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Design and Chemoenzymatic Synthesis of Thiooligosaccharide Inhibitors of 1,3:1,4-β-D-Glucanases

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Abstract—A successful chemoenzymatic synthesis of oligosaccharides with an interglucosidic sulfur atom as inhibitors of 1,3:1,4-β-D-glucanases is described. The key compound **3a** was synthesized from acetylated 1-thio-β-laminaribiose **4** and the methyl 4′-*O*-triflyl-lactoside **5**. After de-*O*-acylation, the tetrasaccharide **3b** was used as an acceptor and glucose-1-P as a donor in a phosphorolytic elongation catalysed by cellodextrin phosphorylase from *Clostridium thermocellum*. The expected pentasaccharide **2a** and hexasaccharide **1** were isolated in 56% and 13% yield, respectively. As expected, the thiooligosaccharides **1**, **2a**, and **3b** were resistant to enzymatic cleavage by 1,3:1,4-β-D-glucanase isolated from *Bacillus licheniformis*. Furthermore, they have been shown to act as competitive inhibitors of the hydrolysis of the chromophoric trisaccharide substrate **11** by this enzyme.

Introduction

The major polysaccharide of endosperm cell walls of barley is 1,3:1,4-β-D-glucan, which is mainly formed of cellotetraosyl units linked through β 1→3 bonds.[1] The enzymatic hydrolysis of this polysaccharide is an early event in the germination process. It can be achieved by endogenous enzymes with three different specificities: 1,4-β-glucan 4-glucanohydrolase (EC 3.2.1.4), 1,3-β-glucan 3-glucanohydrolase (EC 3.2.1.39) and 1,3:1,4-β-glucan 4-glucanohydrolase (EC 3.2.1.73). The latter is the most efficient and has a strict specificity: it cleaves 1,4-β-linkages of laminaribiosyl units,[2,3] as do the microbial 1,3:1,4-β-glucanases.[4] The specificity of these enzymes and the deduced minimum binding requirements have been inferred from the structure of the final products of barley β-glucan, 3-*O*-β-cellobiosyl-D-glucopyranose and 3-*O*-β-cellotriosyl-D-glucopyranose.[4]

Recent progress on the structure–function relationships in plant and microbial 1,3:1,4-β-glucanases include: identification of the catalytic residues by site-directed mutagenesis[5] or specific labeling,[6] determination of the tertiary structure of a covalent enzyme–inhibitor complex,[7] as well as the use of synthetic fluorogenic oligosaccharides.[8] However, all these studies, provided only little information on the amino acids involved in the binding of the substrate in the various subsites, in particular the ones of the acceptor part of the active site.

The aim of this paper is to report the design and the chemoenzymatic synthesis of inhibitors for biochemical and X-ray studies of these β-glucanases.

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Results and Discussion

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 scissile oxygen(s).[9] From the early results it was known that 1,3:1,4-β-glucanases hydrolyse only the glyconic bond of laminaribiosyl residues,[3,4] so we decided to introduce one thiolinkage between the laminaribiosyl unit and the C-4′ of methyl β-cellobioside. Furthermore, recent docking experiments of a hexasaccharide into the postulated active site of the *Bacillus licheniformis* enzyme,[7,10] and kinetic parameters obtained from synthetic substrates of various lengths suggested that the donor part of the active site is formed by four subsites for glucosyl units.[11] For these reasons the synthesis of the hexasaccharide **1** was undertaken.

After examination of possible retrosynthetic pathways, we have selected, for the reasons described thereafter, the chemoenzymatic approach presented in Figure 1.

We have recently reinvestigated the transglucosylation reactions catalysed by cellodextrin phosphorylase from *Clostridium thermocellum* by using a series of cellobiosyl residues as glycosyl acceptors, and glucose-1-P as glucosyl donor.[12] We were also interested to know whether this enzyme could accommodate into its active site a laminaribiosyl unit and elongate this acceptor to afford compounds **2** and **1** starting from the acceptor **3b**. We had also in mind an alternative approach using cellulases in hydroorganic medium for the transglycosylation of β-cellobiosyl or β-lactosyl fluorides onto the acceptor **3b**, under conditions already reported by us and others.[13–15] The synthesis of the fully protected tetrasaccharide **3a** should be achieved by thioglycosylation involving the two disaccharides **4** and **5**. It was

obvious that the starting molecule for the preparation of **4** was the octa-*O*-acetyl laminaribiose **6**.

