Cyclophellitol and its derivatives: synthesis and application as beta-glycosidase inhibitors

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7 Cellobiosyl Cyclophellitol and 4′-Deoxycellobiosyl Cyclophellitol Aziridine Derivatives

Synthesis and Evaluation Inhibitors and Activity-Based Probes for *H. Jecorina* Cellulases

7.1 Introduction

The filamentous fungus *Hypocrea jecorina* (*H. jecorina*), previously reported as *Trichoderma reseei* (*T. reseei* is the predominant source of cellulases for the industrial production of bio-ethanol from cellulose.[1-5] The cellulolytic system secreted by this fungus consists of three general classes of enzymes: cellobiohydrolases (CBH, EC 3.2.1.91), endoglucanases (EG, EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). The major enzyme components of this system are CBHI, CBHII, EGI and EGII. Together they

display strong synergy in the hydrolysis of cellulose, the most abundant renewable carbon source.[6-14] EGI and EGII act randomly along the cellulose chains by hydrolyzing internal glycosidic linkages and thereby creating new cleavage sites for exoglucanases CBHI and CBHII. CBHI and CBHII act on the ends of cellulose to release cellobiose, which in turn is converted to glucose by β-glucosidase. The difference in substrate selectivity is reflected by the architecture of their active site. EGs have a canyon-like active site, allowing random binding of cellulose. In contrast, CBHs have a tunnel-shaped active site and only a single cellulose chain is permitted to thread in the active site tunnel. Eventually, this results in the release of cellobiose at the end of the tunnel. All of these enzymes catalyze the hydrolysis of the 1,4-β-glucosidic linkages by either a double displacement mechanism (CBHI, EGI and EGII) or a single displacement mechanism (CBHII).[15]
The cellulotyic system of *H. jecorina* is applied in the industrial production of bio-ethanol with the commonly encountered problem of "dying off" of one or multiple cellulases over time as described in Chapter 6. Therefore, methodologies to accurately assess and quantify active CBHI, CBHII, EGI and EGII, and determine their relative concentrations in a complex mixture are needed. Chapter 6 describes the synthesis of cellobiosyl cyclophellitol and its BODIPY derivatives as potential mechanism-based inhibitor and activity-based probe (ABP) to profile active *H. jecorina* cellulases. It was envisioned that the aziridine analogues of (BODIPY) cellobiosyl cyclophellitol are suitable for the same purpose.

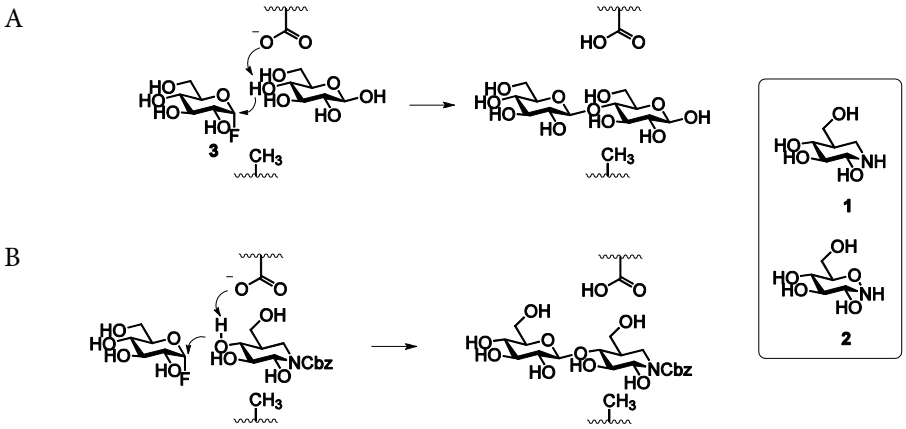
This Chapter details the synthesis of mechanism-based inhibitors and ABPs based on cyclophellitol aziridine using cellobiose as a scaffold. The kinetic parameters of these compounds along with cellobiosyl cyclophellitol (Chapter 6) were evaluated on CBHI, CBHII, EGI and EGII from *H. jecorina*. Furthermore, the labeling efficiency of the designed ABPs towards the four cellulases is described.

7.2 Results and Discussion

The synthetic route towards cellobiosyl cyclophellitol (Chapter 6) proved to be suboptimal regarding the overall yield and the number of steps. This is a common problem in the synthesis of oligosaccharides that in some cases, especially in the construction of 1,4-β-glycosides, can be circumvented using glycosynthase-assisted procedures.[16,17] Glycosynthases are hydrolytically inactive β-glycosidases in which the active site carboxylate nucleophile is replaced by a non-nucleophilic amino acid residue

(Ala or Gly).[18] Consequently, the hydrolytic activity of the enzyme is ablated (Figure 1A). Glycosynthase-mediated procedures have led to the development numerous 1,4-β-linked glycosides, including some di, -tri-and tetrasaccharides (1,4-β-glucosides) of

Figure 1. Glycosynthase-mediated transglycosylation. (A) General mechanism and (B) Synthesis of Cbz-protected isofagamine disaccharides with isofagamine **1** and tetrahydrooxazine **2**.



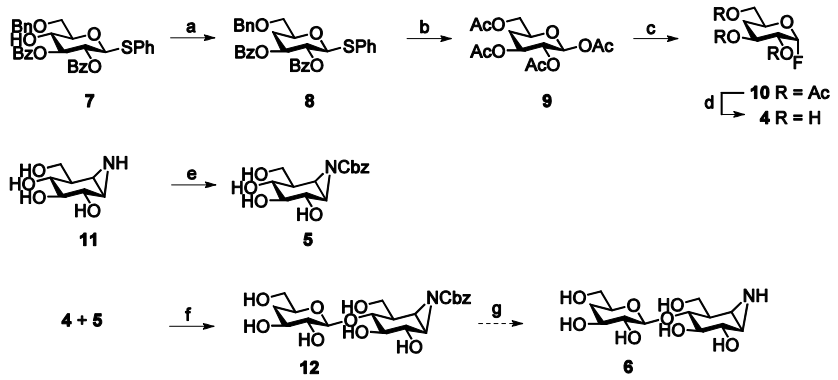
potent glucosidase inhibitors isofagamine **1** and tetrahydrooxazine **2**.[19] These studies employed in general α-glucopyranosyl fluoride (GlcF, **3**) as activated donor and *N*-Cbz protected 1-deoxynojirimycin analogues as acceptor (Figure 1B). The Cbz function was chosen as protective group to mask the basicity of the iminosugars, resulting in a reduced affinity for the enzyme and concomitant substrate inhibition. Additionally, the aromatic group of the Cbz function increased the binding affinity for the aglycon site. With these studies in mind it was envisioned that it would be feasible to synthesize cellobiosyl cyclophellitol aziridine derivatives as cellulase inhibitors and ABPs using glycosynthase-assisted methodology when suitable donors and acceptors are selected.

