Inhibitors and probes targeting PslG

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CHAPTER 6

Summary and future prospects

Summary

This dissertation describes the synthesis of activity-based probes (**1**, **3** and **4**), oligosaccharides (**5** and **6**) and inhibitors (**7**, **8**, **9**, **10** and **11**) based on the Psl repeating unit for the interrogation of PslG (Figure 1 and Figure 2). The synthetic strategies towards the probes, inhibitors and natural substrates all used similar glycosylation protocols and sequences. To obtain the 1,2-cis linkages for the D-mannose residues, the β-Crich mannosylation method was employed, which gave most of the glycosylation products with excellent β-selectivity. In only two glycosylations an α/β mixture was obtained, however in one case these were separable and all building blocks were readily available. The glycosylation of target compound **3** required more effort, however this problem was solved by employing a more reactive acceptor. The 1,2-trans linkages were all obtained by employing glycosyl donors carrying participating protecting groups. The cyclophellitol derivatives were all made using existing routes, after which late stage protecting group manipulations allowed for their regioselective glycosylation. Eventually optimal conditions were found for each cyclophellitol derivative. The deoxynojirimycin moieties in **10** and **11** were efficiently installed using a reduction-double oxidation-double reductive amination sequence on the reducing end glucose moiety before final, global deprotection.

The probes synthesized in chapter 2 were used to determine the hydrolysis site in Psl by PslG. An ABPP experiment using probes **1**, **3** and **4** revealed that only probe **4** bound to the enzyme, indicating PslG has endo-glucosidase activity. This is opposed to what was reported in the literature, where endomannosidase activity was ascribed to PslG.[1] In chapter 3 this result was corroborated by a cleavage experiment using synthetic decasaccharide **6**. Upon treatment of **6** with PslG, only fragments with a terminal D-glucose residue at the reducing end were observed, supporting the results from Chapter 2. Then a set of three inhibitors were synthesized in chapter 4 for crystallization studies to visualize the natural binding mode of Psl. Here it was found that trisaccharide **7** and tetrasaccharide **8** showed almost no inhibition of PslG even after 24 hours (Figure 3), this in contrast to pentasaccharide **9** which proved to be a potent PslG inactivator. Crystal structures, obtained after co-crystallization with either **7** or **9** revealed that trisaccharide **7** and pentasaccharide **9** occupy the enzyme active site with the same conformation, with both compounds covalently bound to Glu276 (the postulated nucleophilic residue of PslG) at the pseudo-anomeric centre after nucleophilic displacement of the epoxide. In chapter 5 a set of two potential non-covalent inhibitors (**10** and **11**) were synthesized, but neither of the two

compounds were shown to be competitive PslG inhibitors in a competitive activity-based protein profiling assay.

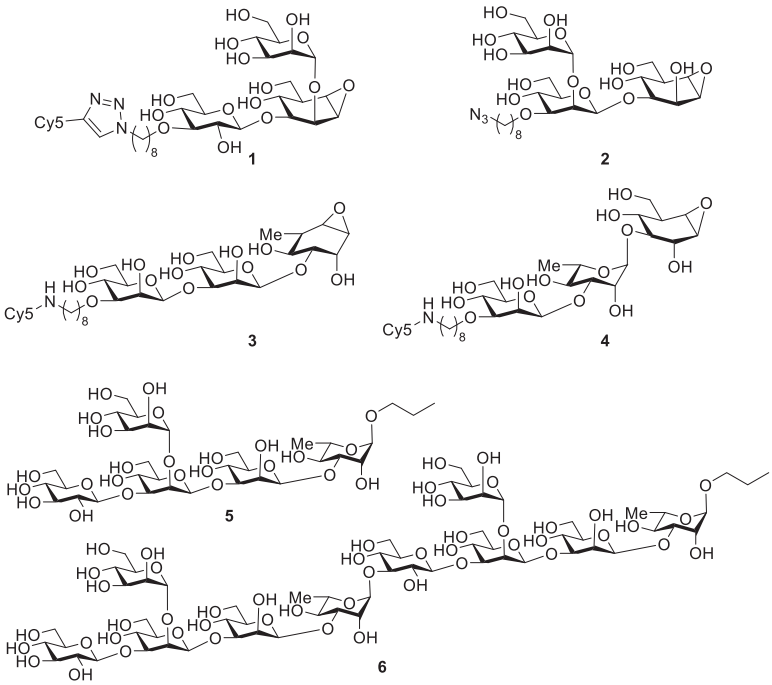


Figure 1. Compound **1**-**4** were used for the initial cleavage site determination of PslG using ABPP. In this Thesis the synthesis of the three ABPs **1**, **3** and **4** is described, whereas that of trisaccharide **2** was described previously.[1] Then Psl based compounds **5** and **6** were synthesized to corroborate the result of chapter 2 by analyzing PslG digested fragments

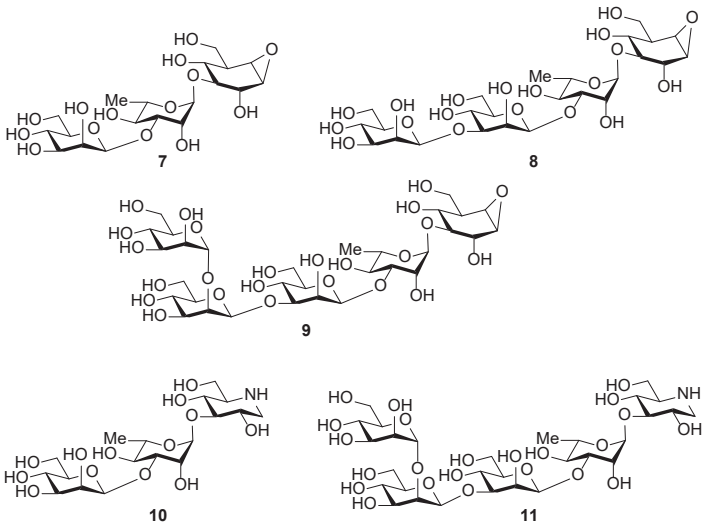


Figure 3. The inhibitors that were synthesized in this dissertation. Inhibitors **7**, **8** and **9** were used in competitive ABPP to determine their activity versus probe **4**. It was observed that inhibitor **9** inhibited PslG much more potently than inhibitors **7** and **8**. No inhibition was observed when deoxynojirimycin derivatives **10** and **11** were evaluated against covalent probe **4**.

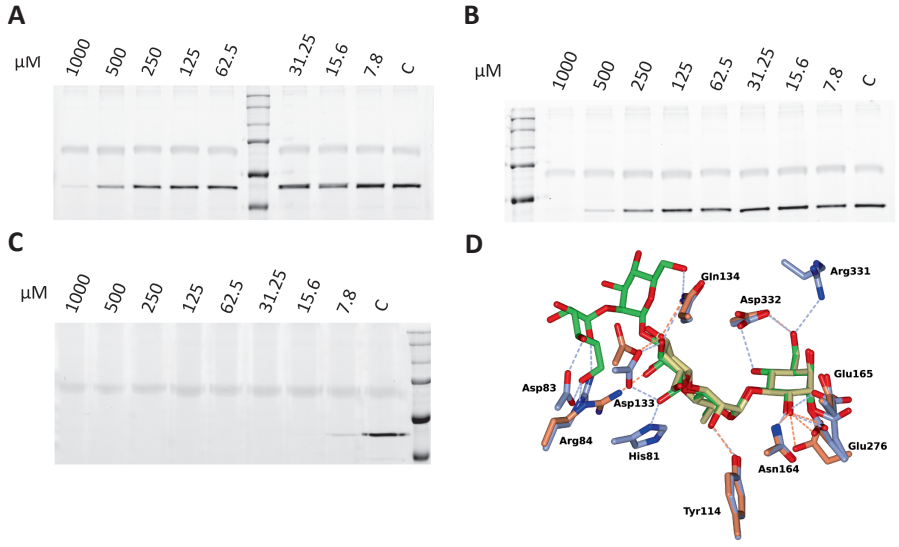
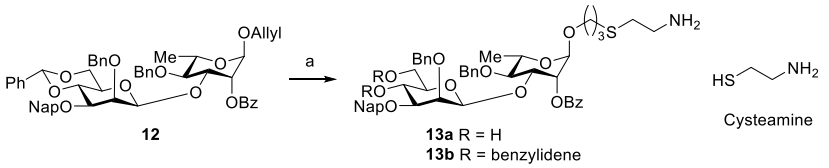