We decided to survey the available methods for the preparation of this acetylated disaccharide. It has been obtained by acetolysis of its corresponding methyl α-glycoside resulting from the condensation of two glucosyl derivatives. Under the best conditions, the overall yield did not exceed 45–55%, starting from the protected glucosyl acceptor methyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside.[16] However, laminaribiose octaacetate was also obtained in ~30%

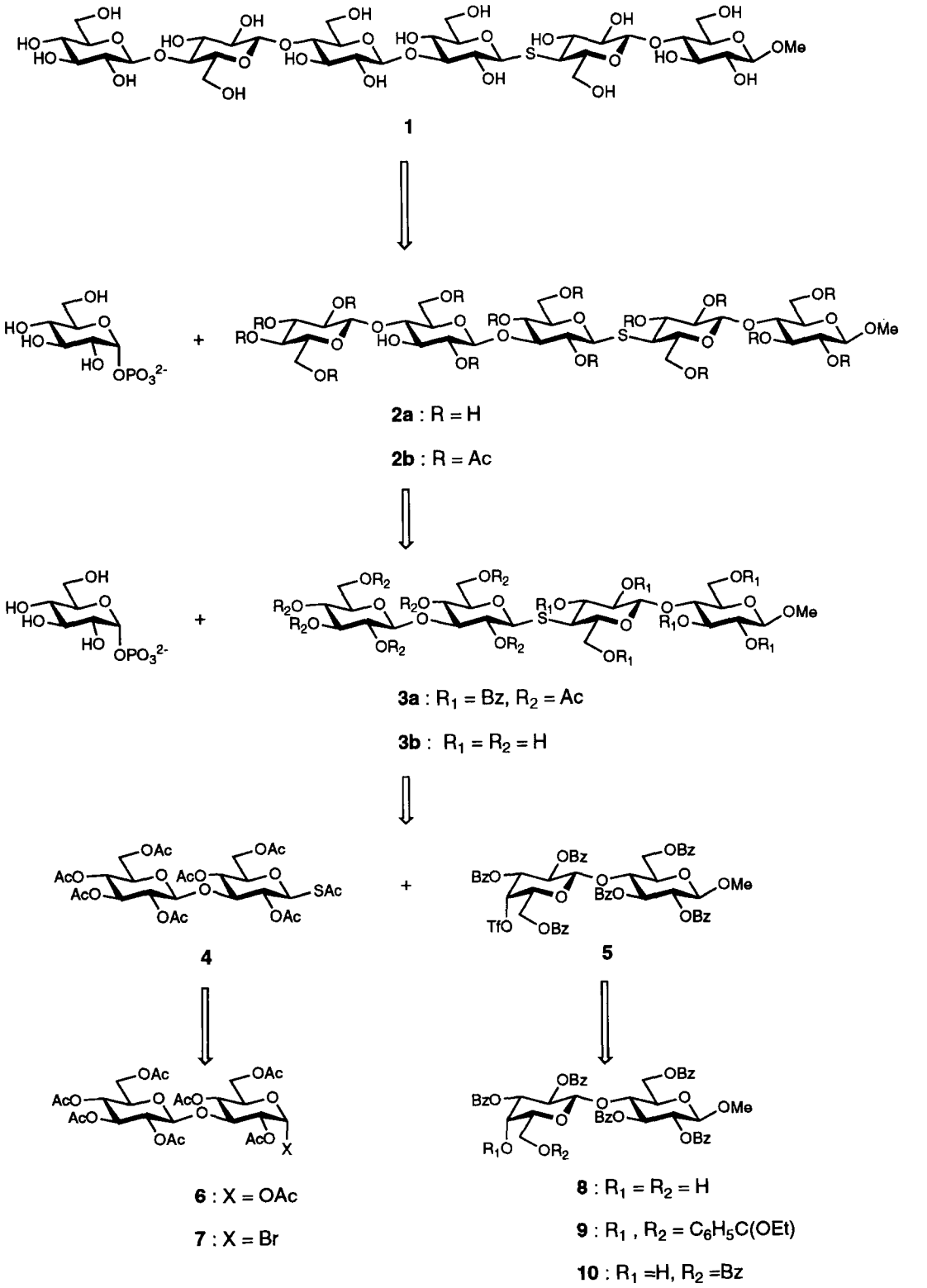


Figure 1. Retrosynthetic pathway for the synthesis of compounds **1**, **2a** and **3b**.

yield by acetolysis of curdlan,[17] laminaran, and pachyman,[18] or after acetylation of the oligosaccharides obtained either by enzymatic hydrolysis of curdlan (50% yield)[17] or acid hydrolysis of laminaran[19] and pachyman[20] (~10% yield). After this survey we thought that the chemical syntheses were time-consuming and the acetolysis of polysaccharides could be the best method. Since laminarin, curdlan, or pachyman were not available to us, we turned our attention to another bacterial polysaccharide, the scleroglucan which consists of a β(1→3)-Glc backbone branched with β(1→6)-Glc units.[21] Acetolysis of a commercially available compound which contains 60–75% of scleroglucan, under essentially the conditions already described for curdlan, afforded the expected α-laminaribiose octaacetate **6** in 23% yield. By conventional treatment with hydrogen bromide in glacial AcOH, this disaccharide was then converted into the corresponding glycosyl bromide **7**. Per-acetylated 1-thiolaminaribiose **4** was obtained in 58% from **6**, by reaction of the bromide **7** with tetrabutylammonium thioacetate in toluene, as already described in the synthesis of thiocellodextrins.[22]