7.2.1 Synthesis of 4'-deoxycellobiosyl cyclophellitol Cbz-aziridine as mechanism-based inhibitor

Glycosynthase-mediated transformations with GlcF **3** as activated donor are often accompanied with the formation of tri-and tetrasaccharides, since the initial product (disaccharide) serves as a good or even better acceptor for the enzymatic reaction.[19] Therefore, 4-deoxyglucopyranosyl fluoride (4-deoxyGlcF) **4** was selected as donor to prevent transglycosylation. In the same line as Cbz-protected iminosugars **1** and **2**, cyclophellitol Cbz-aziridine **5** was selected as acceptor for the synthesis of 4'-deoxycellobiosyl cyclophellitol aziridine **6**.

Donor 4-deoxyGlcF **4** was synthesized from thioglucoside **7**[20] as presented in Scheme 1. 4-Deoxy thioglucoside **8** was formed in two steps by transformation of **7** into the Barton-Mccombie precursor with 1,1'-thiocarbonyldiimidazole and ensuing radical deoxygenation with AIBN and tributyltin hydride. NIS-mediated hydrolysis of the thio functionality in **8** was followed by the removal of benzoyl protective groups under Zemplén conditions. Ensuing hydrogenolysis of the benzyl ethers ($Pd(OH)_2$, H_2, MeOH) generated 4-deoxyGlc, which was treated with a solution of acetic anhydride in pyridine to furnish **9** in an overall yield of 53% over four steps. Treatment of **9** with HF/pyridine generated protected 4-deoxyGlcF **10** and subsequent deacetylation (NH_3, MeOH) provided **4**, which was used in the enzymatic glycosylation in its crude form. Acceptor cyclophellitol Cbz-aziridine **5** was constructed from cyclophellitol aziridine **11** (See Chapter 3) by treatment of **11** with a pre-activated ester solution of benzyl alcohol and 4-nitrophenylchloroformate.

Scheme 1. Synthesis of 4-deoxyGlcF **4**, cyclophellitol Cbz-aziridine **5** and 4'-deoxycellobiosyl cyclophellitol aziridine **6**.



Reagents and conditions: (a) i. 1, 1'-thiocarbonyldiimidazole, DCE, 80 °C, ii. Bu_3SnH, AIBN, toluene, 110 °C, 95%; (b) i. NIS, TFA, DCM, 0 °C, ii. NaOMe, MeOH, iii. 10% $Pd(OH)_2$, H_2, MeOH, iv. Ac_2O, pyridine, 53% over four steps; (c) HF/pyridine, 0 °C, 98%; (d) NH_3, MeOH, 0 °C to rt, quant; (e) i. BnOH, 4-nitrophenyl chloroformate, pyridine, DCM, ii. activated ester, Et_3N, DMF, iii. HPLC (acetonitrile/50 mM aqueous NH_4HCO_3), 42%; (f) i. Abg2F6 glycosynthase, 100 mM phosphate buffer, pH 7, ii. HPLC (acetronitrile/50 mM aqueous NH_4HCO_3), 59%; (g) NH_4OH.

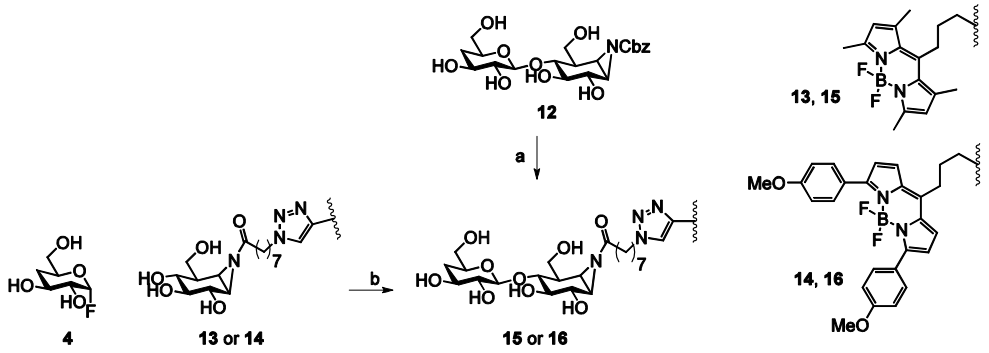
Incubation of 4-deoxyGlcF **4** and cyclophellitol Cbz-aziridine **11** in an aqueous phosphate buffer (100 mM, pH 7) with the glycosynthase Abg2F6[21] (developed from a β-glucosidase from *Agrobacterium sp.*) yielded 4'-deoxycellobiosyl cyclophellitol Cbz-aziridine **12** in 59% yield after HPLC purification under basic conditions (gradient 50

mM NH_4HCO_3/acetonitrile). Basic conditions were required to preserve the labile acyl aziridine moiety (see also Chapter 4 and 5). Removal of the Cbz protective group in **12** with NH_4OH gave 4'-deoxycellobiosyl cyclophellitol aziridine **6**, which was difficult to isolate. Attempts to isolate the compound by gel filtration (HW-40) and reverse-phase column chromatography (Sep-pak 18) proved abortive since ^1H NMR analysis indicated almost complete disappearance of the aziridine proton peaks after purification. As such 4'-deoxycellobiosyl Cbz-cyclophellitol aziridine **12** will be evaluated as potential cellulase inhibitor.

