PERKIN 1

Enzymic synthesis of hemithiocellodextrins

Vincent Moreau and Hugues Driguez *,†

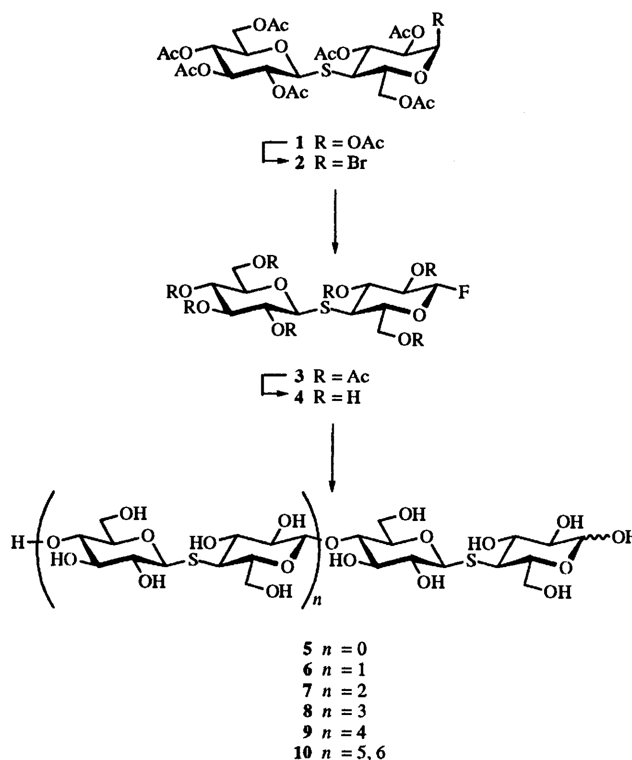
Centre de Recherches sur les Macromolécules Végétales, CNRS,‡ BP 53, 38041 Grenoble Cedex 9, France

A commercial cellulase mixture was used for the enzymic oligomerisation of 4-thio-β-cellobiosyl fluoride 4 in a buffer/organic solvent system and led to the formation of water-soluble hemithiocellodextrins 6–10 with DP 4-14.

Introduction

Owing to the complex architecture of cellulose, the most abundant polysaccharide on Earth, the use of substrate analogues has been a rewarding approach for studies on the mechanism of recognition and action of cellulolytic enzymes.

Most previously studied artificial substrates were hydrolysed at one or several positions depending mainly on their length, but also on the specificity of the cellulase used.[1] We have been involved for several years in the syntheses and uses of substrate analogues which can form reversible enzyme–inhibitor complexes: the thiooligosaccharides in which sulfur atom(s) replace the interglycosidic oxygen(s). The stepwise synthesis in the *S*-series of cello-trio-, -tetra- and -penta-osides was achieved in excellent yields.[2,3] We have shown that these compounds can be used as ligands for affinity chromatography[2] or for the mapping of the active site of cellulases of various families.[3] It was also reported[2] that 1- and 4-thiocellobiosides which possess only a normal glycosidic linkage prone to cleavage either between the sugar residues or at the aglyconic position, respectively, were not hydrolysed by the cellulase cellobiohydrolase I (CBHI) from *Trichoderma reesei.* The preparation of the title compounds was undertaken to elucidate whether this preferential binding in the active site of CBHI, an enzyme which may act on dimers, can be found in other cellulases which require higher oligosaccharides for recognition.



Scheme 1

Results and discussion

Hemithiocellodextrins can be considered as 4-thiocellobiosyl repeating units linked by β(1→4) oxygen linkages, or as cellobiosyl repeating units linked by sulfur bridges. Several laboratories have attempted to prepare cellodextrins following a stepwise chemical synthesis, but the low reactivity of the equatorial 4-OH of the glucopyranosyl residue requires more powerful acceptors such as derivatives of 1,6-anhydro-β-D-glycopyranose[4,5] or 2,3,6-tri-*O*-benzyl-glucosides.[6] These compounds are difficult to obtain, and the extension of this approach using 4-thiocellobiosyl units as donor and acceptor molecules was not explored. Therefore, we have investigated alternative procedures based on enzymic catalysis. The chemoenzymic route using cellulases appears to be the most promising approach for the synthesis of the former type of thiooligosaccharides described above.