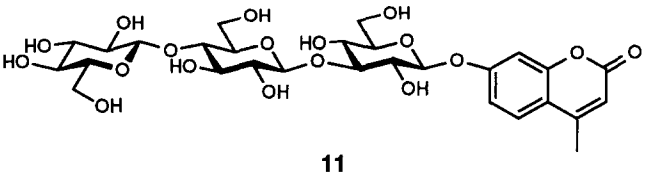
Conversion of methyl β-lactoside into methyl β-lactoside hexabenzoate **10** could be realized via either $3^{II},4^{II}$-*O*-isopropylidene or $4^{II},6^{II}$-*O*-benzylidene protected derivatives.[23,24] In our hands, the synthesis of **10** was best achieved starting from $4^{II},6^{II}$-di-OH-β-lactoside pentabenzoate **8** obtained in high yield by improvement of standard procedures,[25] and then controlled acid hydrolysis[26] of $4^{II},6^{II}$-*O*-(ethyl orthobenzoyl) intermediate **9** which was not fully characterized (overall yield from methyl β-lactoside 35% versus 10.5% in reference 24). The high selectivity observed for the ring opening of 4,6-*O*-orthobenzoate lactoside **9** should be favorably compared to the mixture obtained when methyl β-galactosides were used under similar conditions.[27]

The coupling of 1-thioglycose **4** with the triflate **5** in HMPA and in the presence of cysteamine and dithioerythritol (DTE) conditions we developed for efficient syntheses of thiooligosaccharides[22,28] gave a disappointing yield (~30%) of the expected tetrasaccharide **3a**. When the condensation was repeated in DMF, in the presence of diethylamine as recently reported,[29] a slight improvement was observed (~40%), but some 1,2-*cis*-thioglycoside was also formed (~3–8%), which hampered the isolation of pure **3a**. Two successive chromatographies were necessary to obtain pure **3a**. Alternatively, de-*O*-acylation of crude **3a** gave **3b**, which was purified by HPLC on μ-Bondapak NH_2 column. From the pure **3b** recovered, one estimated that the purity of **3a** was up to 80% after the first purification. The tetrasaccharide structure of **3b** was evident from its ^1H NMR spectrum which shows four distinct β-anomeric protons. The proton H^{III}-1 was easily attributed from its splitting ($J_{1,2}=10.0$ Hz), whereas all the others had coupling constants of 8.3 Hz. As expected, the interglycosidic proton H^{II}-4 involved in the thiolinkage was shielded at δ 2.79 ppm and appeared as a double doublet ($J_{3,4}=J_{4,5}=10.7$ Hz).

At this stage, we would like to investigate the efficiency of cellodextrin phosphorylase to elongate the non-reducing end of compound **3b**. From our previous work,[12] it was known that this synthesis required the removal of all glucose produced as side product in the hydrolysis of glucose-1-P to avoid the formation of cellodextrins. For this reason, incubation of **3b**, glucose-1-P (10 equiv), and cellodextrin phosphorylase was performed in the presence of glucose oxidase and catalase. Under the conditions used, a HPLC profile of the reaction mixture recorded after 24 h shows the transformation of **3b** into a compound with a longer retention time. The pentasaccharide **2a** was isolated by HPLC in 56% yield or under its acetylated form **2b** in 55% yield. For unknown reasons, **2a** was not a good primer for the phosphorolytic synthesis of **1**, since elongation of **1** occurred when most of **2a** was still unreacted. The incubation was then stopped when the presence of a heptasaccharide was detected on the HPLC chromatogram. At this stage **1** was isolated in 13% yield, while **2a** was recovered in 80% yield.

We first confirmed by HPLC and TLC that thiooligosaccharides **1**, **2a**, and **3b** were not hydrolysed by *B. licheniformis* 1,3:1,4-β-glucanase[11] after a 20 h incubation time. This expected result was a prerequisite to any further inhibition studies using 4-methylumbelliferyl *O*-β-D-glucopyranosyl (1→4)-*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside **11** (G4G3G-MeUmb) as a substrate[8] (Fig. 2). Under the conditions used for the K_I determinations, we controlled that the thiooligosaccharides were not able to be acceptors in transglycosylation reactions involving the trisaccharide product G4G3G resulting of the hydrolysis of the aglycon of **11**. Enzyme inhibition experiments were carried out at pH 7.2 and 55 °C, conditions of maximum activity on its natural substrates. The thiooligosaccharides are competitive inhibitors of the hydrolysis of the chromophoric trisaccharide **11** as shown in the Dixon plot for **3b** (Fig. 3) as an example. The inhibition constants (K_I) are 3.5±0.6 mM, 6.1±0.8 mM and 8.7±1.8 mM for compounds **1**, **2a**, and **3b**, respectively.