7.2.2 Synthesis of 4'-deoxycellobiosyl BODIPY-cyclophellitol aziridines as ABPs

The synthetic routes towards 4'-deoxycellobiosyl BODIPY-cyclophellitol aziridines **15** and **16** are depicted in Scheme 2.

Scheme 2. Synthesis of 4'-deoxycellobiosyl BODIPY-cyclophellitol aziridines **15** and **16**.



Reagents and conditions: (a) i. NH_4OH, H_2O, ii. BODIPY-spacer **17** or **18**[22], 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), DMF, 0 °C, iii. HPLC (25 mM aqueous NH_4HCO_3/acetonitrile), **15**: 16%, **16**: 12%; (b) i. Abg2F6 glycosynthase, 100 mM phosphate buffer, pH 7, DMSO, ii. HPLC (25 mM aqueous NH_4HCO_3/acetronitrile), **15**: 15%.

With the mechanism-based inhibitor **12** in hand, ABP **15** and **16** were obtained by a two-step procedure. The carbamate protective group in **12** was first removed by treatment with NH_4OH and the liberated free aziridine moiety was then uneventfully functionalized with either BODIPY spacer **17** or **18**[21] using EEDQ as coupling reagent. Purification by HPLC utilizing a 25 mM aqueous NH_4HCO_3 and acetonitrile gradient furnished the target ABPs **15** and **16** in moderate yields.

Next, the question was raised whether the BODIPY-functionalized 4'-deoxy cellobiosyl cyclophellitol aziridines **15** and **16** could be directly generated from 4-deoxyGlcF **4** and BODIPY-cyclophellitol aziridine **13** and **14**[23] when reacting with Abg2F6 synthase. To this end, **13** was first dissolved together with 4-deoxyGlcF **4** in a solution of sodium phosphate buffer (100 mM, pH 7) and DMSO (3/2; v/v). DMSO was needed to solubilize the BODIPY acceptors. When fluorodonor **4** and **13** were reacted in equimolar amounts (10 mM as final concentration) with Abg2F6, the enzyme started to precipitate from the reaction mixture and no conversion was observed after two days. Optimal enzymatic glycosylation conditions were obtained by lowering the final reaction concentration of **13** (from 10 mM to 2 mM) and **13** was fully converted into target compound **15** after two days although some enzyme precipitation was still observed. However, using identical conditions for the synthesis of ABP **16** proved abortive in that starting material **14** was not soluble in the DMSO/phosphate buffer (3/2; v/v). Attempts to decrease the final concentration of **14** down to 0.5 mM or increase the DMSO level did not result in the formation of ABP **16**.

7.2.3 Biological evaluation of cellobiosyl cyclophellitol and 4'-deoxycellobiosyl cyclohellitol aziridine analogues

The potential inhibitory potential of 4'-deoxycellobiosyl cyclophellitol aziridine **12** and cellobiosyl cyclophellitol **19** (see Chapter 6) towards the four *H. jecorina* cellulases (CBHI, CBHII, EGI and EGII) was assessed by determining the Michaelis-Menten parameters. To this end, the enzymes were incubated with a concentration series of the inhibitor for different time intervals. Subsequently, aliquots of the inactivation mixture were assayed, after specific time intervals with 2,4-dinitrophenylcellobioside (DNPC) as substrate, and the release of DNP group was monitored over time. Plotting the inactivation rate constants against the inhibitor concentration (Figure 2) allowed the determination of the inactivation kinetic parameters (k_i and K_i) as summarized in Table 1.

Time-dependent inactivation was observed for cellobiosyl cyclophellitol **19** towards the four enzymes, CBHI, CBHII, EGI and EGI. The kinetic data also illustrated that cellobiosyl cyclophellitol **19** inactivates endoglucanases EGI and EGII more effectively (~10-60 fold) than cellobiohydrolases CBHI and CBHII as a possible consequence of the more open-spaced active site of endoglucanases. Of particular interest is the selective EGII activation by 4'-deoxycellobiosyl cyclophellitol Cbz-aziridine **12**, which inhibits EGII in a comparable efficiency as cellobiosyl cyclophellitol **19** with a k_i/K_i value of 2.8 mM^{-1} min and 1.48 mM^{-1} min respectively. Bearing an aromatic moiety at a

different position compared to the universal cellulase substrate 2,4-dinitrophenylcellobioside (DNPC) resulted in a complete loss of activity towards CBHI, CBHII and EGI, indicating that their active sites are quite restrictive to some modifications.

Figure 2. Inactivation plots of **12** and **19** towards CBHI, CBHII, EGI and EGII of *H. jecorina*. Inactivation enzyme assays were carried out at 30 °C in a 50 mM sodium citrate buffer, pH 5.0 containing BSA (0.1% w/v) at a range of inhibitor concentration and in the presence of DNPC (5 mM) as substrate.

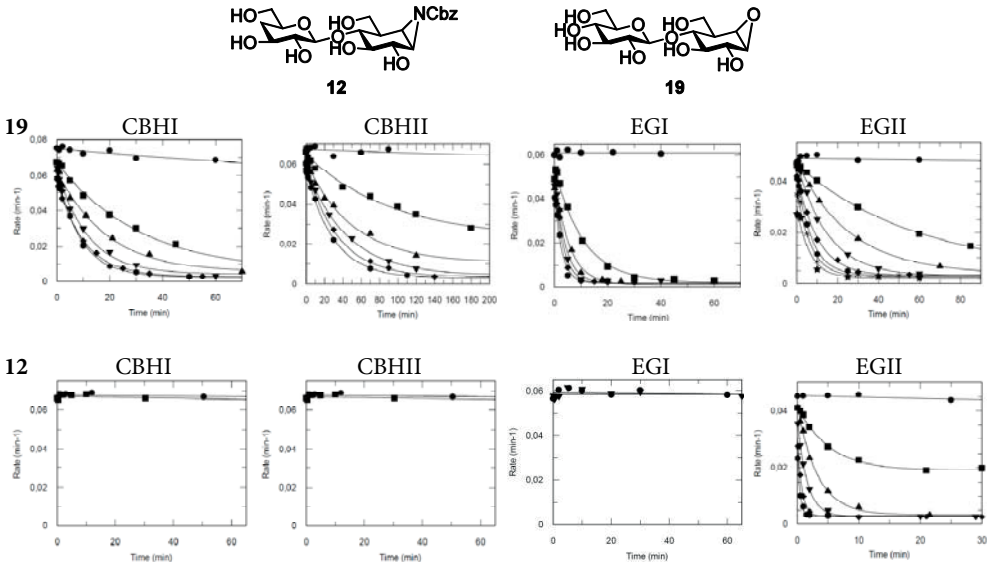


Table 1. Michaelis-Menten kinetic parameters of **12** and **19**.