Following the pioneering work of Hehre and co-workers who showed that maltooligosaccharides were obtained by transglycosylation of α-maltosyl fluoride using α-amylases,[7] Kobayashi *et al.* have recently reported the synthesis of cellodextrins from β-cellobiosyl fluoride and cellulases in hydroorganic solution.[8–10] These authors have also shown[10] that cellulases can catalyse the transfer of β-lactosyl fluoride to various acceptors. Based on our previous studies in which we reported that 4-thio-α-maltosyl fluoride may act as a donor and an acceptor in the enzymic reaction catalysed by CGTase,[11] we decided to investigate the synthesis of hemithiocellodextrins using cellulases and 4-thio-β-cellobiosyl fluoride **4** as glycosyl donor and acceptor.

The acetylated compound **3** was obtained by conventional synthesis from the corresponding acetylated α-bromide **2** prepared from peracetylated 4-thiocellobiose **1** (Scheme 1).[2] After conventional de-*O*-acetylation of compound **3**, a solution of unblocked fluoride **4** in a mixture of acetonitrile and buffer was treated with cellulases from *Trichoderma veride* in buffer to obtain a final CH_3CN/buffer ratio of 5:1. The course of the reaction was monitored by high-performance liquid chromatography (HPLC) using a μ-Bondapak NH_2 column. After the mixture had been stirred for 12 h at 30 °C, the enzymes were inactivated by boiling and were eliminated by filtration of the warm water suspension obtained after evaporation off of the acetonitrile.

To the filtrate was added methanol; the precipitate was filtered off, dissolved in warm water and freeze-dried (F1). The

† Fax number: (33) 76-54-72-03. E-mail: hdriguez@cermav.grenet.fr
‡ Affiliated with the Université J. Fourier, Grenoble.

filtrate was evaporated; the residue was dissolved in water and also freeze-dried (F2). These two fractions were analysed by gel permeation chromatography (GPC) and HPLC. F2 consisted of a mixture of hemithiocellodextrins DP4–14, and F1 contained the higher oligosaccharides DP8–20. The hemithiocellodextrins from F2 were separated using size-exclusion chromatography (SEC) on a P2 biogel column with water as eluent.

The structure of these compounds has been confirmed by ^{13}C NMR spectroscopy. The signals at δ_C ~85 and ~48, respectively, are assigned to C-1 and C-4 carbon atoms involved in the thioglycosidic linkages. The signal at δ_C ~ 103 is ascribed to C-1 carbon atoms linked through β(1→4) oxygen bonds to the next glycosyl unit. Signals at δ_C ~62 are due to C-6 carbon atoms which are not involved in interglycosidic linkages, which should give rise to C-6 atoms deshielded to 67–70.[12]

It is worthy of note that hemithiocellodextrins up to DP20 are water-soluble since it is known that cellooctaose is almost completely water-insoluble.[13] This insolubility of natural cellodextrins and celluloses I and II was attributed to intra- and inter-hydrogen-bond networks which stabilise the overall architecture of these compounds. The crystal structure of β-cellotetraose, which has been recently solved, demonstrates unambiguously the tight network of intramolecular hydrogen bonds between O-3--O-5′ and O-2--O-6′ and intermolecular bonds which connect molecules into two adjacent chains.[14,15] Since it has been recently proposed that the interruption of H-bonds may introduce a distortion in the packing of the chains of oligo- and poly-saccharides,[16] it is highly possible that the water solubility of hemithiocellodextrins is due to such a phenomenon. In the hemithiocellodextrin series there may be two reasons for an 'unusual' packing: (i) a weaker exo-anomeric effect is expected, so these compounds are more flexible;[17,18] (ii) the C–S bonds are longer than their corresponding C–O bonds,[19] so the ideal distances for the establishment of strong hydrogen bonds in two successive residues cannot occur. The behaviour of these compounds in enzymic media will be described in due course.

Experimental

NMR spectra were recorded in D_2O (external reference) with a Bruker 300 AC spectrometer. Mass measurements were performed on a Nermag R-1010C spectrometer in the fast-atom bombardment (FAB) mode. Optical rotations were measured with a Perkin-Elmer 241 polarimeter and $[\alpha]_D$-units are recorded in units of 10^{-1} deg cm^2 g^{-1}. Mps were measured on a Büchi 535 apparatus. Microanalyses were performed by the Microanalytical laboratory of the CNRS (Vernaison). For flash chromatography, Merck Silica Gel 60 (230–400 mesh) was used. Light petroleum refers to the 60–80 °C fraction. The course of the enzymic reactions was monitored by HPLC on an analytic μ-Bondapak NH_2 column (Waters, Milford, MA, USA) with MeCN–water (70:30) as the eluent. The separation of various hemithiocellodextrins was achieved on a Biogel P2 column (ϕ 1.5 × 210 cm) with water at 60 °C as eluent.

Cellulase Onozuka R-10 from *T. viride* was a gift from Yakult, Tokyo (Japan).