The binding site cleft is composed of six to seven subsites, each one accommodating a glucopyranosyl unit of the bound carbohydrate: subsites −I to −IV on the donor part or nonreducing end from the site of cleavage, and subsites +I, +II, or +III on the acceptor part. The K_I values obtained here are in the range of those obtained for G4G4G3G ($K_I=2.3$ mM) and G4G3G ($K_I=12$ mM).[11] Evaluation of the contribution of subsite −III and −IV to binding free energy as $\Delta(\Delta G_b)=-RT\ \ln(K_{I,1}/K_{I,2})$, where $K_{I,1}$ and



11

Figure 2. Methylumbelliferyl-β-D-trisaccharide as chromophoric substrate for 1,3:1,4-β-glucanase from *B. licheniformis*.

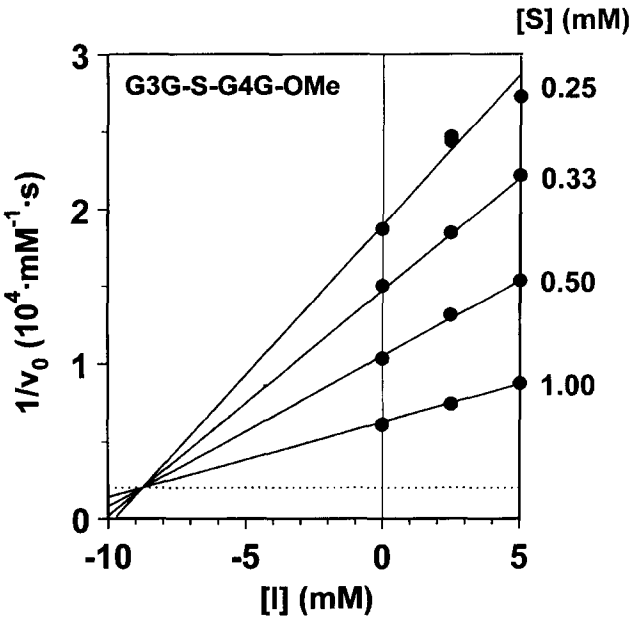


Figure 3. Dixon plot. Concentrations of the substrate **11** and the inhibitor **3b** were as indicated. The reaction was initiated by adding the substrate and allowed to proceed for 10 min at 55 °C.

$K_{I,2}$ are the inhibition constants of two inhibitors of the same series differing in one glucopyranose unit, gives values of -0.23 kcal mol^{-1} and -0.37 kcal mol^{-1} for subsites $-$III and $-$IV, respectively, when using the thiooligosaccharides **1**, **2a**, and **3b**. However, $\Delta(\Delta G_b)$ assigned to subsite $-$IV calculated from the K_I values of G4G4G3G and G4G3G is -1.1 kcal mol^{-1}.[11] This difference may indicate that the binding of both series of competitive inhibitors are somewhat different due to the replacement of a sulfur for an oxygen atom, or that the contribution of each subsite to binding depends on the binding to other distant subsites, i.e. +I and +II. These results raise the question of whether subsite binding shows some kind of cooperative behavior. On the other hand, being an *endo*-depolymerase (catenase), and because of the multiplicity of potential modes of binding, thermodynamic measurements are highly ambiguous by themselves. Structural work is required to analyse binding modes and further dissect the contributions to free energy of interaction.

Conclusions

A straightforward strategy was used in this work for the obtainment of rather long oligosaccharides which are resistant to enzymatic hydrolysis. Despite the low affinities of this set of inhibitors for the bacterial enzyme, crystallization studies with the enzyme to attempt the X-ray structure determination of an enzyme–inhibitor complex are undertaken. To design better inhibitors, the role of subsites +I and +II in substrate or inhibitor binding requires further analysis. Fully enzymatic data of 1,3:1,4-β-glucanase with compounds **1**, **2a**, and **3b** as inhibitors and improvement of the thioglycosylation of $^4C_1$ acceptors bearing equatorial aglycons are in progress.