Figure 2. In-gel competitive ABPP evaluation of compounds **6**, **7** and **8**, in which the inhibitors were used at varying concentrations while 10 μM of probe **4** was used. **A**) SDS-PAGE gel for inhibitor **1**. **B**) SDS-PAGE gel for inhibitor **2**. **C** SDS-PAGE gel for inhibitor **3**. **D**) Overlay of structures with inhibitor **7** (yellow) and **9** (green) with side chains and hydrogen bonds in coral and ice blue for **7** and **9** respectively.

The probes synthesized in chapter 2 may be of future use in several biological experiments. Firstly, probe **4** could be added to *Pseudomonas aeruginosa* PAO1 (a common lab strain) to study the localization of PslG at different stages of biofilm development. For this, it first needs to be assessed whether compound **4** is cell permeable, and/or whether it penetrates *P. aeruginosa* biofilms. Moreover, it may, like inhibitors **7**-**11**, be used to study the influence of PslG inhibition on biofilm development. This would add to earlier reports describing gene knockout studies. In case the probe or inhibitors would show inhibition of biofilm development, PslG could be further pursued as an antibiotic target, with the mechanism-based PslG inactivators described in this Thesis featuring as good starting points for inhibitor development. Secondly, in case PslG itself becomes a component of a therapeutic treatment, probe **4** could be used as a quality control tool. Moreover, probe **4** could also be used in the further development of PslG, by probing its activity, in search of more stable variants of PslG in order to increase its efficacy. Lastly, all probes could be used to find other Psl degrading enzymes. For example, it has been observed that Psl could induce lysis in *Staphylococcus aureus*, therefore it is reasonable to assume that other organisms developed a mechanism to break down Psl by employing a glycoside hydrolase. Compounds **10** and **11** could be better analysed for their inhibition potential by synthesizing a fluorescent substrate for PslG, this substrate would likely require at least a trisaccharide as recognition moiety.

Pentasaccharide **5** and decasaccharide **6** could be used for additional studies towards the development of monoclonal antibodies (mAbs) or to discover their binding epitopes.[2] Prior to deprotection the allyl group can be converted into a linker containing a primary amine in order to conjugate Psl to a carrier protein. After conjugation of the Psl fragments, the conjugate can be used for vaccine development. The primary amine would be obtained by using a radical addition to the allyl using cysteamine (Scheme 1). Initial attempts to install cysteamine using AIBN as radical initiator on model compound **12** yielded small amounts of compound **13** with concomitant recovery of most of the starting material **12**. It was speculated that this was due to degradation of the cysteamine reagent, which can form S-S bonds over time. This was then verified via NMR, and it was observed that half of the cysteamine reagent had indeed formed dimers. Adding more reagent in order to install the linker had no effect, thus freshly purchased cysteamine was used and this afforded product **13a** and **13b** in a combined yield of 99%.



Scheme 1. MeOH, $HS(CH_2)_2NH_3Cl$, AIBN, 65 °C, 44% of **13a** and 55% of **13b**.

Potential probes and inhibitors to target the glycoside hydrolases from the Pel operon and the alginate operon

The *P. aeruginosa* enzyme, PelA is essential for biofilm formation and is encoded by the Pel operon. PelA has a dual mode of action and combines deacetylase activity with glycoside hydrolase activity.[3,4] It has been determined that the deacetylase activity of PelA is required for biosynthesis of Pel.[3] Recently, the role of the PelA hydrolase ($PelA_h$) activity in the Pel biosynthesis has been investigated, and it was shown that lack of $PelA_h$ activity increases adhesion to cells and alters biofilm phenotype. As well, a higher host-killing rate was observed, indicating that the $PelA_h$ functionality alters virulence.[5] A study into the molecular mechanism of $PelA_h$ showed that $PelA_h$ is able to process GalNAc oligosaccharides but not GalN oligosaccharides, and that the minimum substrate size for recognition was 7 residues. Moreover, it was found that $PelA_h$ activity increased when a larger amount of GalNAc residues were deacetylated in the oligosaccharide. Furthermore, due to lack of sequence similarity with other glycoside hydrolases a new GH family was formed, GH166. Eventually, $PelA_h$ was classified as a retaining α-1,4-N-acetylgalactosaminidase. Having a probe and inhibitor would allow to further study $PelA_h$ and its role in the biosynthesis of Pel. In Scheme 2 putative $PelA_h$ inhibitor **32a** and probe **33** are shown based on design parameters as depicted in Figure 4.

For the design of inhibitor **32a** and probe **33** several considerations are relevant (Figure 4). Due to the presence of an NH_2 in the inhibitor, an NH_2 cannot be used as a handle to selectively attach a tag, as in chapter 2. Furthermore, for the formation of the α-glycosidic linkage ideally an N_3 is used so that the building block resembles the donor employed by Wang *et al*.[6] Therefore, an alkyne appears most appropriate as a click handle to install the reporter tag. For the synthesis of the D-galactosamine cyclophellitol two strategies can be employed: installation of the NHAc either late stage (after synthesizing the D-galactose cyclophellitol) or at an early stage (when the starting material still contains a

furanose ring). Here a synthetic route is described using early-stage installation of an N_3 moiety, since more literature procedures can be found on similar compounds as described in Scheme 2.

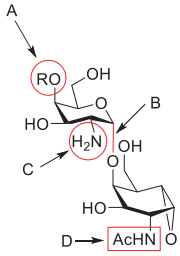
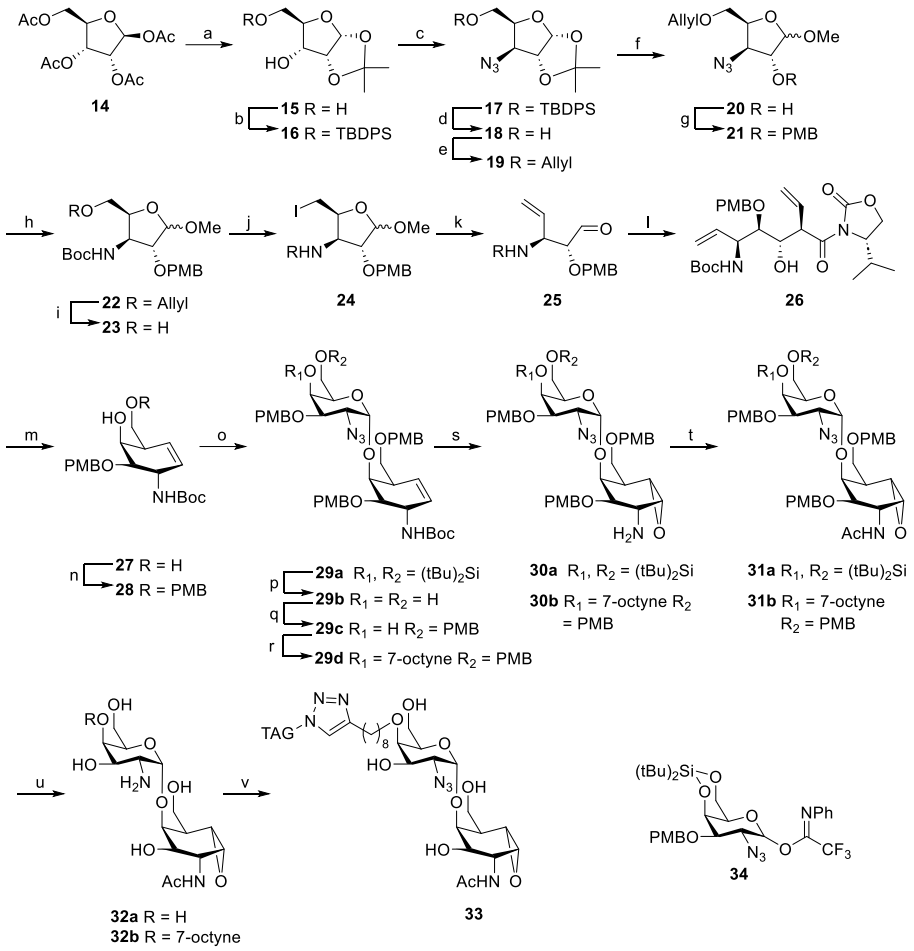