2,3,6-Tri-*O*-acetyl-4-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-4-thio-β-D-glucopyranosyl fluoride 3
Crude 4-thiocellobiosyl bromide **2** was prepared from peracetylated 4-thiocellobiose **1** (1.65 g, 2.3 mmol) as already described[2] and without purification was treated with silver fluoride (2.23 g, 17.6 mmol) in dry acetonitrile (112 cm^3). After 1.5 h at room temperature, the reaction mixture was filtered through a Celite bed. The residue obtained upon concentration of the filtrate was subjected to flash chromatography [ethyl acetate–light petroleum (1:1)]. The fluoride **3** was obtained (1.42 g, 91%), mp 188 °C (from diethyl ether) (Found: C, 47.6; H, 5.4; F, 2.4; S, 4.3. $C_{19}H_{35}FO_{16}S$ requires C, 47.70; H, 5.39; F, 2.90; S, 4.90%); $[\alpha]_D$ −31 (*c* 0.74, $CHCl_3$); $\delta_C(CDCl_3)$ 170.23, 169.90, 169.29, 169.17 and 169.10 (CO), 107.80 and 104.91 (C-1), 81.79 (C-1^2), 75.77, 74.21, 73.54, 72.56, 72.20, 70.11, 69.35, 69.23 and 68.15 (C-2, -2^2, -3, -3^2, -4^2, -5 and 5^2), 63.16 and 62.19 (C-6 and -6^2), 44.99 (C-4) and 20.68, 20.51, 20.44 and 20.37 (CH_3).

4-*S*-β-D-Glucopyranosyl-4-thio-β-D-glucopyranosyl fluoride 4
Compound **3** (1.26 g, 1.92 mmol) was deacetylated by treatment with sodium methoxide (1 mol dm^{-3} in methanol; 1 cm^3) in methanol (230 cm^3) for 3.5 h at 0 °C. The mixture was neutralised with Amberlite IRN 77 (H^+) resin, the resin was removed by filtration, and the filtrate was concentrated. The residue (696 mg, 100%) was characterised by its MS spectrum: *m/z* 359 $[M + H]^+$ and 341 $[M - F + H]^+$.

Enzymic synthesis of hemithiocellodextrins 5–10
Fluoride **4** (696 mg, 1.9 mmol) was dissolved in a mixture of acetonitrile (66 cm^3) and acetate buffer (pH 5.0; 50 mmol dm^{-3}, 11 cm^3), then cellulases (51 mg) in the same buffer (2 cm^3) were added, and the mixture was stirred at 30 °C for 12 h. The reaction mixture was heated to 100 °C for 10 min, then was evaporated to dryness. The residue was suspended in warm water (10 cm^3; 60 °C) and the proteins were eliminated by filtration. Methanol (50 cm^3) was added to the filtrate, and the precipitate (F1) was separated from the filtrate, which was evaporated (F2). F1 and F2 were dissolved in warm water and freeze-dried. F1 (300 mg, 41%) consisted of DP 8–20 and in F2 (400 mg, 57%) were found DP 2–14. The hemithiocellodextrins of F2 were separated using SEC on a Biogel P2 column with water as eluent. Pure oligomers were eluted in this reverse order:

4-Thiocellobiose 5. (74 mg, 10.5% Yield) identified by comparison to an authentic sample.[2]

4′-*O*-[4-Thiocellobiosyl]-β-thiocellobiose 6. (30 mg, 4.5% Yield) (Found: C, 39.2; H, 6.3; S, 8.3. $C_{24}H_{42}O_{19}S_2 \cdot 2H_2O$ requires C, 39.20; H, 6.20; S, 8.70%); *m/z* 699 $[M + H]^+$; $[\alpha]_D$ −22 (*c* 0.5, water); $\delta_C(D_2O)$ 103.68 (C-1^3), 97.09 (C-1α), 93.57 (C-1β), 85.25 (C-1^4), 85.15 and 85.11 (C-1^{2}α,β), 81.24, 80.00, 79.66, 78.52, 77.98, 77.85, 77.04, 76.84, 75.84, 74.35, 74.11, 73.74, 73.15 and 70.84 (C-2, -2^2, -2^3, -2^4, -3, -3^2, -3^3, -3^4, -4^2, -4^4, -5, -5^2, -5^3 and -5^4), 62.88, 62.77, 62.25 and 61.54 (C-6, -6^2, -6^3 and -6^4) and 48.57 and 48.39 (C-4 and -4^3).