Experimental

General methods and materials

NMR spectra were recorded in D_2O (external reference) or in $CDCl_3$ (internal TMS) with a Bruker 300 AC spectrometer; H and C atoms of glucopyranosyl units are indicated with a superscript roman number increasing from the reducing end (C^{II}-1 indicates the anomeric carbon of the unit next to the reducing end). Low-mass and high-mass resolution measurements were performed on a Nermag R-1010C and VG ZAB-SEQ spectrometers, respectively; NH_3-isobutane and glycerol matrix were, respectively, used for DCI and FAB(+) modes. Optical rotations were measured at room temperature (20 °C) with a Perkin–Elmer 241 polarimeter. For flash and open chromatographies, Merck Silica Gel 60 (0.040–0.063 mm) and (0.063–0.2 mm) were used, respectively. Light petroleum refers to the 60–80 °C fraction. The course of the enzymatic reaction was monitored by HPLC on an analytical μ-Bondapak NH_2 column (Waters, Milford, Massachusetts, U.S.A.) with MeCN:water (75:25 v/v). The free oligosaccharides **1** and **2a** were purified by HPLC on μ-Bondapak NH_2 column (10 μm, 19×150 mm, Waters) using the same eluent. The commerciably available Actigum CS6 containing scleroglucan was a gift of Sanofi BioIndustries Paris (France). Production, purification, and activity of cellodextrin phosphorylase were accomplished as already published.[12] *B. licheniformis* 1,3:1,4-β-glucanase was produced and purified as already described.[4]

1,2,4,6-Tetra-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranose (6). To a stirred suspension of Actigum CS6 (20 g) in acetic anhydride (130 mL) was added dropwise a solution of concd H_2SO_4 (3.5 mL) in acetic anhydride (10 mL) at 0 °C. The reaction was stirred for 5 days at 50–55 °C, then the same amount of acid was added at 0 °C, and the reaction was heated again at 50–55 °C for an additional 24 h. The acid was neutralized by adding anhyd NaOAc (10 g). The mixture was then poured into ice–water (600 mL), stirred overnight and extracted with $CHCl_3$ (3×500 mL). The organic phase was washed successively with brine, aq $NaHCO_3$, and water, and dried and concentrated. Flash chromatography of the residue (first ethyl acetate:light petroleum 1:2 then 1:1) afforded compound **6** (6.8 g, 23%); mp 83–88 °C (from EtOH), $[\alpha]_D$ +22° (*c* 0.96, $CHCl_3$); lit.[17]: mp 78–80 °C, $[\alpha]_D$ +22.1° (*c* 0.3, $CHCl_3$).

2,4,6-Tri-*O*-acetyl-1-S-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (4). To a toluene solution (100 mL) of tetrabutylammonium thioacetate obtained from thioacetic acid (1 mL, 13.5 mmol) and tetrabutylammonium hydroxide (17 mL, 13.6 mmol) as already described,[22] hepta-acetyl-laminaribiosyl bromide **7**[17] prepared from **6** (2.5 g, 3.6 mmol) was added. The mixture was stirred overnight and concentrated, the residue was dissolved in dichloromethane, and the organic phase was treated as described for **6**. Evaporation of the solvent, treatment

under reflux in methanol (100 mL) for 5 min with charcoal, filtration (Celite), evaporation of the filtrate, and flash chromatography (ethyl acetate:light petroleum 1:1.5) of the residue, gave compound **4** (1.5 g, 58%); mp 172 °C (from ether); $[\alpha]_D$ −22° (*c* 0.52, $CHCl_3$); anal. calcd for $C_{28}H_{38}O_{18}S$: C, 48.41; H, 5.47; S, 4.16; found: C, 48.30; H, 5.56; S, 4.36%; FABMS(+): *m/z* 733 $(M+K)^+$, 618 $(M-SAc)^+$; ^1H NMR ($CDCl_3$): δ 5.15–5.03 (m, 3H, H-1, H-2, H^{II}-3), 5.02 (dd, 1H, H^{II}-4, $J_{3,4}=J_{4,5}=9.4$ Hz), 4.96 (dd, 1H, H-4, $J_{3,4}=J_{4,5}=9.7$ Hz), 4.86 (dd, 1H, H^{II}-2, $J_{1,2}=J_{2,3}=8.5$ Hz), 4.58 (d, 1H, H^{II}-1), 4.34 (dd, 1H, H^{II}-6a, $J_{6a,6b}=12.7$, $J_{6a,5}=4.7$ Hz), 4.16 (dd, 1H, H-6a, $J_{6a,6b}=12.6$, $J_{6a,5}=4.7$ Hz), 4.08 (dd, 1H, H-6b, $J_{6b,5}=2.3$ Hz), 4.03 (dd, 1H, H^{II}-6b, $J_{6b,5}=2.3$ Hz), 3.93 (dd, 1H, H-3, $J_{2,3}=8.8$ Hz), 3.75 (m, 1H, H-5), 3.65 (m, 1H, H^{II}-5); ^{13}C NMR ($CDCl_3$): δ 192.4 ($S\underline{C}OCH_3$), 170.6–168.9 ($O\underline{C}OCH_3$) 100.9 (C^{II}-1), 80.2 (C-1), 80.0, 76.4, 72.9, 71.8, 71.0, 70.6, 68.0, 67.6 (C-2–5, C^{II}-2–5), 61.9, 61.6 (C-6, C^{II}-6), 30.8 ($S\underline{C}OCH_3$), 20.8–20.0 ($O\underline{C}O\underline{C}H_3$).

Methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3-di-*O*-benzoyl-β-D-galactopyranosyl)-β-D-glucopyranoside (8). Methyl $4^{II},6^{II}$-*O*-benzylidene lactoside pentabenzoate[25] (7 g, 7.26 mmol) was treated with 35% aq tetrafluoroboric acid (2 mL) for 3 h as described.[30] After work up, flash chromatography (ethyl acetate:light petroleum 1:1) of the residue afforded **8** (4.8 g, 75%); mp 206 °C (EtOH); $[\alpha]_D$ +91° (*c* 0.56, $CHCl_3$); lit.[23]: mp 202–203 °C (EtOH); $[\alpha]_D$ +91° (*c* 1.0, $CHCl_3$).

Methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl)-β-D-glucopyranoside (10). Compound **8** (467 mg, 0.53 mmol) and camphorsulfonic acid (30 mg) were dissolved in acetonitrile (7.5 mL) and triethyl orthobenzoate (1 mL, 4.35 mmol) was added. Treatment of crude **9** as essentially described for galactosides,[27] followed with flash chromatography (ethyl acetate:chloroform 4:26), yielded compound **10** (386 mg, 74%); mp 122–124 °C (EtOH); $[\alpha]_D$ +60° (*c* 0.52, $CHCl_3$); lit.[24]: mp 120 °C (EtOH) $[\alpha]_D$ +65° (*c* 1.0, $CHCl_3$).

Methyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-S-(2, 4, 6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*O*-(2, 3, 6-tri-*O*-benzoyl-4-thio-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (3a). Methyl 4^{II}-*O*-triflyl-lactoside **5** obtained from compound **10** (2.75 g, 2.8 mmol) as already described for the corresponding galactoside[22] was added to a solution of fully acetylated thiolaminaribiose **4** (1.94 g, 2.8 mmol) in dry *N*,*N*-dimethylformamide (25 mL) containing diethylamine (6 mL). The resulting mixture was stirred under argon at rt for 15 h, then diluted with ethyl acetate (400 mL) and extracted with water (2 × 300 mL). The extract was dried, concentrated and partially purified by flash chromatography (ethyl acetate:chloroform 1:5). Impure **3a** was isolated (2.29 g, 50%). Two additional open column chromatographies (ethyl acetate:light petroleum 1:1.2) were needed to prepare an analytical sample of **3a**; mp 140 °C (EtOH); $[\alpha]_D$ +15° (*c* 0.7, $CHCl_3$); anal. calcd for $C_{81}H_{82}O_{33}S$: C, 60.21; H, 5.11; S, 1.98%; found: C, 60.54; H, 5.24; S, 2.18%; FABMS(+): *m/z* 1654 $(M+K)^+$; ^{13}C NMR ($CDCl_3$): δ 170.4–168.9 ($O\underline{C}OCH_3$), 165.9–164.7 ($O\underline{C}OC_6H_5$), 133.5–128.1 (aromatics), 101.7, 100.8 (C-1, C^{II}-1, C^{IV}-1), 81.5 (C^{III}-1), 79.9 (C^{III}-3), 76.7, 75.5, 74.4, 73.2, 73.1, 72.9, 72.4, 71.9, 71.7, 71.1, 70.8, 70.1, 67.9, 67.5 (C-2–5, C^{II}-2,3,5, C^{III}-2,4,5, C^{IV} 2–5) 63.6, 62.6, 61.8, 61.7 (C-6, C^{II}-6, C^{III}-6, C^{IV}-6), 56.9 ($O\underline{C}H_3$), 46.2 (C^{II}-4), 20.6–20.1 ($OCO\underline{C}H_3$).

Methyl *O*-β-D-glucopyranosyl-(1→3)-S-β-D-glucopyranosyl-(1→4)-*O*-(4-thio-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranoside (3b). A stirred solution of impure compound **3a** (225 mg) in dry methanol (15 mL) was treated with 1 N methanolic sodium methoxide (500 μL). The solution was stirred at rt for 15 h, then neutralized (Amberlite IRN $77H^+$), filtered, and the filtrate concentrated to dryness. The residue was dissolved in water, freeze-dried, and subjected to HPLC (μ-Bondapak NH_2 column using acetonitrile:water 70:30 as eluent). The expected compound **3b** was obtained pure (80 mg, 41% from **10**); $[\alpha]_D$ −15° (*c* 0.5, H_2O); HRFABMS, calcd for $C_{25}H_{44}O_{20}S$ (M+H): 697.2211; found: 697.2225; ^1H NMR (D_2O): δ 4.64 (d, 1H, H^{IV}-1, $J_{1,2}=8.3$ Hz), 4.54 (d, 1H, H^{III}-1, $J_{1,2}=10$ Hz), 4.37 (d, 1H, H^{II}-1, $J_{1,2}=8.3$ Hz), 4.27 (d, 1H, H-1, $J_{1,2}=8.3$ Hz), 3.45 (s, 3H, OCH_3), 2.79 (dd, 1H, H^{II}-4, $J_{4,3}=J_{4,5}=10.7$ Hz); ^{13}C NMR (D_2O): δ 104.5, 104.2, 103.7 (C-1, C^{II}-1, C^{IV}-1), 86.7, 85.1, 80.9, 80.1, 79.9, 77.5, 77.0, 76.2, 75.8, 75.8, 74.3, 74.1, 73.6, 71.1, 69.4 (C-2–5, C^{II}-2,3,5, C^{III}-1–5, C^{IV}-2–5), 62.8, 62.3, 62.2, 61.5 (C-6, C^{II}-6, C^{III}-6, C^{IV}-6), 58.7 ($O\underline{C}H_3$), 48.3 (C^{II}-4).