Figure 4. Considerations to be made for the construction of an inhibitor and probe for $PelA_h$. At position A, a reporter tag can be installed. At position B, an 1,2-cis glycosidic linkage has to be made. This is most efficiently done with a silylidene group bridging the 4 and 6-OH and an N_3 group at position C. Lastly, the AcHN group at position D can be installed either early or late stage.

Potential synthetic route towards inhibitor 32a and probe 33

The synthesis of inhibitor **32a** and probe **33** would commence from per-*O*-acetyl-β-D-ribofuranoside, which can be transformed into compound **16**, by following the synthesis as outlined in the Thesis of Vincent Lit.[7] Aldehyde **25** would then be obtained combining synthetic work as described by Lit as well as Sangsuwan *et al*.[7,8] The free hydroxyl in **16** can be replaced with an azide by triflating the 3-position and subsequent substitution with NaN_3 to obtain **17**. Subsequently, the TBDPS group in **17** can be replaced with an allyl group to obtain compound **19**. Then the isopropylidene can be removed using CSA in MeOH to obtain compound **20**, after which protecting the 2-position with a PMB group would result in compound **21**. Subsequent azide reduction and Boc protection results in compound **22**. Subsequent de-allylation, followed by an Apple-like reaction then delivers iodide **24**. Vasella fragmentation would then result in aldehyde **25**.[8] Using an Evans auxiliary, the aldehyde can be alkylated selectively to obtain compound **26**, which can be turned into galactosamine cyclophellitol precursor **27** after removal of the chiral auxiliary and ring-closing metathesis.[9] Using Taylor's catalyst building block **28** can be obtained, and this building block can be coupled with donor **34** to obtain **29a**.[6,10] Subsequent protecting group manipulations would then yield **29d**, which may be used to install a reporter tag. Alternatively, intermediate **29c** may be used to further elongate the disaccharide. Using NIS and AcOH in DCM and subsequent treatment with NaOH would result in epoxides **30a** and **30b**, and subsequent acetylation would then result in **31a** and **31b**.[11] The alkyne moiety could be affected during the NIS/AcOH step, therefore the protecting group manipulations could also be done on compound **31a** in order to obtain **31b**. Complete deprotection would then result in inhibitor **32a** and alkyne **32b**, and the latter can be converted into probe **33** by employing click chemistry.



Scheme 2. Potential synthesis towards putative inhibitor **32a** and probe **33**. a) I) I2, anhydrous acetone; II) NaOMe, MeOH; b) TBDPS-Cl, imidazole, DMF; c) I) Tf_2O, pyridine, DCM, -15 °C; II) NaN_3, DMF; d) TBAF, THF; e) allyl bromide, NaH, TBAI, DMF; f) CSA, MeOH, 50 °C; g) PMB-Cl, NaH, TBAI, DMF; h) I) PPh_3, THF, Et_3N H_2O; II) Boc_2O, MeOH; i) PdCl2, MeOH/DCM; j) I2, imidazole, PPh3, THF, 60 °C; k) Zn, AcOH, THF/H_2O, 40 °C; l) Evans auxiliary, Bu_2BOTf, Et_3N; m) I) 2 M $LiBH_4$, Et_2O; II) Grubbs 2nd generation, DCM, 40 °C; n) PMB-Cl, 2-ADB, K_2CO_3, KI, CH_3CN, 60 °C; o) **34**, TfOH (or TMSOTf), DCM; p) HF-pyridine, THF; q) PMB-Cl, 2-ADB, K_2CO_3, KI, CH_3CN, 60 °C; r) I) Tf_2O, pyridine, 7-octynol, DCM; II) **29c**, NaH, 7-octyn-OTf; s) I) NIS, AcOH, DCM; II) NaOH, DMF; t) Ac_2O, THF, Et_3N; u) I) HF-pyridine, THF; II) PPh_3, THF, Et_3N H_2O; III) TFA, DCM (or H_2O) or DDQ, H_2O; v) Cy5-azide, sodium ascorbate, $CuSO_4$.

Alginate is a naturally occurring polymer consisting of 1,2-cis linked D-mannuronic acids and D-guluronic acids. Alginate is widespread in nature and can be found in many different organisms, including bacteria, plants and seaweed. Commercial alginate is commonly obtained from brown seaweed and has a

plethora of applications: it is used as an additive in dehydrated products, in the manufacturing of paper and textile, for waterproofing and fireproofing of fabrics, as a thickening agent in the food industry and as an additive in the pharmaceutical industry. Coinciding with the widespread nature of alginate, the enzyme responsible for degradation of alginate, alginate lyase, is also found in multiple organisms. Alginate lyases utilize an $E1_{cb}$ mechanism (Figure 5),[12] in which first the C-5 hydrogen is activated by a positively charged metal ion or residue located close to the carboxylic acid. Then a base removes the C-5 hydrogen after which the resulting electron pair flows towards the carboxylic acid forming a double bond between C-5 and C-6. This negative charge is stabilized by the same positive moiety that is responsible for the H-5 activation. Then the π-electrons in the double bond between C-5 and C-6 flow towards C-4, forming a double bond between C-4 and C-5, breaking the C-4, O-4 bond thus eliminating the carbohydrate present at the C-4 position.

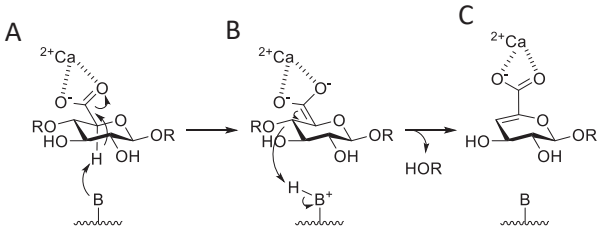


Figure 5. Mechanism of a polysaccharide lyase, the Ca^{2+} is shown as the positive ion, but this could also be another metal ion or a protonated arginine side chain.