Compound 7. (50 mg, 7.5% Yield) (Found: C, 35.9; H, 5.6; S, 7.6. $C_{36}H_{62}O_{28}S_3 \cdot 9H_2O$ requires C, 35.95; H, 6.71; S, 8.00%); $[\alpha]_D$ −41 (*c* 0.5, water); $\delta_C(D_2O)$ 103.69 (C-1^3 and -1^5), 97.09 (C-1β), 93.56 (C-1α), 85.25 and 85.10 (C-1^2, -1^4 and -1^6), 81.24, 80.00, 79.66, 78.52, 77.98, 77.86, 77.03, 76.84, 75.84, 74.10, 73.88, 73.73, 73.14 and 70.84 (C-2, -2^2, -2^3, -2^4, -2^5, -2^6, -3, -3^2, -3^3, -3^4, -3^5, -3^6, -4^2, -4^4, -4^6, -5, -5^2, -5^3, -5^4, -5^5 and -5^6), 62.8, 62.76, 62.25 and 61.55 (C-6, -6^2, -6^3, -6^4, -6^5, -6^6) and 48.58 and 48.39 (C-4, -4^3 and -4^5).

Compound 8. (38 mg, 5.7% Yield) (Found: C, 38.3; H, 6.2; S, 8.3. $C_{48}H_{82}O_{37}S_4 \cdot 7H_2O$ requires C, 38.29; H, 6.43; S, 8.52%); *m/z* 1379 $[M + H]^+$; $[\alpha]_D$ −64 (*c* 0.5, water); $\delta_C(D_2O)$ 103.69 (C-1^3, -1^5 and -1^7), 97.09 (C-1β), 93.68 (C-1α), 85.25 and 85.1 (C-1^2, -1^4, -1^6 and -1^8), 81.24, 80.00, 79.66, 78.52, 77.95, 77.85, 77.03, 76.84, 75.84, 74.36, 74.10, 73.88, 73.73, 73.15 and 70.84 (C-2, -2^2, -2^3, -2^4, -2^5, -2^6, -2^7, -2^8, -3, -3^2, -3^3, -3^4, -3^5, -3^6, -3^7, -3^8, -4^2, -4^4, -4^6, -4^8, -5, -5^2, -5^3, -5^4, -5^5, -5^6, -5^7 and -5^8), 62.74, 62.25 and 61.55 (C-6, -6^2, -6^3, -6^4, -6^5, -6^6, -6^7 and -6^8) and 48.57 and 48.38 (C-4, -4^3, -4^5 and -4^7).

Compound 9. (34 mg, 5.0% Yield) (Found: C, 37.7; H, 6.15; S, 8.4. $C_{60}H_{102}O_{46}S_5 \cdot 11H_2O$ requires C, 37.57; H, 6.52; S, 8.36%); *m/z* 1741 $[M - OH + K]^+$; $[\alpha]_D$ −48 (*c* 0.48, water); $\delta_C(D_2O)$ 103.69 (C-1^3, -1^5, -1^7 and -1^9), 97.09 (C-1β), 93.57 (C-1α), 85.25 and 85.09 (C-1^2, -1^4, -1^6, -1^8 and -1^{10}), 81.24, 80.01, 79.66, 78.52, 77.95, 77.03, 76.84, 75.84, 74.35, 74.09, 73.88, 73.73, 73.15 and 70.84 (C-2, -2^2, -2^3, -2^4, -2^5, -2^6, -2^7, -2^8, -2^9, -2^{10}, -3, -3^2, -3^3, -3^4, -3^5, -3^6, -3^7, -3^8, -3^9, -3^{10}, -4^2, -4^4, -4^6, -4^8,

-4[10], -5, -5[2], -5[3], -5[4], -5[5], -5[6], -5[7], -5[8], -5[9] and -5[10]), 62.74, 62.26 and 61.55 (C-6, -6[2], -6[3], -6[4], -6[5], -6[6], -6[7], -6[8], -6[9] and -6[10]) and 48.57 and 48.38 (C-4, -4[3], -4[5], -4[7] and -4[9]).

Compound 10. (80 mg, 20% Yield) identified as a mixture of DP 12–14 by analytical HPLC; $\delta_C(D_2O)$ 103.70 (C-1[3], -1[5], -1[7], -1[9], -1[11] and -1[13]), 85.09 (C-1[4], -1[6], -1[8], -1[10] and -1[12]), 79.99, 79.68, 77.97, 77.08, 75.87, 74.14 and 73.17 (C^{2-10}, -3^{2-10}, -4[2], -4[4], -4[6], -4[8], -4[10] and -5^{2-10}), 62.79 and 61.60 ($C\text{-}6^{2-10}$) and 48.39 (C-4[3], -4[5], -4[7], -4[9] and -4[11]).

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