studied the synergy of various cellulases by use of several different ratios of two enzymes[17]. In the present work, bacterial microcrystalline cellulose was incubated with mixtures containing various ratios of enzymes purified in this work, and the resulting profile was identical to the one already reported[17].

EXPERIMENTAL

General methods. — Optical rotations were measured at 20° with a Perkin–Elmer polarimeter. The ^{1}H-n.m.r. spectra (Table I) were recorded at 250 MHz and the ^{13}C-n.m.r. spectra at 25.18 MHz. Assignments were confirmed by double irradiation. T.l.c. was performed on silica gel (Merck F 254, Merck, Darmstadt, Germany). Purification was achieved by column chromatography on Kieselgel 60 (70–230 mesh). "Usual work-up" means that, after dilution with dichloromethane, the organic phase was successively washed with ice-cold aqueous solutions of 10% $KHSO_4$, saturated $NaHCO_3$, and with ice-cold water. Aqueous washings were then reextracted with dichloromethane. Organic solutions were dried (Na_2SO_4) and evaporated under reduced pressure.

*Methyl 2,3,6-tri-*O*-benzoyl-4-*S*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-4-thio-α-*D*-glucopyranoside* (**1**). — 2,3,4,6-Tetra-*O*-acetyl-1-*S*-acetyl-β-D-glucopyranose[12] (5.2 g, 12.8 mmol) was dissolved in methanol (80 mL) containing (M) sodium methoxide (14 mL). After being kept overnight at room temperature, the solution was concentrated and the residue dried *in vacuo* in the presence of P_2O_5. It was then suspended in *N'*, *N''*, *N'''*-hexamethylphosphoramide (50 mL) containing methyl 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-α-D-galactopyranoside[13] (7.82 g, 15.5 mmol). After 4 h at room temperature and overnight acetylation with 1:1 (v/v) acetic anhydride–pyridine (40 mL), the usual extraction process and purification on a silica gel column with 5:5:1 (v/v) ethyl acetate–hexane–dichloromethane gave **1** (8.2 g, 75%), m.p. 185–188° (diethyl ether), $[\alpha]_D$ +62° (*c* 1.4,chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.5 (C-4), 81.4 (C-1'), and 97.3 (C-1); lit.[11] m.p. 194–195° (ethanol), $[\alpha]_D$ +60° (*c* 1, chloroform).

Anal. Calc. for $C_{42}H_{44}O_{17}S$: C, 59.15; H, 5.20; S, 3.70. Found: C, 59.17; H, 5.35; S, 3.75.

*1,2,3,6-Tetra-*O*-acetyl-4-*S*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-4-thio-α-*D*-glucopyranose* (**2**). — Acylated compound **1** (3.4 g, 4 mmol) was treated in methanol (200 mL) with M sodium methoxide (4.8 mL). After neutralization with Amberlite IR-120 (H^+) cation-exchange resin, filtration, and concentration to dryness, the residue was acetylated with 1:1 (v/v) pyridine–acetic anhydride (30 mL). After the usual workup, the crude compound was treated with 7:300:700 (v/v) H_2SO_4–acetic acid–acetic anhydride (147 mL) for 24 h at room temperature. After coevaporation with toluene in the presence of sodium acetate (4 g) and the usual workup, column chromatography (3:2, v/v, ethyl acetate–hexane) of the mixture gave **2**, contaminated with its β anomer. Crystallization from ether gave pure **2** (2.3 g, 83%), m.p. 175–177°, $[\alpha]_D$ +23° (*c* 0.9, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.1 (C-4), 82.4 (C-1'), and 89.6 (C-1).

Anal. Calc. for $C_{28}H_{38}O_{18}S$: C, 48.41; H, 5.51; S, 4.62. Found: C, 48.65; H. 5.25; S, 4.47.

4-S-β-D-Glucopyranosyl-4-thio-D-glucopyranose (4-thiocellobiose, **3**). — Peracetylated **2** (100 mg, 0.14 mmol) was treated in methanol (30 mL) with M sodium methoxide in methanol (80 μL). Neutralization and freeze-drying gave amorphous **3** (50 mg, 97%), $[\alpha]_D$ −11.5° (*c* 0.37, water); ^{13}C-n.m.r. (D_2O): δ 47.9 (C-4), 84.7 (C-1α), 93.0 (C-1α), and 96.5 (C-1β); lit. [12] $[\alpha]_D$ −16° (*c* 1, water).

Anal. Calc. for $C_{12}H_{22}O_{10}S \cdot 1.5\ H_2O$: C, 37.40; H, 6.49; S, 8.32. Found: C, 37.83; H, 6.37; S, 8.36.