Since the occurrence and usage of alginate is widespread, alginate lyases have a large biotechnological role. Moreover, their use is postulated for the treatment of chronic *P. aeruginosa* infections in patients suffering from cystic fibrosis, where *P. aeruginosa* predominantly uses alginate to build its biofilm.[13] Interestingly, *P. aeruginosa* is able to produce an alginate lyase as well, AlgL, and the *algL* gene can be found on the operon that encodes for enzymes involved in the biosynthesis of alginate.[14] The requirement of AlgL for alginate biosynthesis has been under debate, however a recent study from Gheorghita *et al.* was able to explain the observed literature discrepancies, by showing that AlgL is not required for alginate biosynthesis but is required for cell viability.[15] First, using point mutations they found that tyrosine 256 and arginine 249 are required for enzymatic function. The tyrosine acts as an acid/base residue while the arginine stabilizes the negative charge in the carboxylic acid of alginate during catalysis. Subsequently, using *algL* deletion strains or point mutated AlgL, they observed cell death due to cell lysis. It was previously hypothesized by Bakkevig *et al*. and Jain *et al*. that AlgL was required to maintain homeostasis in the periplasm during alginate production, which was supported by Gheorghita *et al*.[15–17] However,

Wang *et al*. reported that AlgL is not necessary for cell viability.[18] Gheorghita and co-workers could also verify this result, and showed that expression of the entire AlgU/T regulon restored cell viability even with *algL* deletion. This indicates that there are enzyme(s) present in the AlgU/T regulon that can compensate for the *algL* deletion. These results indicate that alginate lyase activity in *P. aeruginosa* is required for cell viability, thus indicating that inhibition of lyase activity can be an interesting therapeutic strategy.

Since polysaccharide lyases have a non-covalent intermediate the classical retaining glycosidase warhead will likely not apply. The lyase AlgL utilizes a tyrosine residue to remove the proton from the C-5 carbon. A common group of warheads used to target tyrosine residues are arylfluorosulphates. This warhead could be placed on O-3 of an alginate dimer resulting in potential inhibitor **35** (Figure 6). At this position it may be in close enough proximity of tyrosine 67, which is located opposite from active site tyrosine 256.[15,19] It could potentially also react with another tyrosine (259) which is also in close proximity to tyrosine 256, or it could react less selectively with another nucleophile in the active site. In order to verify this approach, docking studies with; for example, PyMOL and the crystal structures obtained by Gheorghita *et al*.[15] could be performed. Other potential inhibitors could be based on β-lactam antibiotics, more specifically the carbacephem group which have a general moiety as in **36** (Figure 6). Compounds **37** and **38** resemble potential AlgL inhibitors where an iminosugar is added as recognition group. The chloride moiety in **37** and **38** would function as a leaving group after opening of the 4-membered ring.[20–22]

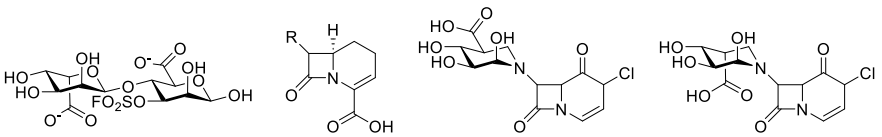
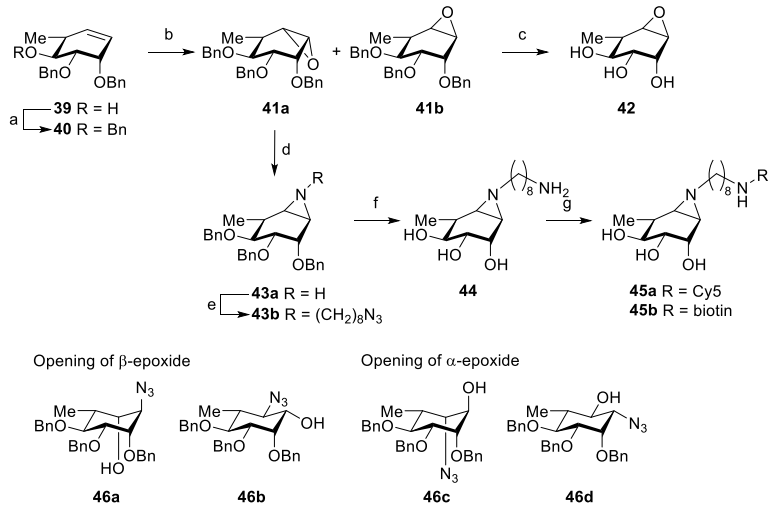


Figure 6. Potential inhibitors for AlgL. Compound **35** resembles a part of alginate where a fluorsulfonyl is attached at the 3-position as warhead. Compounds **37** and **38** resemble potential inhibitors based on the carbacephem β-lactam antibiotics, where compound **36** presents a general scaffold for the carbacephem β-lactam group. In compounds **37** and **38** the R group is either a D-mannuronic or L-guluronic acid, the chloride could function as a leaving group after opening of the 4-membered ring. Possibly the 2-OH groups in compounds **37** and **38** could be removed to stabilize the recognition moiety. [20–22]

Synthesis of a potential retaining rhamnosidase probe and inhibitor

During the synthesis of probe **3**, intermediate **39** was obtained, and this intermediate could be turned into a potential α-rhamnosidase probe and inhibitor (Scheme 3). To this end, compound **39** was benzylated using BnBr, NaH and TBAI to obtain compound **40**. Compound **40** was then epoxidized to quantitatively obtain **41**, as a separable α/β mixture. However, on NMR no clear difference was observed in the J-coupling between the protons of the α and β-epoxides. Therefore, epoxide **41** was opened using $LiClO_4$ and NaN_3, which resulted in **46a** and **46b** or **46c** and **46d**. NMR analysis then indicated, for one of the two obtained compounds, the presence of two di-axial H-H couplings at position 7, indicating the presence of either **46b** or **46d**. COSY NMR studies showed an additional coupling between the H-1 and OH proton indicating that **46b** had formed when the epoxide was opened. After determining the stereochemistry of the formed epoxides, compound **41b** could be deprotected using Birch conditions to obtain putative α-rhamnosidase inhibitor **42**. After obtaining compounds **46a** and **46b**, they were treated with PPh_3 to obtain aziridine **43a** in 23% over 2 steps. Then a spacer containing a primary amine was installed on the aziridine to obtain **43b** in 64%. Treatment of **43b** with PPh_3 on beads and subsequent dissolving metal reduction then yielded **44**. Finally, a Cy5 was installed using a condensation protocol to obtain **45a** in 18% over 3 steps.



Scheme 3. a) BnBr, TBAI, NaH, quant.; b) m-CBPA, DCM, quant., α:β, 2:1; c) Na, NH_3, t-BuOH, THF -60 °C, 63%; d) I) MeCN, $LiClO_4$, MeCN, 80 °C, 65%; II) MeCN, PPh_3 on beads, 60 °C, 36%; e) $TfO(CH_2)_8N_3$, DCM, DIPEA, 64%; f) I) MeCN, PPh_3 on beads, 70 °C; II) Na, NH_3, t-BuOH, THF, -60 °C; g) Cy5-COOPFP, DIPEA, H_2O, DMF, 18% over 3 steps.