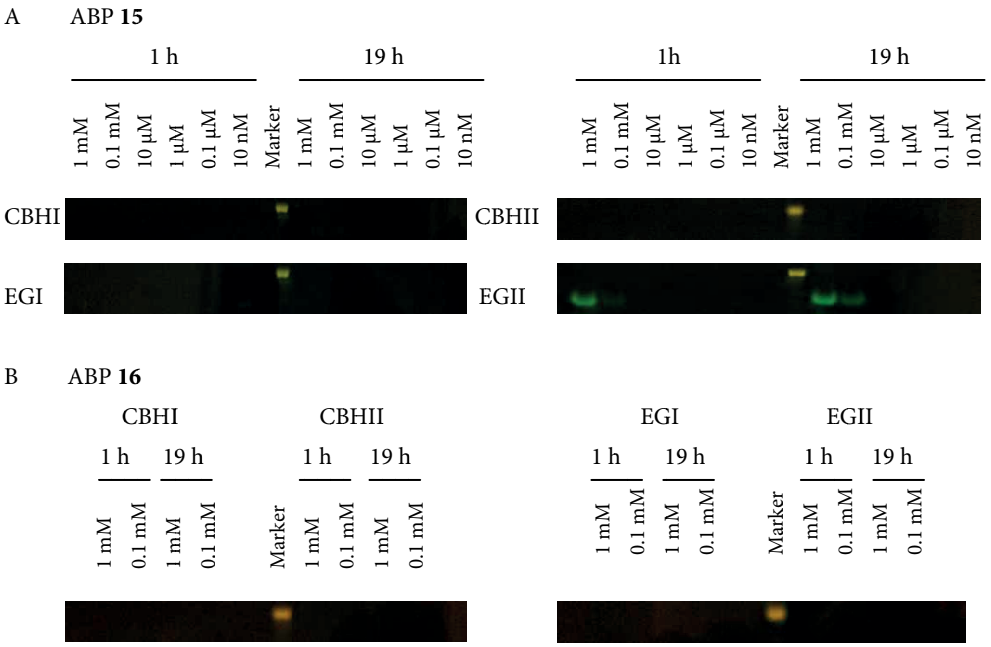
Compound	Enzyme	k_i (min)	K_i (mM)	k_i/K_i ($mM^{-1}.min^{-1}$)
Cellobiosyl cyclophellitol **19**	CBHI	0.16	1.01	0.16
	CBHII	0.036	0.74	0.049
	EGI	0.56	0.56	1.00
	EGII	0.17	0.078	2.8
4'-deoxycellobiosyl cyclophellitol Cbz-aziridine **12**	CBHI	-*	-*	-*
	CBHII	-*	-*	-*
	EGI	-*	-*	-*
	EGII	4.94	3.34	1.48

* No inactivation was observed at [I] = 1 mM

Without a sufficient amount of ABP **15** and **16** in hand to determine the corresponding binding affinity and rate of inactivation, the attention was then focused on the labeling

ability of **15** and **16** on the four cellulases from *H. jecorina*. The cellulases were incubated with different concentrations of the probes, varying from 10 nM to 1 mM (ABP **15**) and 0.1 mM to 1 mM (ABP **16**) at 30 °C for 19 h. Aliquots at two different intervals, 1 h and 19 h were taken and analyzed on SDS-page gels (Figure 3). In line with the inhibition kinetics of 4'-deoxycellobiosyl cyclophellitol Cbz-aziridine **12**, labeling was only observed for EGII with ABP **15**. However, no comparable labeling of EGII was observed with the more bulky ABP **16** illustrating that subtle alteration in the inhibitor or ABP can have deleterious effect on the enzyme activity.

Figure 3. Labeling efficiency of ABP **15** and ABP **16** towards CBHI, CBHII, EGI and EGII of *H. jecorina*. (A) Labeling of cellulases (1 mg/mL) with 10 nM-1 mM of ABP **15** for 1 h and 19 h; (B) Labeling of cellulases (1 mg/mL) with 1 mM of 0.1 mM of ABP **16** for 1 h and 19 h. All gels are 4-12% Bis-Tris Plus SDS gel with fluorescent readout.



7.3 Conclusion

This Chapter describes the glycosynthase-mediated synthesis of 4'-deoxycellobiosyl cyclophellitol aziridines analogues as mechanism-based inhibitors and ABPs for CBHI, CBHII, EGI and EGII of the filamentous fungus *H. jecorina*. This enzymatic strategy

simplifies the development of cellobiosyl cyclophellitol aziridine analogues by minimizing the elaborated functionalization steps, a common problem in the synthesis of oligosaccharides. Kinetic evaluation of cellobiosyl cyclophellitol **19** (Chapter 6) and 4'-deoxycellobiosy cyclophellitol Cbz-aziridine **12** revealed that **19** was able to inhibit the four cellulases broadly, while **12** was a selective EGII inhibitor. In the same line, only EGII was selectively labeled by ABP **15**. Modification of the reporter group in **15** into a more bulky BODIPY as in **16** was detrimental for the labeling of EGII. These results imply that by successful introduction of reporter group on the cellobiosyl cyclophellitol scaffold leads to a broad-spectrum-based ABP for the EGs and CBHs of *H. jecorina* with different efficiency, while tuning the substituents on the aziridine moiety opens up the possibility to develop specific and/or broad spectrum based probes for the *H. jecorina* cellulases. Further studies will have to justify these assumptions, but it is evident that cellobiosyl cyclophellitol **19** (Chapter 6) and cellobiosyl cyclophellitol aziridine (this Chapter) along with their analogues are promising starting points for the development of a full set of selective and broad-spectrum inhibitors and ABPs for *H. jecorina* cellulases, which ultimately will have to result in optimal industrial production of bio-ethanol from biomass.