4-Aminophenyl 2,3,6-tri-O-acetyl-4-S-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,4-dithio-β-D-glucopyranoside (**4**). — HBr in acetic acid (33%, 7 mL) was added to a cold solution of acetylated **2** (500 mg, 0.72 mmol) in dichloromethane (20 mL), and the mixture was stirred for 2 h at 0°. After the usual workup and evaporation, the crude bromide **5** was dissolved in acetone (20 mL) and a water solution (14 mL) of K_2CO_3 (440 mg, 3.2 mmol) and 4-aminobenzenethiol (400 mg, 3.2 mmol) were added. The mixture was stirred overnight at room temperature, and then evaporated. After the usual workup, column chromatography (1:1, v/v, ethyl acetate–hexane) of the residue gave **4** which crystallized from ether (450 mg, 67%), m.p. 104–106°, $[\alpha]_D$ −32° (*c* 1.17, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.3 (C-4), 81.5 (C-1′), and 86.4 (C-1).

Anal. Calc. for $C_{32}H_{41}O_{16}NS_2$: C, 50.58; H, 5.44; N, 1.84; S, 8.44. Found: C, 50.51; H, 5.34; N, 1.83; S, 8.26.

4-Aminophenyl 4-S-β-D-glucopyranosyl-1,4-dithio-β-D-glucopyranoside (**6**). — Compound **4** (200 mg) was deacetylated by treatment with sodium methoxide in methanol, as described for compound **3**, to yield **6** (110 mg, 90%), $[\alpha]_D$ −63° (*c* 0.82, water); ^{13}C-n.m.r. (D_2O): δ 47.7 (C-4), 84.5 (C-1′), and 88.6 (C-1).

Anal. Calc. for $C_{18}H_{27}O_9NS_2 \cdot 1.5\ H_2O$: C, 43.89; H, 6.14; N, 2.84; S, 13.02. Found: C, 43.83; H, 6.06; N, 2.81; S, 12.45.

4-Acetamidophenyl 4-S-β-D-glucopyranosyl-1,4-dithio-β-D-glucopyranoside (**7**). — A solution of **4** (500 mg, 0.65 mmol) in 1:1 (v/v) pyridine–acetic anhydride (2 mL) was stirred overnight. Evaporation and column chromatography (5:1, v/v, ethyl acetate–hexane) of the residue yielded the peracetylated derivative **8** (510 mg). Catalytic *O*-deacetylation afforded **7** (300 mg, 90%), $[\alpha]_D$ −38.5° (*c*, 0.44 water); ^{13}C-n.m.r. (D_2O): δ 47.7 (C-4), 84.6 (C-1′), and 88.0 (C-1).

Anal. Calc. for $C_{20}H_{29}O_{10}NS_2$: C, 44.18; H, 6.17; N, 2.76; S, 11.24. Found: C, 44.05; H, 5.85; N, 2.52; S, 11.64.

4-Methylumbelliferyl 2,3,6-tri-O-acetyl-4-S-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-4-thio-β-D-glucopyranoside (**9**). — K_2CO_3 (1.5 g, 10.8 mmol) and 4-methylumbelliferone (1.9 g, 10 mmol) in acetone (45 mL) were added to a solution of the crude bromide **5** (1 g, 1.44 mmol) in acetone (4.5 mL). The mixture was stirred at room temperature for 15 h, and treated and purified as described for the preparation of **4** to yield **9** (550 mg, 46%), which crystallized from ethanol, m.p. 210°, $[\alpha]_D$ −33.5° (*c*, 0.83 chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.2 (C-4), 81.7 (C-1′), and 103.9 (C-1).

Anal. Calc. for $C_{36}H_{42}O_{19}S$: C, 53.26; H, 5.34; S, 3.95. Found: C, 53.35; H, 5.19; S, 4.04.

4-Methylumbelliferyl 4-S-β-D-glucopyranosyl-4-thio-β-D-glucopyranoside (**10**). — Catalytic *O*-deacetylation of **9** (300 mg) gave **10** (170 mg, 89%), m.p. 240° (methanol), $[\alpha]_D$ −50.5°(*c* 0.11, pyridine); ^{13}C-n.m.r. (C_5D_5N): δ 45.5 (C-4), 85.6 (C-1′), and 104.2 (C-1).

Anal. Calc. for $C_{22}H_{28}O_{12}S$: C, 51.15; H, 5.46; S, 6.21. Found: C, 51.28; H, 5.53; S, 6.17.

2,3,6-Tri-O-*acetyl-1*-S-*acetyl-4*-S-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*glucopyranosyl-1,4-dithio-β*-D-*glucopyranose* (**11**). — Crude bromide **5** obtained from **2** (3 g, 4.3 mmol) was treated with tetrabutylammonium thioacetate in toluene as described for the thiomaltose series[13]. Column chromatography in ether afforded pure **11** (1.9 g, 63%), $[\alpha]_D$ − 37° (*c* 1.0, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.3 (C-4), 80.0 (C-1), and 81.2 (C-1′).