After obtaining probe **45a** and inhibitor **42**, they were tested in *Aspergillus niger* secretomes. Secretomes were obtained by growing *A. niger* mutants: MW2.1#112, MW3.8#1 and EA21.6 on fructose, these mutant randomly express enzymes. Alternatively, *A. niger* (strain N402) was grown on different carbon sources, since by using different carbon sources *A. niger* expresses specific enzymes to efficiently break down the complex structures in the carbon source. The carbon sources used for this experiment were: fructose, pectin from apple, rhamnogalacturonan, L-rhamnose and guar gum. After obtaining the eight different secretome samples, samples were incubated with probe **45a**. Subsequently the samples were denatured and developed on an SDS-PAGE gel, after which the wet gel slap was scanned for in-gel fluorescence (Figure 7). In this experiment, any fluorescent protein band indicates reaction of the respective probe with an enzyme. As can be seen in Figure 7A there are many enzymes labelled in different mutants and when *A. niger* is grown on different carbon sources. This was not expected since there are no retaining exo-rhamnosidases described in the Cazy database.[23] The observed enzyme labelling in Figure 7A could therefore also be aspecific labelling. Therefore, a competitive ABPP experiment was run on several selected mutants and carbon sources: MW3.8#1 (lane 2 in Figure 7C), pectin from apple (lane 6 in Figure 7C) and guar gum (lane 9 in Figure 7C). First each secretome was incubated with 1 mM of inhibitor **42** for 2 hours, then probe **45a** was added and the samples were denatured and developed on an SDS-PAGE gel, after which the wet gel slap was scanned for in-gel fluorescence. In this experiment, any lack of fluorescent labelling of a protein band indicates reaction of the inhibitor. As can be observed in Figure 7C no inhibition of the labelled proteins was observed using inhibitor **42**. It has to be noted that there was labelling observed of different enzymes in lanes where the samples were denatured prior to the start of the experiment. Since no evidence of specific or covalent labelling was observed using this competitive ABPP experiment a pH dependent experiment was run. The pH of secretome samples from pectin apple were adjusted towards pH 2 or pH 9 using 1 M HCl or 1 M NaOH respectively. Then the samples were incubated with probe **45a** and the samples were denatured and developed on an SDS-PAGE gel, after which the wet gel slap was scanned for in-gel fluorescence (Figure 7E). As can be observed in Figure 7E the amount of labelling of a specific enzyme around 100 kDa differed by changing the pH values. There was optimal binding at pH 4, and to a lesser extent at pH 5 and 6. At pH 3 and pH 7 a small amount of labelling was observed, at pH 2, 3, 8 and 9 no labelling was observed. This does indicate that the labelling of the enzyme could be in a covalent manner since the amount of labelling differs at different pH values. However, more experiments are needed in order to

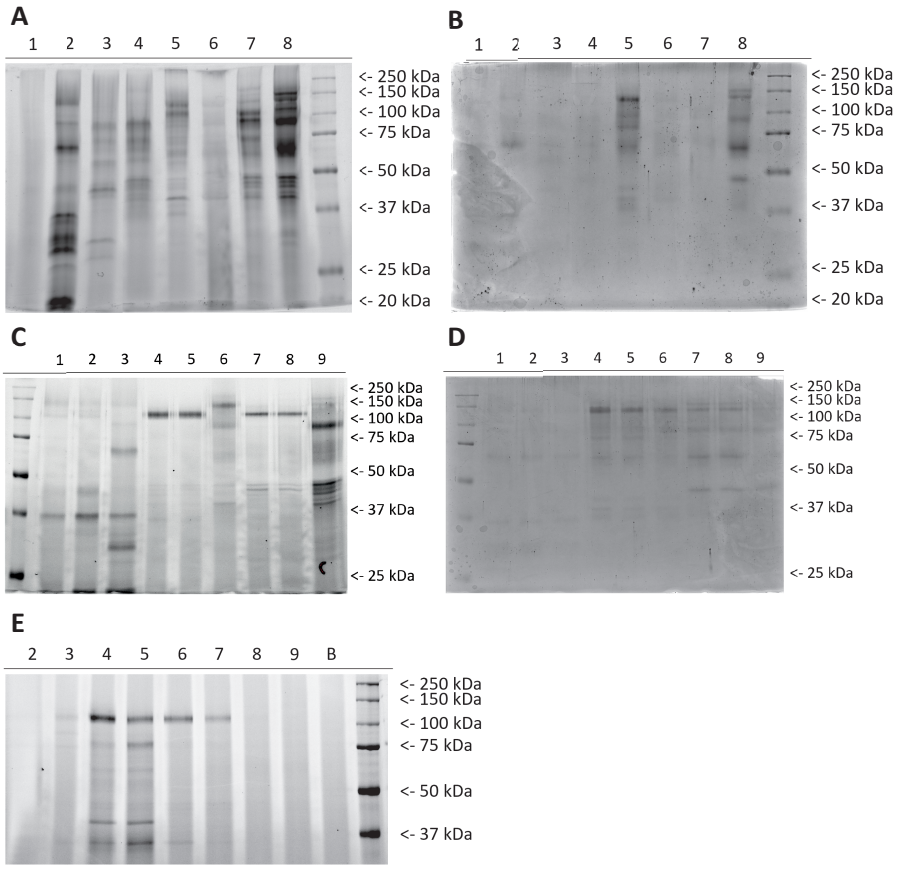
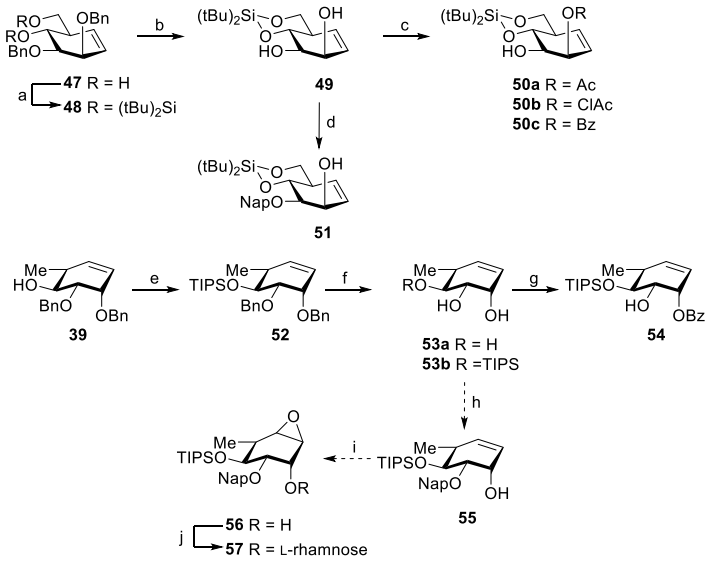


Figure 7. **A**) Fluorescent gel using 10 µM of probe **45a** with different *A. niger* mutants and *A. niger* (strain N402) grown on various carbon sources. 1) MW2.1#112; 2) MW3.8#1; 3) EA21.6; 4) fructose; 5) pectin from apple; 6) rhamnogalacturonan; 7) L-rhamnose monohydrate; 8) guar gum. **B**) Coomassie stain from the gel shown in **A**. **C**) competitive ABPP using 1 mM of inhibitor **42** and 10 µM of probe **45a**. For lanes 1-3 mutant MW3.8#1 is used, in lane 1 there is only probe, in lane 2 inhibitor + probe and lane 3 is the boiled control prior to inhibition. For lanes 4-6 pectin apple is used as carbon source, in lane 4 there is only probe, in lane 5 inhibitor + probe and lane 6 is the boiled control prior to inhibition. For lanes 7-9 guar gum is used as carbon source, in lane 7 there is only probe, in lane 8 inhibitor + probe and lane 9 is the boiled control prior to inhibition. **D**) Coomassie stain from the gel shown in **C**. **E**) Fluorescence imaging of gels using secretomes grown on pectin apple, then adjust to the corresponding pH values using 1 M HCl or 1 M NaOH, after which the samples were incubated with 10 µM of probe **45a**.

identify probe-reacted proteins. This could be done by making biotin probe **45b**. Using biotin probe **45b** a pull down could be performed in order to enrich the covalently bound enzymes, after a trypsin digest the peptide fragments could be obtained. These results could then be cross-referenced against the expected transcribed enzymes from *A. niger* in order to determine which enzyme is labelled by rhamnosidase probe **45b**. Moreover, probe **45a** and putative biotin probe **45b** could be used to discover enzymes from other sources as well, such as the gut microbiome.