Experimental section

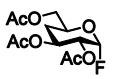
General methods: All reagents and solvents were of a commercial grade and used as received unless stated otherwise. THF and dichloromethane were stored over flamed-dried 3Å molecular sieves. All reactions were performed under an inert atmosphere unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Reactions were monitored by TLC analysis using aluminum sheets pre-coated with silica gel 60 with detection by UV-absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}.H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4.H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C or by spraying with 20% sulfuric acid in ethanol followed by charring at ~150 °C. Column chromatography was performed using Screening Device silica gel in the indicated solvents. ^{1}H NMR, ^{13}C NMR, COSY and HSQC spectra were recorded on a Bruker DMX-400 (400/100 MHz), Bruker AV-400 (400/100 MHz) and Bruker AV-600 (600/150 MHz) spectrometer in the given solvent. Chemical shifts are reported as δ-values in ppm relative to the chloroform residual solvent peak or tetramethylsilane (TMS) as internal standard or the signal of the deuterated solvent. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. High resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). HPLC-MS purifications were performed on an Agilent Technologies 1200 series automated HPLC system with a Quadropole MS 6130, equipped with a semi-preparative Gemini C18 column (Phenomenex, 250 x 10, 5μ particle size).

Phenyl 2,3-di-*O*-benzoyl-6-*O*-benzyl-4-deoxy-1-thio-β-D-glucopyranoside 8

1,1'-Thiocarbonyldiimidazole (1.71 g, 9.6 mmol) was added to a solution of thioglucoside **7**[20] (2.74 g, 4.79 mmol) in DCE (25 mL). After stirring for 3 h at 80 °C, the reaction was concentrated under reduced pressure and the crude product was purified over a small pad of silica (petroleum ether/EtOAc, 60:40→40:60) giving the Barton-McCombie intermediate, which was redissolved in toluene (95 mL). After the addition of Bu_3SnH (1.93 mL, 7.19 mmol) and AIBN (39 mg, 0. 24 mmol), the solution was stirred at 100 °C for 18 h and concentrated *in vacuo*. Purification by silica column chromatography (petroleum ether/EtOAc, 80:20) yielded **8** (2.51 g, 4.53 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (d, *J* = 7.6 Hz, 2H, H_{Ar}Bz), 7.93 (d, *J* = 8.0 Hz, 2H, H_{Ar}Bz), 7.52-7.50 (m, 2H, H_{Ar}Bz), 7.46-7.40 (m, 2H, H_{Ar}Bz), 7.34-7.26 (m, 9H, H_{Ar}Bz, H_{Ar}Bn), 7.23-7.22 (m, 3H, H_{Ar}Bn), 5.42 (dd, *J* = 2.8, 8.4 Hz, 2H, H-2, H-3), 4.95 (d, *J* = 9.2 Hz, 1H, H-1), 4.56 (s, 2H, CH_2Bn), 3.95-3.92 (m, 1H, H-5), 3.66 (dd, *J* = 6.0, 10.4 Hz, 1H, H-6), 3.58 (dd, *J* = 4.0, 10.4 Hz, 1H, H-6), 2.41 (dd, *J* = 2.0, 12.4 Hz, 1H, H-4), 1.85-1.77 (m, 1H, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.8, 165.4, 137.3, 135.6, 135.6, 133.7, 133.1, 133.1, 128.8, 128.8, 128.2, 128.2, 127.9, 127.7, 127.5, 86.4, 76.6, 73.6, 73.2, 71.2, 66.0, 32.9. HRMS: found 555.1836 $[M+H]^+$, calculated for $[C_{33}H_{31}O_6S]$ 555.1836.

2,3,6-tri-*O*-acetyl-4-deoxy-1-acetyl-β-D-glucopyranoside 9

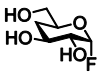
N-iodosuccinimide (1.53 g, 6.80 mmol) and TFA (0.50 mL, 6.80 mmol) were added to a cooled solution of **8** (2.51 g, 4.53 mmol) in DCM (45 mL) and H_2O (4.5 mL). The mixture was stirred at 0 °C for 1 h before being quenched with 10% sodium thiosulphate, extracted with EtOAc, washed with $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was taken up in MeOH (50 mL). A catalytic amount of NaOMe was added and the solution was stirred at ambient temperature for 1 h. The mixture was neutralized with Amberlite IR-120 H^+, filtered and concentrated *in vacuo*. The crude product was purified over short pad of silica (EtOAc/MeOH, 80:20) before being redissolved in MeOH (50 mL). A catalytic amount of $Pd(OH)_2$ was added and the mixture was stirred under H_2 atmosphere for 18 h, filtered over a plug of celite and concentrated under reduced pressure. Finally, the crude product was again taken up in pyridine (15 mL) and Ac_2O (10 mL). After stirring at ambient temperature for 4 h, the reaction was quenched with 1 M HCl, extracted with EtOA, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica column chromatography (petroleum ether/EtOAc, 60:40→40:60) yielded **9** (791 mg, 2.38 mmol, 53%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.26 (d, *J* = 4.0 Hz, 1H, H-1α), 5.59 (d, *J* = 8.0 Hz, 1H, H-1β), 5.22 (dt, *J* = 4.0, 16.0 Hz, 1H, H-3α), 5.02-4.91 (m, 3H, H-3β, H-2α and β), 4.37-4.03 (m, 3H, H-5α, H-6α and β), 3.87-3.82 (m, 1H, H-5β), 2.19-2.17 (m, 2H, H-4α and β), 2.05-1.93 (m, 24H, CH_3OAc), 1.64-1.52 (m, 2H, H-4α and β). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.4, 170.1, 169.9, 169.4, 168.8, 92.0, 90.0, 71.0, 70.3, 70.0, 67.6, 67.0, 65.6, 65.0, 64.8, 64.6, 32.2, 32.0, 32.0, 20.7, 20.5, 20.44. HRMS: found 333.1178 $[M+H]^+$, calculated for $[C_{14}H_{21}O_9]$ 333.1180.