Methyl *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl)-(1→3)-S-β-D-glucopyranosyl-(1→4)-*O*-(4-thio-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranoside (2a). Method 1. To a solution of tetrasaccharide **3b** (175 mg, 0.25 mmol) in Tris–HCl buffer (11.6 mL, 50 mM, pH 7.5) and water (38.5 mL) containing EDTA (5 mM) and DTT (2.5 mM) was added cellodextrin phosphorylase (930 μL, 1.55 U), glucose oxidase (465 μL, 1 mg mL^{-1}, 9.3 U), catalase (230 μL, 1 mg mL^{-1}, 2530 U), and then Glc-1-P disodium salt hydrate (765 mg, 2.5 mmol). The resulting solution was stirred at 25 °C for 24 h, then heated at 100 °C for 2 min. The reaction mixture was filtered and treated with some TMD-8 resin. After removal of the resin, the solution was freeze-dried, and the solid redissolved into a minimum of water. HPLC purification afforded pure **2a** (120 mg, 56%): $[\alpha]_D$ −13° (*c* 0.55, H_2O); HRFABMS, calcd for $C_{31}H_{54}O_{25}S$ (M+H): 859.2736, found: 859.2753; ^1H NMR (D_2O, 353 K): δ 4.65 (dd, 1H, H^{IV}-1, $J_{1,2}=8.3$ Hz), 4.52 (d, 1H, H^{III}-1, $J_{1,2}=9.9$ Hz), 4.38, 4.37 (2d, 2H, H^{II}-1, H^{V}-1, $J_{1,2}=8.3$ Hz), 4.25 (d, 1H, H-1, $J_{1,2}=8.3$ Hz), 3.45 (s, 3H, OCH_3), 2.79 (dd, 1H, H^{II}-4, $J_{3,4}=J_{4,5}=10.7$ Hz); ^{13}C-NMR (D_2O): δ 104.5, 104.0, 103.9, 103.7 (C-1, C^{II}-1, C^{IV}-1, C^{V}-1), 86.6, 85.1, 81.0, 80.1, 78.0, 77.5, 77.0, 76.3, 76.2, 75.8, 75.6, 74.7, 74.3, 74.1, 73.6, 70.9, 69.4 (C-2–5, C^{II}-2,3,5, C^{III} 1–5, C^{IV}2–5,

C^{V}-2–5), 62.8, 62.2, 62.1, 61.5 (C-6, C^{II}-6, C^{III}-6, C^{IV}-6, C^{V}-6), 58.7 ($O\underline{C}H_3$), 48.3 (C^{II}-4).

Method 2. After the incubation time, the reaction mixture was freeze-dried, and then dissolved in pyridine (25 mL)–anhydride acetic (14 mL). After stirring at rt for 15 h, methanol (14 mL) was added at 0 °C, the solution evaporated to dryness, and the residue dissolved in dichloromethane. The organic solution was washed successively with ice-cold aq $KHSO_4$ (10%), ice-cold aq $NaHCO_3$, and water. After drying and concentration, the crude **2b** was subjected to flash chromatography (acetone:cyclohexane 1:2): **2b** was obtained (211 mg, 55%); mp 137–140 °C (EtOH); $[\alpha]_D$ −36 ° (*c* 1.08 $CHCl_3$); anal. calcd for $C_{63}H_{87}O_{41}S$: C, 49.38; H, 5.72; S, 2.09; found: C, 49.47; H, 5.74; S, 2.08%; DCIMS: *m/z* 1549 $(M + NH_4)^+$; ^{13}C NMR ($CDCl_3$): δ 170.4, 170.3, 170.2, 170.1, 169.8, 169.5, 169.4, 169.2, 169.0 ($O\underline{C}OCH_3$), 101.4, 100.9, 100.8, 100.2 (C-1, C^{II}-1, C^{IV}-1, C^{V}-1), 81.5, 80.0, 77.2, 76.3, 76.2, 75.7, 73.8, 73.0, 72.9, 72.8, 72.7, 72.2, 72.0, 71.6, 71.5, 71.0, 70.2, 67.9, 67.8 (C-2–5, C^{II}-2,3,5, C^{III}-1–5, C^{IV}-2–5, C^{V}-2–5), 63.6, 62.2, 62.0, 61.5 (C-6, C^{II}-6, C^{III}-6, C^{IV}-6, C^{V}-6), 56.9 (OCH_3), 45.9 (C^{II}-4), 20.8, 20.7, 20.5, 20.4, 20.3 ($OCOCH_3$). Deacetylation of **2b** as already described for **3b** afforded **2a** in quantitative yield.