Late stage deprotection and protection strategies of cyclophellitol based compounds

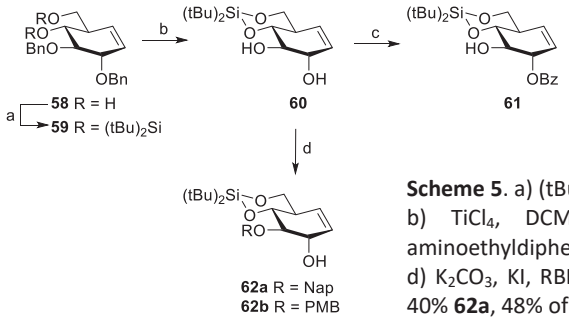
During the synthesis of probes **1** and **3** late-stage protection-deprotection had to be investigated on D-mannose cyclophellitol and L-rhamnose cyclophellitol (Scheme 4). Compound **50c** had already been made previously by de Boer.[24] After obtaining **47** from the cyclophellitol route pioneered by Madsen and co-workers,[25] a silylidene was installed to obtain **48**. Then, after debenzylation using $TiCl_4$, compound **49** could be obtained using borinate catalysis. In this work it was found that **51** could be obtained in good yield when **49** was treated with Bu_2SnO and subsequent addition of NapBr and TBABr at 60 ^oC in toluene. A similar route could be applied to rhamnose cyclophellitol precursor **39**, by installing a TIPS group, giving **52**, and subsequent debenzylation to obtain **53b**. Treating **53b** with the same borinate catalyst resulted in compound **54** in good yield. Attempts to selectively naphthylate compound **53a** all failed (chapter 2), however using building block **53b**, and the conditions to selectively install a naphthyl group on **49** (manno compound) may result in **55**. Having the 2-OH free would then allow for selective epoxidations, to form compound **56**, and subsequent glycosylation, to form **57**. This would then result in inhibitors and probes to target endo-rhamnosidases.



Scheme 4. a) $(tBu)_2Si(OTf)_2$, imidazole, DMF, 0 °C -> rt, 86%; b) $TiCl_4$, DCM, -20 °C, 84%; c) for **50a**: I) trimethylorthoacetate, pTsOH, MeCN; II) 80% AcOH, 99%; for **50b**: I) 2-chloro-1,1,1-trimethoxy-ethane, pTsOH, MeCN; II) 80% AcOH, 90%, 2-OClAc:3-OClAc, 1:6.5; for **50c**: DIPEA, BzCl, 2-ADB, MeCN, 65%; d) I) Bu_2SnO, toluene, 140 °C; II) TBABr, NapBr, 60 °C, 89%; e) TIPSOTf, 2,6-lutidine, DCM, 0 °C, 98%; f) $TiCl_4$, DCM, 79%; g) DIPEA, BzCl, 2-ADB, MeCN, 81%; h)) I) Bu_2SnO, toluene, 140 °C; II) TBABr, NapBr, 60 °C; i) I) m-CPBA, DCM, II) suitable L-rhamnose donor.

The synthetic strategies outlined above could also be applied to D-glucose cyclophellitol and D-galactose cyclophellitol (Scheme 5). After obtaining compound **58** from the known cyclophellitol route, it can be silylated using $(tBu)_2Si(OTf)_2$ and imidazole to obtain **59**, which can be transformed into diol **60** by using $TiCl_4$. From here the 2-position can be efficiently benzoylated in 4 hours using Taylors catalyst and DIPEA to obtain **61**.[10] When the same reaction is performed without the catalysts, **61** is obtained in 58%, together with starting material **60**, but the reaction requires 30 hours. Attempts to naphthylate the 3-position using Bu_2SnO resulted in low yields. Alternatively, naphthylation using Taylors catalyst, NapBr (1.2 eq.) KI and K_2CO_3 (1.2 eq.) in MeCN at 60 °C gave **62a** in 40% together with 48% recovered starting material. This result could not be improved by prolonged stirring or adding more catalyst. Using PMBBr instead of NapBr also did not gave more conversion towards **62b**. During all of the attempts to selectively protect the 3-position no alkylation of the 2-position was observed, therefore it would be interesting to see the result of this reaction when more equivalents of NapBr and K_2CO_3 would be used. The same methodology outlined above could also be applied to D-galactose cyclophellitol, this would then allow

to target endo-galactosidases by elongation from either the 2 or 3-position. Alternatively, selective epoxidation could be performed using the allylic alcohol in compounds **62a** and its galactose derivative. Additionally, the above-mentioned methodology could be applied to different types of cyclophellitol derivatives as well, such as L-fucose.



Scheme 5. a) $(tBu)_2Si(OTf)_2$, imidazole, DMF, rt, 84%; b) $TiCl_4$, DCM, -20 °C, 75%; c) BzCl, 2-aminoethyldiphenyl-borinate (2-ADB), DIPEA, 74%; d) K_2CO_3, KI, RBR, 2-ADB, MeCN, 60 °C, for NapBr: 40% **62a**, 48% of **60**; for PMB-Cl: 53%.

Acknowledgments

Massimo Tedeschi is acknowledged for providing the *A. niger* secretome samples, and Douwe Osinga for his synthetic efforts in the protecting group manipulations of the D-glucose and D-galactose building blocks.

Experimental

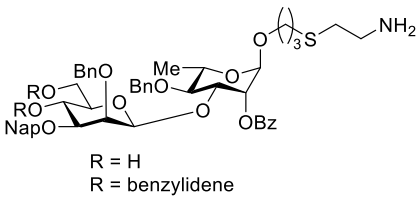
General experimental

Chemicals were purchased from Sigma Aldrich, Acros, Fluorochem, VWR, Carbosynth, Fischer Scientific and Merck. Chemicals were used as received unless stated otherwise. Toluene, DCM, THF, DMF and ACN were stored over flame-dried molecular sieves (either 3 or 4Å) before use. All reactions were performed under a nitrogen atmosphere unless stated otherwise. Thin layer chromatography was performed on Merck aluminium sheets (Silica gel 60 F254). For initial UV detection a lamp set to 254 nm was used after which spots were further visualized by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid or a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, followed by charring at 150 °C. Flash column chromatography was performed using Screening Device B.V. silica gel (particle size of 40 – 63 µm, pore diameter of 60 Å) with the indicated eluents. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-300 (300 and 75 MHz respectively), Bruker AV-400 (400 and 101 MHz respectively), Bruker AV-400WB (400 and 101 MHz respectively), a Bruker AV-500 (500 and 126 MHz respectively), a Bruker AV-600 (600 and 151 MHz respectively) or a Bruker-850

(800 and 200 MHz respectively) spectrometer in the given solvent. Chemical shifts are reported in ppm (δ) relative to the residual solvent peak or tetramethyl silane (0 ppm) as internal standard and coupling constants are given in Hz. An Äkta explorer (GE Healthcare) using 1.6x60 cm Toyopearl HW-40S resin was used for gel filtration. Elution of the compounds was done with a solution of 1% AcOH in ACN/H2O, 1/9, v/v for acid labile compounds or 150 mM solution of NH_4HCO_3 for base labile compounds. Refractive index was used to analyse fractions. For reverse phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi-preparative column (Gemini C18, 250 x 10 mm, 5 µm particle size, Phenomenex) was used. LC/MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 5 µm particle size, Phenomenex), coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI+). The applied buffers were H_2O, MeCN and 1% aqueous TFA. High-resolution mass spectrometry (HRMS) analysis was performed with a LTQ Orbitrap mass spectrometer (Thermo Finnigan). The LTQ Orbitrap is equipped with an electron spray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL/min, capillary temperature 250 °C) with a resolution R = 60000 at m/z 400 (mass range m/z = 150 – 2000) and dioctyl phthalate (m/z = 391.28428) as a lock mass. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

NMR assignments are done as follows: the anomeric C/H is denoted as 1 and numbered through the ring, ending with C-6/H-6 at the primary alcohol. Cyclophellitol compounds are numbered the same method and the additional ring C/H is denoted as 7. Each consecutive carbohydrate is assigned an additional ‘ (so the 2nd carbohydrate residue has one ‘ and the 3rd carbohydrate has two “ etc.).