2,3,6-tri-*O*-acetyl-4-deoxy-β-D-glucopyranosyl fluoride 10

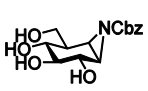
HF/pyridine (4.85 mL) was added to a plastic vial containing peracetylated **9** (791 mg, 2.38 mmol) at 0 °C and the reaction was stirred at 0 °C for 5 h. The solution was quenched by adding the solution dropswise to a mixture of solid $NaHCO_3$ and ice, until bubbling

ceased. The mixture was diluted with saturated aqueous $NaHCO_3$, extracted with EtOAc, dried over $MgSO_4$ and concentrated under reduced pressure. Purification by silica column chromatography (petroleum ether/EtOAc, 70:30→60:40) provided **10** (682 mg, 2.33 mmol, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.68 (dd, *J* = 2.4, 52.3 Hz, 1H, H-1), 5.24 (dt, *J* = 5.2, 11.2 Hz, 1H, H-3), 4.83 (ddd, *J* = 2.4, 10.0, 24.0 Hz, 1H, H-2), 4.23-4.20 (m, 1H, H-5), 4.12-4.04 (m, 2H, H-6), 2.19 (ddd, *J* = 2.0, 4.8, 10.0 Hz, 1H, H-4), 2.05 (s, 3H, CH_3OAc), 2.03 (s, 3H, CH_3OAc), 1.98 (s, 3H, CH_3OAc), 1.61 (q, 1H, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.3, 170.0, 169.7, 105.7, 103.5, 70.8, 70.6, 69.7, 67.8, 66.5, 64.7, 31.6, 20.6, 20.4, 20.3. HRMS: found 293.0959 $[M+H]^+$, calculated for $[C_{12}H_{18}O_7F]$ 293.0958.



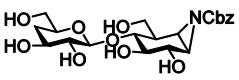
4-deoxy-β-D-glucopyranosyl fluoride 4

Ammonia was bubbled through a solution of **10** (146 mg, 0.5 mmol) in MeOH (10 mL) at 0 °C for 10 min. The reaction was allowed to come to ambient temperature. After stirring for 4 h, the reaction was concentrated and the crude **4** was used without further purification. HRMS: found 167.0702 $[M+H]^+$, calculated for $[C_6H_{12}O_4F]$ 167.0714.



7-(*N*-carboxylbenzyl)-cyclophellitol aziridine 5

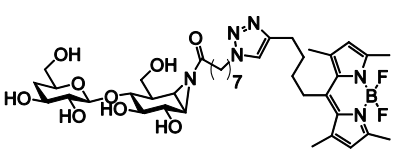
To a solution of benzyl alcohol (28 µL, 0.28 mmol) and 4-nitrophenyl chloroformate (105 mg, 0.32 mmol) in DCM (6 mL) was added pyridine (104 µL, 1.3 mmol). After stirring for 4 h at ambient temperature, the reaction mixture was quenched with brine and extracted with EtOAc, before being filtered over $MgSO_4$ and concentrated under reduced pressure. The crude product was taken up again in DMF (1 mL) and Et_3N (0.2 mL). A solution of cyclophellitol aziridine **11** (see Chapter 3, 46 mg, 0.26 mmol) in DMF (1.0 mL) was added to the pre-activated ester solution and the reaction mixture was stirred for 18 h at ambient temperature before being concentrated under reduced pressure. Purification by HPLC (50 mM NH_4HCO_3/acetonitrile) and lyophilization furnished **11** as a white powder (30 mg, 0.11 mmol, 42%). ^{1}H NMR (400 MHz, MeOD): δ 7.39-7.29 (m, 5H, H_{Ar}Bz), 5.12 (dd, *J* = 12.4, 14.0 Hz, 2H, CH_2Cbz), 4.03 (dd, *J* = 4.4, 10.4 Hz, 1H, H-8), 3.78 (dd, *J* = 7.6, 16.0 Hz, H-8), 3.70 (d, *J* = 8.0 Hz, 1H, H-2), 3.19 (dd, *J* = 8.0, 10.0 Hz, 1H, H-3), 3.10 (t, *J* = 9.6 Hz, 1H, H-4), 3.03 (dd, *J* = 2.8, 9.6 Hz, 1H, H-6), 2.74 (d, *J* = 6.0 Hz, 1H, H-1), 1.98-1.90 (m, 1H, H-5). ^{13}C NMR (100 MHz, MeOD): δ 164.6, 137.4, 129.6, 129.3, 129.1, 129.0, 79.0, 73.3, 69.3, 63.6, 45.1, 43.7, 42.2. HRMS: found 310.1288 $[M+H^+]$, calculated for $[C_{15}H_{19}NO_5]$ 310.1285.