Methyl *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*S*-β-glucopyranosyl-(1→4)-*O*-(4-thio-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranoside (1). To a solution of pentasaccharide **2a** (100 mg, 0.11 mmol) in Tris–HCl buffer (15 mL, 50 mM, pH 7.5) and water (17.6 mL) containing the same concentrations of EDTA and DTT as above, glucose oxidase (212 μL, 4.25 U) and catalase (125 μL, 1375U), was added Glc-1-P (350 mg, 1.1 mmol) and cellodextrin phosphorylase (410 μL, 0.7 U). The resulting solution was stirred at 25 °C until the HPLC tracing indicated the formation of heptasaccharide. The reaction mixture, treated as described above in method 1, led to **1** (16 mg, 13.5%); $[\alpha]_D$ −59 ° (*c* 0.32, H_2O); HRFABMS, calcd for $C_{37}H_{64}O_{30}S$ (M+H): 1021.3261; found: 1021.3281; ^1H NMR (D_2O, 353 K): δ 4.63 (δ, 1H, H^{IV}-1, $J_{1,2}$=7.9 Hz), 4.49 (d, 1H, H^{III}-1, $J_{1,2}$=9.9 Hz), 4.4–4.34 (m, 3H, H^{VI}-1, H^{V}-1, H^{II}-1), 4.23 (d, 1H, H-1, $J_{1,2}$=7.9 Hz), 3.42 (s, 3H, OCH_3), 2.75 (dd, 1H, H^{II}-4, $J_{3,4}$=$J_{4,5}$=10.7 Hz); ^{13}C NMR (D_2O): δ 104.5, 104.0, 104.0, 103.8, 103.7 (C-1, C^{II}-1, C^{IV}-1, C^{V}-1, C^{VI}-1), 86.6, 85.2, 81.0, 80.1, 80.0, 79.9, 78.0, 77.4, 77.0, 76.3, 76.2, 75.9, 75.8, 75.5, 74.7, 74.6, 74.4, 74.3, 74.1, 73.6, 70.9, 69.4 (C-2–5, C^{II}-2,3,5, C^{III}1–5, C^{IV}-2–5, C^{V}-2–5 , C^{VI}-2–5), 62.7, 62.2, 62.0, 61.5, 61.4, 61.1 (C-6, C^{II}-6, C^{III}-6, C^{IV}-6, C^{V}-6, C^{VI}-6), 58.7 (OCH_3), 48.3 (C^{II}-4).

Enzymatic studies

In a final volume of 1 mL, the thiocompounds **1** (0, 0.25, and 0.50 mM), **2a** (0, 0.50, 1.00 and 1.50 mM) and **3b** (0, 0.25, 5.0, and 10.0 mM) were each preincubated for 5 min at 55 °C in 6.5 mM citrate-87 mM phosphate buffer (pH 7.2) in the presence of $CaCl_2$ (0.1 mM). Then, the enzyme was added to a final 150 nM concentration and after 3 min the enzymatic reaction was started by adding the substrate **11** (0.17, 0.25, and 0.50 mM for **1**; 0.20, 0.25, 0.33, 0.50, and 1.00 mM for **2a**; 0.25, 0.33, 0.50, and 1.00 mM for **3b**). The release of 4-methylumbelliferone was monitored by UV spectrophotometry at 365 nm ($\Delta\varepsilon_{365}$=5440 $M^{-1}cm^{-1}$) for 10 min.[11] The K_I values were determined by nonlinear regression to the competitive inhibition model $v = V_{max}.[S]/([S]+K_M\ (1+\ [I]/K_I)$ linearized as $v = V_{max} - K_M\ (v/[S]) - (K_M.v.[I]/K_I[S])$, where 1/[S] and [I] are the independent variables and V_{max}, K_M and K_I are the fitting parameters.

The lack of hydrolysis of compounds **1**, **2a**, and **3b** under the same experimental conditions, as well as the absence of transglycosylation products when incubating **2a** and the substrate **11** with the enzyme, were assessed by TLC and HPLC monitoring (Aminex HPX-42A (BioRad) column, elution: H_2O, 0.6 mL min^{-1}, 85 °C, refractive index detector) for 20 h.

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