2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-naphthyl-β-D-mannose-(1->3)-1-*O*-(2-aminoethyl)thio)propoxy)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnose (13a and 13b)

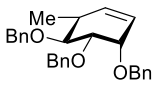


R = H
R = benzylidene

Compound **12** (0.088 g, 0.1 mmol) was dissolved in MeOH (0.8 mL, 0.125 M) and flushed with Argon for 10 min. Freshly bought cysteamine-HCl (0.17 g, 1.5 mmol, 15 eq.) and AIBN (0.1 mL, 0.2 M, 0.2 eq.) were added. The mixture was heated to 65 °C and after stirring for 24 hours TLC indicated full conversion. The mixture was diluted with DCM and washed with 1 M NaOH, brine, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Column chromatography (1% -> 5% MeOH in DCM then 10% MeOH, 1% Et_3N in DCM) yielded compound **13a** (0.042 g, 0.044 mmol) in 44% and compound **13b** (0.048 g, 0.055 mmol) in 55%. With benzylidene; ^1H NMR (400 MHz, $CDCl_3$) δ = 8.16 – 7.07 (m, 27H,

Aromatic), 5.54 (s, 1H, benzylidene), 5.32 (dd, *J*=3.5, 1.7, 1H, H-2), 4.86 (d, *J*=1.7, 1H, H-1), 4.78 – 4.71 (m, 2H, H-1', CH_2-Bn/Nap), 4.71 – 4.51 (m, 5H, CH_2-Bn/Nap), 4.27 (dt, *J*=9.7, 5.6, 1H, H-6), 4.16 (dd, *J*=9.5, 3.5, 1H, H-3), 4.10 (t, *J*=9.6, 1H, H-4'), 3.84 – 3.73 (m, 3H, H-5, H-6', CH_2-linker), 3.72 (d, *J*=3.1, 1H, H-2'), 3.61 (t, *J*=9.5, 1H, H-4), 3.51 (dt, *J*=9.9, 6.1, 1H, CH_2-linker), 3.42 (dd, *J*=9.9, 3.1, 1H, H-3'), 3.30 (dt, *J*=11.9, 6.1, 1H, H-5'), 3.10 (q, *J*=7.3, 2H, CH_2-linker), 2.95 – 2.88 (m, 2H, CH_2-linker), 2.62 (d, *J*=14.3, 2H, CH_2-linker), 1.92 – 1.83 (m, 2H, CH_2-linker), 1.37 (t, *J*=7.3, 3H, H-6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.8, 138.5, 138.1, 137.6, 135.8, 133.2, 132.9, 130.1, 129.8, 128.8, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.9, 127.8, 127.6, 127.4, 127.2, 126.2, 126.0, 126.0, 125.8, 125.4 (Aromatic), 103.4 (Benzylidene), 101.3 (C-1), 97.1 (C-1'), 81.5 (C-4), 78.3 (C-3'), 78.3 (C-4') 77.7 (C-3), 75.9 (C-2'), 75.3 (CH_2-Bn/Nap), 74.3 (CH_2-Bn/Nap), 73.5 (C-2), 72.2 (CH_2-Bn/Nap), 68.5 (CH_2-linker), 67.7 (C-5), 67.5 (C-5'), 66.1 (C-6') , 39.3 (CH_2-linker), 29.2 (CH_2-linker), 29.1 (CH_2-linker), 28.3 (CH_2-linker), 18.2 (C-6). Without benzylidene; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 – 6.95 (m, 22H), 5.45 (dd, *J* = 3.5, 1.7 Hz, 1H, H-2), 4.75 (d, *J* = 1.7 Hz, 1H, H-1), 4.69 (d, *J* = 11.5 Hz, 1H, CH_2-Bn/Nap), 4.65 (s, 1H, H-1'), 4.63 – 4.42 (m, 5H, CH_2-Bn/Nap), 4.14 (dd, *J* = 9.5, 3.4 Hz, 1H, H-3), 3.86 – 3.69 (m, 5H, H-5, H-6 (2x), H-4'/H-5', CH_2-linker (1x), 3.61 (d, *J* = 2.9 Hz, 1H, H-2'), 3.56 (t, *J* = 9.5 Hz, 1H, H-4), 3.52 – 3.40 (m, 1H, CH_2-linker (1x)), 3.26 – 3.19 (m, 2H, H-3', H-4'/H-5'), 2.89 (td, *J* = 6.6, 2.0 Hz, 2H, CH_2-linker), 2.69 – 2.64 (m, 2H, CH_2-linker), 2.58 (t, *J* = 7.1 Hz, 2H, CH_2-linker), 1.82 (q, *J* = 6.5 Hz, 2H, CH_2-linker), 1.33 (d, *J* = 6.3 Hz, 3H, H-6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 138.5, 138.1, 135.8, 133.4, 133.1, 132.8, 129.8, 129.7, 128.5, 128.5, 128.0, 128.0, 127.8, 127.6, 127.3, 127.2, 126.2, 126.1, 125.8, 125.6 (Aromatic), 103.1 (C-1'), 97.3 (C-1), 81.4 (C-3'/C-4'/C-5'), 81.2 (C-4), 77.8 (C-3), 76.9 (C-3'/C-4'/C-5'), 75.4 (C-2'), 75.3 (CH_2-Bn/Nap), 74.1 (CH_2-Bn/Nap), 73.4 (C-2), 72.0 (CH_2-Bn/Nap), 67.9 (C-5/C-4'/C-5'), 66.8 (C-5/C-4'/C-5'), 66.0 (CH_2-linker), 61.8 (C-6'), 40.2 (CH_2-linker), 32.9 (CH_2-linker), 29.2 (CH_2-linker), 28.4 (CH_2-linker), 18.1 (CH_2-linker).

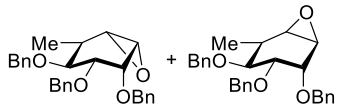
2,3,4-tri-*O*-benzyl-L-rhamnose-cyclohexene (40)



Compound **39** (0.084 g, 0.26 mmol) was co-evaporated one time with toluene and dissolved in dry DMF (1.3 mL, 0.2 M) under N_2 and cooled to 0 °C. BnBr (0.046 mL, 0.39 mmol, 1.5 eq.), TBAI (4 mg, 0.013 mmol, 0.05 eq.) and NaH (0.02 g, 0.052 mmol, 2 eq.) were added. After stirring for 24 hours additional BnBr (0.046 mL, 0.39 mmol, 1.5 eq.) and NaH (0.016 g, 0.039 mmol, 1.5 eq.) were added. After stirring for an additional 6 hours, the reaction was cooled to 0 °C and quenched with H_2O. The mixture was then diluted with Et_2O and washed with H_2O (3x), brine, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Column chromatography (5% -> 20% Et_2O in pentane) yielded compound **40** (0.11 g, 0.26 mmol) quantitatively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.22 (m, 15H, Aromatic), 5.67 (ddd, *J* = 9.8, 5.1, 2.6 Hz, 1H, H-1), 5.57 (dd, *J* = 9.8, 2.3 Hz, 1H, H-7),