7-(*N*-carboxylbenzyl)-4'-deoxycellobiosyl cyclophellitol aziridine 12

A solution of cyclophellitol Cbz-aziridine **5** (10 mg, 32 µmol), crude 4-deoxyGlcF **4** (10.7 mg, 64 µmol), glycosynthase Abg2F6 (1mg/mL) in a sodium phosphate buffer (3.2 mL, 100 mM, pH 7) was stirred at ambient temperature for two days before being concentrated under reduced pressure. HPLC purification (50 mM $NaHCO_3$/acetonitrile) afforded **12** (8.6 mg, 19 µmol, 59%) as a white solid. ^{1}H NMR (600 MHz, D_2O): δ 7.53-7.46 (m, 5H, H_{Ar}Cbz), 5.24 (s, 2H, CH_2Cbz), 4.69 (d, *J* = 8.0 Hz, 1H, H-1'), 4.10 (d, *J* = 8.0 Hz, 1H, H-2), 4.07 (dd, *J* = 3.6, 10.8 Hz, 1H, H-7), 3.86-3.78 (m, 2H, H-7, H-5'), 3.74-3.72 (m, 2H, H-3', H-6'), 3.64-3.58 (m, 2H, H-3, H-6'), 3.37 (t, *J* = 10.0 Hz, 1H, H-4), 3.30-3.24 (m, 2H, H-2', H-6), 2.94 (d, *J* = 6.0 Hz, 1H, H-1), 2.18-2.12 (m, 1H, H-5), 2.02-1.98 (m, 1H, H-4'), 1.52-1.45 (m, 1H, H-4'). ^{13}C NMR (150 MHz,

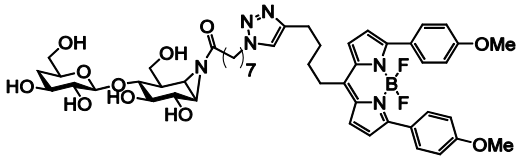
D_2O): δ 164.0, 135.4, 128.9, 128.8, 128.1, 102.9, 85.5, 75.2, 72.8, 71.2, 70.2, 69.0, 66.0, 63.5, 61.5, 42.6, 41.9, 41.0. HRMS: found 456.1862 $[M+H]^+$, calculated for $[C_{21}H_{30}NO_{10}]$ 456.1864.



BODIPY-4'-deoxycellobiosyl cyclopellitol aziridine derivative 15

Strategy 1: Disaccharide **12** (2.9 mg, 6.4 μmol) was dissolved in H_2O (0.1 mL) and 28% NH_4OH (0.2 mL) was added. The solution was stirred at ambient temperature for 2 h and concentrated under reduced pressure. The crude product was taken up in DMF (0.1 mL) and a pre-activated mixed anhydride BODIPY solution (1M, 6.4 μL, 6.4 μmol) was added at 0 °C and subsequently stirred for 18 h at 0 °C. The pre-activated mixed anhydride solution (1 M) was prepared by dissolving EEDQ (3.2 mg, 12.8 μmol) and BODIPY[22] (6.6 mg, 12.8 μmol) in DMF (12.8 μL) and the reaction was stirred at room temperature for 2 h before use. After TLC analysis showed full consumption, the volatiles were removed under reduced pressure and the crude was purified by HPLC (50 mM aqueous NH_4HCO_3/acetonitrile) to yield **15** (0.9 mg, 1.03 μmol, 16%) as an orange solid.

Strategy 2: A solution of BODIPY-cyclophellitol aziridine **13**[23] (10.3 mg, 15.4 μmol), crude 4-deoxyGlcF **4** (12.8 mg, 77 μmol), glycosynthase Abg2F6 (2 mg/mL) in DMSO (3.5 mL) and a sodium phosphate buffer (4.2 mL) was stirred at ambient temperature for two days before being concentrated *in vacuo*. The crude was filtered, concentrated under reduced pressure and subjected to HPLC purification (50 mM aqueous NH_4HCO_3/acetonitrile) to yield **15** (1.94 mg, 2.38 μL, 15%). ^{1}H NMR (800 MHz, MeOD): δ 7.75 (s, 1H, CH_{trz}), 6.12 (s, 2H, H_{Ar}), 4.47 (d, *J* = 7.7 Hz, 1H, H-1'), 4.36 (t, *J* = 6.8 Hz, 2H, CH_2N_{trz}), 4.09 (dd, *J* = 4.3, 10.0 Hz, 1H, H-7), 3.89 (d, *J* = 7.7 Hz, 1H, H-2), 3.68-3.65 (m, 2H, H-7, H-5'), 3.64-3.60 (m, 2H, H-3', H-6'), 3.59-3.56 (m, 1H, H-6'), 3.41 (dd, *J* = 7.7, 10.2 Hz, 1H, H-3), 3.18 (t, *J* = 10.2 Hz, 1H, H-2'), 3.15 (dd, *J* = 8.5, 9.4 Hz, 1H, H-4), 3.05-3.03 (m, 3H, H-6, CH_2alkyl), 2.79 (t, *J* = 6.8 Hz, 2H, CH_2alkyl), 2.74 (d, *J* = 6.0, 1H, H-1), 2.44 (s, 6H, CH_3Me), 2.40 (s, 6H, CH_3Me), 2.03-1.99 (m, 1H, H-5), 1.93-1.84 (m, 8H, H-4', CH_2alkyl), 1.68-1.64 (m, 3H, CH_2alkyl), 1.57-1.55 (m, 3H, CH_2alkyl), 1.36-1.24 (m, 10H, H-4', CH_2alkyl). ^{13}C NMR (200 MHz, MeOD): δ 188.4, 155.0, 148.5, 147.9, 142.2, 132.6, 123.4, 122.6, 105.4, 105.4, 89.0, 77.4, 74.4, 73.9, 73.9, 72.8, 72.0, 67.8, 65.4, 64.4, 64.3, 63.5, 63.4, 51.2, 49.5, 49.4, 49.3, 45.3, 42.1, 40.8, 36.7, 36.1, 32.3, 31.1, 30.8, 29.9, 29.6, 29.1, 27.2, 25.9, 25.8, 16.5, 14.4. HRMS: found 817.4480 $[M+H]^+$, calculated for $[C_{40}H_{60}BF_2N_6O_9]$ 817.4477.