Anal. Calc. for $C_{28}H_{38}O_{17}S_2$: C, 47.35; H, 5.39; S, 9.03. Found: C, 47.00; H, 5.60; S, 8.66.

Methyl S-*(2,3,4,6*-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-*benzoyl-4-thio-α*-D-*glucopyranoside* (**12**). — Methanolic M sodium methoxide (4 mL) was added to a solution of **11** (1.9 g, 2.7 mmol) in methanol (95 mL). The mixture was stirred overnight at room temperature under N_2, evaporated, coevaporated with toluene, and dried *in vacuo* in the presence of P_2O_5. The resulting salt was added as a powder to a solution of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-α-D-galactopyranoside[13] (2.7 g, 5.3 mmol) in *N′, N″, N‴*-hexamethylphosphoramide (10 mL). The mixture was stirred for 20 h at room temperature, and then treated as described for the preparation of **1**. The residue was purified by chromatography (3:2, v/v, ethyl acetate–hexane) to yield **12** (1.7 g, 58%), $[\alpha]_D$ + 8.4° (*c* 0.95, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.4 (C-4, 4′), 80.9, 82.0 (C-1, 1″), and 97.4 (C-1).

Anal. Calc. for $C_{54}H_{60}O_{24}S_2$: C, 56.04; H, 5.22; S, 5.54. Found: C, 55.92; H, 5.37; S, 5.60.

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)-2,3,6-tri*-O-*acetyl-4-thio-α*-D-*glucopyranoside* (**13**). — *O*-Deacylation and *O*-acetylation of **12** (1 g, 0.86 mmol), as described for **1**, led to a residue that gave **13** (0.5 g, 60%) after purification by chromatography (3:1, v/v, ethyl acetate–hexane); compound **13** crystallized from ether, m.p. 118–121°, $[\alpha]_D$ − 9.5° (*c* 0.94, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.2 (C-4, 4′), 81.2, 81.9 (C-1′, 1″), and 97.1 (C-1).

Anal. Calc. for $C_{39}H_{59}O_{24}S_2$: C, 48.24; H, 5.60; S, 6.60. Found: C, 48.38; H, 5.77; S, 6.49.

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)-1,2,3,6-tetra*-O-*acetyl-4-thio-β*-D-*glucopyranose* (**14**). — Acetolysis of **13** (0.5 g), as described for the preparation of **2**, gave **14** (0.48 g, 94%; 10:1 α-to-β anomeric mixture by ^{1}H-n.m.r.), $[\alpha]_D$ − 14.5° (*c* 0.83, chloroform); ^{13}C-n.m.r. ($CDCl_3$: δ 46.0, 46.4 (C-4,4′), 81.8, 82.2 (C-1′,1″), 89.5 (C-1α), and 91.5 (C-1β).

Anal. Calc. for $C_{40}H_{54}O_{25}S_2$: C, 48.09; H, 5.45; S, 6.41. Found: C, 47.96; H, 5.40; S, 6.36.

4-Aminophenyl S-*(2,3,4,6-tetra*-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-1,4-dithio-β*-D-*glucopyranoside* (**15**). — Compound **14** (0.7 g, 0.7 mmol) was treated in the usual manner with HBr in acetic acid and then with 4-aminobenzenethiol and K_2CO_3 as described for the preparation of **2**. Column chromatography (5:2, v/v, ethyl acetate–hexane) of the residue gave amorphous **15** (540 mg, 70%), $[\alpha]_D$ − 44° (*c* 1.24, chloroform); ^{13}C-n.m.r. ($CDCl_3$): δ 46.2 (C-4,4′), 80.6, 81.4 (C-1′,1″), and 86.2 (C-1).

Anal. Calc. for $C_{44}H_{57}O_{23}NS_3$: C, 49.66; H, 5.39; N, 1.31; S, 9.04. Found: C, 49.43; H, 5.54; N, 1.33; S, 9.11.

4-Aminophenyl S-*β*-D-*glucopyranosyl-(1→4)*-S-*4-thio-β*-D-*glucopyranosyl-(1→4)-1,4-dithio-β*-D-*glucopyranoside* (**16**). — Catalytic *O*-deacetylation of **15** (4.70 mg, 0.44 mmol) gave **16** in quantitative yield (260 mg, 91%), $[\alpha]_D$ −65° (*c* 0.75, water); ^{13}C-n.m.r. (D_2O): *δ* 47.7 (C-4,4'), 84.3, 84.6 (C-1',1''), and 88.6 (C-1).

Anal. Calc. for $C_{24}H_{37}O_{13}NS_3$: C, 44.77; H, 5.79; N, 2.17; S, 14.90. Found: C, 44.84; H, 5.72; N, 2.07; S, 14.76.