BODIPY-4'-deoxycellobiosyl cyclopellitol aziridine derivative 16

A pre-activated mixed anhydride solution (0.5 M) was prepared by dissolving EEDQ (25 mg, 100 μmol) and BODIPY[22] (67 mg, 100 μmol) in DMF (200 μL) and the reaction was stirred at room temperature for 2 h before use. Disaccharide **12** (5.3 mg, 12 μmol) was dissolved in H_2O (0.2 mL) and 28% NH_4OH (0.4 mL) was added. The solution was stirred at ambient temperature for 2 h and concentrated under reduced pressure. The crude product was taken up in

DMF (0.2 mL) and a pre-activated mixed anhydride BODIPY solution (0.5 M, 12 µL, 6.0 µmol) was added at 0 °C and subsequently stirred at 0 °C. After 30 min activated anhydride solution (0.5 M, 12 µL, 6.0 µmol) was added again and stirred at 0 °C for additional 30 min. After this period, activated solution (0.5 M, 4 µL, 2 µmol) was added again and stirred at 0 °C for 30 min. The reaction was concentrated under reduced pressure and the crude was purified by HPLC (50 mM aqueous NH_4HCO_3/acetonitrile) to yield **16** (1.43 mg, 1.45 µmol, 12%) as a purple solid. ^{1}H NMR (800 MHz, MeOD): δ 7.82 (d, *J* = 6.8 Hz, 3H, H_{Ar}Ph), 7.67 (s, 1H, CH_{trz}), 7.41 (d, *J* = 4.3 Hz, 2H, H_{Ar}Ph), 6.94 (d, *J* = 9.4 Hz, 3H, H_{Ar}Ph), 6.67 (d, *J* = 4.3 Hz, 2H, H_{Ar}Ph), 4.43 (d, *J* = 7.7 Hz, 1H, H-1'), 4.30 (t, *J* = 6.8 Hz, 2H, CH_2NH_{trz}), 4.04 (dd, *J* = 4.3, 10.2 Hz, 1H, H-7), 3.85 (d, *J* = 7.7 Hz, 1H, H-2'), 3.82 (s, 6H, CH_3 OMe), 3.63 (t, *J* = 9.4 Hz, 1H, H-7), 3.59 (ddd, *J* = 5.0, 9.4, 11.0 Hz, 1H, H-3'), 3.55-3.54 (m, 2H, H-6'), 3.51-3.49 (m, 1H, H-5'), 3.38 (dd, *J* = 8.5, 10.2 Hz, 1H, H-3), 3.15 (t, *J* = 10.2 Hz, 1H, H-2'), 3.13 (dd, *J* = 7.7, 9.4 Hz, H-4), 3.04 (t, *J* = 7.7 Hz, 2H, CH_2alkyl), 2.97 (dd, *J* = 2.6, 6.0 Hz, 1H, H-6), 2.76 (t, *J* = 6.8 Hz, 2H, CH_2alkyl), 2.69 (d, *J* = 6.0 Hz, 1H, H-1), 2.43-2.36 (m, 2H, CH_2alkyl), 1.99-1.96 (m, 1H, H-5), 1.85 (m, 6H, H-4', CH_2alkyl), 1.521.49 (m, 1H, CH_2alkyl), 1.37-1.33 (m, 1H, H-4'), 1.27-1.28 (m, 6H, CH_2alkyl). ^{13}C NMR (200 MHz, MeOD): δ 188.4, 162.2, 158.8, 148.6, 146.8, 137.5, 132.2, 132.2, 132.1, 132.1, 128.5, 126.5, 123.3, 121.0, 114.6, 114.6, 114.6, 105.4, 89.0, 77.3, 74.4, 72.8, 72.0, 67.8, 65.4, 64.4, 63.4, 55.8, 51.2, 49.5, 49.4, 45.3, 42.1, 40.8, 36.7, 36.1, 34.1, 31.1, 31.0, 30.4, 29.9, 29.6, 27.2, 25.8, 25.8. HRMS: found 973.4694 $[M+H]^+$, calculated for $[C_{50}H_{64}BF_2N_6O_{11}]$ 973.4698.

Enzyme inhibition kinetics

General: All kinetic studies were carried out on a Cary 4000 UV/VIS spectrophotometer (Agilent Technologies) equipped with a circulating water bath. Quarts cuvettes (200 µL) with a pathlenght 1 cm were used and measurements were carried out at 400 nm. CBHI, CBHII, EGI and EGII were assayed at 30 °C using 2,4-dinitrophenyl-β-cellobioside (DNPC, 5 mM, 3 x K_m, saturating concentration) as substrate and sodium citrate buffer (50 mM, pH 5.0) containing 0.1% BSA.

Method: Samples of the given enzyme were incubated in the sodium citrate buffer in the presence of various concentrations of the inhibitor (**12** or **19**). Aliquots (20 µL) of these inactivation mixture were removed at the indicated time intervals (1 h to 19 h) and diluted into assay cells containing a fixed volume of DNPC (180 µL, 5 mM). The residual enzymatic activity was then determined from the rate of hydrolysis of the substrate, which is directly proportional to the amount of active enzyme. This process was monitored until 80-90% of the enzymatic activity was inactivated. Pseudo-first order rate constants (k_{obs}) of each inactivator concentration were calculated by fitting plots of the residual activity versus time to a single exponential equation using GraFit (Leatherbarrow, R.J. (2012) *GraFit version 7*, Erithacus Softward Ltd., Horley, U.K.). These values of k_{obs} were then fitted to the following equation: $k_{obs} = k_i [I] / K_i + [I]$ to obtain the kinetic parameters k_i and K_i.

Labeling experiments

The four enzymes (EGI, EGII, CBHI and CBHII), each with a concentration of (1 mg/mL) were incubated with the indicated concentrations of the inhibitor (**15** or **16**) at 30 °C for 19 h. At two time points (1 h and 19 h) samples (15 µL) were taken to be loaded on SDS PAGE gels. Samples taken after 1 h incubation were stored at -24 °C prior to loading. Inactivation mixture (15 µL) was treated with NUPage LDS sample buffer (2X, 15 µL) containing β-mercaptoethanol and heated to 90 °C for 3 min.

The obtained samples were loaded (20 µL) on to a Bolt 4-12%Bis-Tris Plus Gel from Life Technologies and ran for 35 min at 165 V using MES running buffer. The fluorescence was visualized by both an illuminated light box and by using a phosphorimager (AlphaImager) prior to staining with coomassie blue (not shown).

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