4-Acetamidophenyl 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-1,4-dithio-β*-D-*glucopyranoside* (**17**). — Compound **16** (102 mg, 0.16 mmol) was treated in the usual manner with acetic anhydride and pyridine to give **17** (159 mg, 87%) after workup and chromatography of the residue (5:2, v/v, ethyl acetate–hexane), $[\alpha]_D$ −35° (*c* 0.76, chloroform); ^{13}C-n.m.r. ($CDCl_3$): *δ* 46.0 (C-4,4'), 80.7, 81.3 (C-1',1''), and 85.7 (C-1).

Anal. Calc. for $C_{46}H_{59}O_{24}NS_3$: C, 49.94; H, 5.37; N, 1.26; S, 8.69. Found: C, 50.82; H, 5.70; N, 1.21; S, 8.45.

4-Acetamidophenyl S-*β*-D-*glucopyranosyl-(1→4)*-S-*4-thio-β*-D-*glucopyranosyl-(1→4)-1,4-dithio-β*-D-*glucopyranoside* (**18**). — *O*-Deacetylation of **17** (100 mg, 0.09 mmol), as described for the preparation of **8**, gave **18** (40 mg, 96%), $[\alpha]_D$ −40° (*c* 0.2, water); ^{13}C-n.m.r. (C_5D_5N): *δ* 44.6 (C-4,4'), 85.3, 85.7 (C-1',1''), and 89.8 (C-1).

Anal. Calc. for $C_{26}H_{39}O_{14}NS_3$: C, 45.53; H, 5.73; N, 2.04; S, 14.02. Found: C, 45.82; H, 5.81; N, 2.01; S, 13.3.

Enzymes and enzymic assays. — The cellulolytic enzymes purified in this work were a commercial preparation from *Trichoderma reesei* (Celluclast™, Novo), which was diluted with water and then precipited with cold acetone and freeze-dried. Pure CBH-I used for comparison was a gift of Dr. M. Schülein from Novo-Nordisk. Protein determination and SDS–poly(acrylamide) gel electrophoresis were performed as previously decribed.[17]

Activities on carboxymethylcellulose were measured by a standard procedure using 3,5-dinitrosalicylic acid reagent[19] to estimate the amount of reducing sugars liberated, substrate (10 mg) and enzymes (10 or 20 μg) in 50 mM sodium acetate buffer (pH 4.8, 2 mL) were incubated for 30 min at 50°. The reaction was stopped by the addition of dinitrosalicylic acid reagent (3 mL). The hydrolysis and synergistic degradation of bacterial microcrystalline cellulose were estimated at various dilutions of CBH I, CBH II, or CX II, as previously described[17] but with a final enzyme concentration of 10μg/mL. The hydrolysis of 4-methylumbelliferyl lactoside was monitored[16] at A_{385} in the absence or in the presence of inhibitors **7** or **18** each at three different concentrations (7.5, 30, and 75μM), and inhibitor **10** (at concentrations of 12, 60, and 120μM).

Preparation and use of affinity columns. — Aniline or the 4-aminophenyl derivatives **6** and **16** (100 mg) were coupled to CH-Sepharose 4B (1.25 g; Pharmacia, Sweden) in the presence of hydrosoluble carbodiimide (150 mg) as recommended by Pharmacia. The residual carboxyl residues were then blocked by reaction with a 5mM tris(hydroxymethyl)aminomethane solution (2.5 mL). The resulting gels (Gels 1, 2, and 3) can be stored and used at 4° without any loss of activity for several years.

A solution of proteins (5 mg) in 10mM sodium acetate buffer, pH 4.8 (200μL) was deposited on a column packed with the appropriate gel (5 μL). Stepwise elution was monitored at A_{280} with an Avicor S spectrophotometer (LKB) and analyzed by SDS–Page electrophoresis. From Gel 2, components of crude, precipitated Celluclast™ were eluted and analyzed. Firstly, proteins (0.24 mg) were eluted with the sodium acetate buffer (12 mL), and nonspecifically adsorbed proteins (0.47 mg) were eluted with the same buffer containing 2M NaCl (8 mL). Desorption of specifically bound proteins (3.2 mg) was achieved by applying the sodium acetate buffer (12 mL) containing both M NaCl and 0.1M lactose, and this lactose fraction was analyzed. Another thightly bound protein fraction (0.4 mg) was eluted with a gradient of 1–10mM cellobiose in sodium acetate buffer containing M NaCl. This cellobiose fraction was also analyzed, and the proteins obtained from two cellobiose elutions were further purified on Gel 3. After this gel had been washed with 2M NaCl and M urea–2M NaCl in sodium acetate buffer, the main protein fraction (560 μg) was eluted with 3M urea–M NaCl in sodium acetate buffer. After each experiment the column was washed with 6M urea in sodium acetate buffer and then reequilibrated in sodium acetate buffer.

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