5.10 – 4.61 (m, 6H, CH_2-Bn), 4.06 (ddd, *J* = 4.9, 3.7, 0.9 Hz, 1H, H-2), 3.69 (dd, *J* = 10.0, 8.1 Hz, 1H, H-4), 3.62 (dd, *J* = 10.0, 3.7 Hz, 1H, H-3), 2.27 (dddt, *J* = 8.2, 5.9, 2.5, 1.3 Hz, 1H, H-5), 1.14 (d, *J* = 7.1 Hz, 3H, H-6). HRMS $[M+Na]^+$ calc. 437.2087 found 437.2083

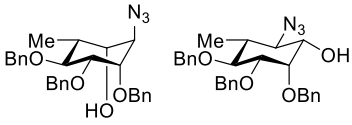
2,3,4-tri-*O*-benzyl-L-rhamno-cyclophellitol (41a, 41b)



41a **41b**

Compound **40** (0.11 g, 0.26 mmol) was dissolved in dry DCM (2.7 mL, 0.1 M) under N_2 and mCPBA (0.094 g, 0.52 mmol, 2 eq.) was added. TLC indicated complete conversion after 24 hours and the crude reaction mixture was evaporated. Column chromatography (5% -> 35% Et_2O in pentane) yielded α-epoxide **41b** (0.075 gr, 0.17 mmol) in 65% and β-epoxide **41a** (0.042 g, 0.098 mmol) in 37%, both with trace amounts of meta-chlorobenzoic acid. α-epoxide: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 – 7.20 (m, 15H, Aromatic), 5.04 – 4.55 (m, 6H, CH_2-Bn), 4.26 (t, *J* = 2.9 Hz, 1H, H-2), 3.69 (dd, *J* = 10.0, 2.9 Hz, 1H, H-3), 3.57 (dd, *J* = 10.1, 8.2 Hz, 1H, H-4), 3.20 (t, *J* = 3.2 Hz, 1H, H-1), 2.86 (d, *J* = 3.6 Hz, 1H, H-7), 2.15 (p, *J* = 7.5 Hz, 1H, H-5), 1.17 (d, *J* = 7.4 Hz, 3H, H-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.6, 138.9, 138.8, 138.7, 134.8, 134.5, 133.9, 131.2, 130.4, 130.3, 130.0, 129.5, 128.6, 128.6, 128.5, 128.4, 128.4, 128.2, 128.1, 128.0, 127.9, 127.7, 127.7, 127.5 (Aromatic), 80.6 (C-3), 80.1 (C-4), 75.3 (CH_2-Bn), 74.2 (CH_2-Bn), 74.0 (CH_2-Bn), 73.2 (C-2), 57.1 (C-7), 54.7 (C-1), 36.7 (C-5), 17.0 (C-6). β-epoxide ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 – 7.19 (m, 15H, Aromatic), 5.00 – 4.49 (m, 6H, CH_2-Bn), 4.02 (t, *J* = 4.8 Hz, 1H, H-2), 3.59 – 3.44 (m, 1H, H-4), 3.36 (dd, *J* = 9.8, 5.0 Hz, 1H, H-3), 3.18 (dd, *J* = 4.7, 3.7 Hz, 1H, H-1), 3.14 (dd, *J* = 3.7, 2.1 Hz, 1H, H-7), 2.00 (ddd, *J* = 8.8, 6.8, 2.1 Hz, 1H, H-5), 1.36 – 1.20 (m, 3H, H-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.8, 138.6, 138.2, 133.8, 130.3, 129.9, 128.5, 128.5, 128.4, 128.4, 128.3, 128.0, 128.0, 127.9, 127.7, 127.7 (Aromatic), 81.0 (C-3), 77.6 (C-4), 75.5 (CH_2-Bn), 72.5 (CH_2-Bn), 71.6 (CH_2-Bn), 70.6 (C-2), 58.3 (C-7), 52.1 (C-1), 37.8 (C-5), 15.2 (C-6).

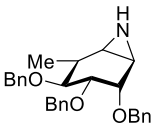
1-azido-2,3,4-tri-*O*-benzyl-L-rhamnose-cyclohexane (46a, 46b)



Compound **41a** (0.108 g, 0.25 mmol) was dissolved in dry MeCN (0.05 M, 5 mL) under N_2 and NaN_3 (0.23 g, 3.5 mmol, 14 eq.), $LiClO_4$ (0.53 g, 5 mmol, 20 eq.) were added and the reaction mixture was heated to 80 °C. TLC-MS indicated complete conversion after 24 hours and the reaction mixture was diluted with Et_2O, quenched with H_2O, after which the mixture was then further diluted with Et_2O, washed with H_2O, brine, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Column chromatography (10% -> 40% Et_2O in pentane) then yielded compound **46**

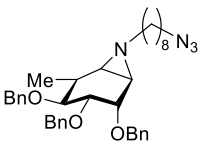
(0.077 g, 0.16 mmol) in 65% as a regio-isomeric mixture. Azide position 7: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 – 7.27 (m, 15H, Aromatic), 5.14 – 4.92 (m, 2H, CH_2-Bn), 4.78 – 4.69 (m, 2H, CH_2-Bn), 4.69 – 4.60 (m, 2H, CH_2-Bn), 4.06 (t, *J* = 2.7 Hz, 1H, H-2), 3.56 (dd, *J* = 10.4, 9.5 Hz, 1H, H-4), 3.44 (dd, *J* = 9.6, 2.3 Hz, 1H, H-3), 3.41 (d, *J* = 8.8 Hz, 1H, H-2), 3.20 (dd, *J* = 11.3, 9.8 Hz, 1H, H-1), 2.31 – 2.22 (m, 1H, 1-OH), 1.42 – 1.33 (m, 1H, H-5), 1.18 (d, *J* = 6.4 Hz, 3H, H-6). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.5, 138.5, 138.2, 128.8, 128.7, 128.6, 128.6, 128.3, 128.3, 128.2, 128.2, 128.0, 128.0, 127.9, 127.9, 127.9, 127.8 (Aromatic), 83.7 (C-3), 80.8 (C-4), 78.1 (C-2), 75.8 (CH_2-Bn), 75.1 (CH_2-Bn), 73.9 (C-1), 73.2 (CH_2-Bn), 67.8 (C-7), 39.3 (C-5), 15.3 (C-6). Azide position 1: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 – 7.17 (m, 15H, Aromatic), 4.78 – 4.49 (m, 6H, CH_2-Bn), 3.97 (t, *J* = 6.7 Hz, 1H, H-2), 3.84 – 3.76 (m, 2H, H-3, H-7), 3.73 (d, *J* = 5.4 Hz, 1H, H-1), 3.66 (t, *J* = 6.3 Hz, 1H, H-4), 2.20 (s, 1H, H-5), 1.19 (d, *J* = 7.2 Hz, 3H, H-6). HRMS $[M+Na]^+$ calc. 496.2207 found 496.2205

L-rhamnose-α-cyclophellitol aziridine (43a)



Compound **46** (0.076 g, 0.16 mmol) was co-evaporated with toluene (1x), dissolved in dry MeCN (1.6 mL, 0.1 M) under N_2, PPh_3 (0.32 mmol, 0.084 g, 2 eq.) was added and the mixture was heated to 60 °C. TLC indicated complete conversion after 24 hours and the reaction mixture was concentrated *in vacuo*. Column chromatography (10% -> 50% EtOAc in pentane) yielded compound **43a** (0.025 g, 0.057 mmol) in 36%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.23 (m, 15H, Aromatic), 5.00 – 4.53 (m, 6H, CH_2-Bn), 4.23 (t, *J* = 2.7 Hz, 1H, H-2), 3.70 (dd, *J* = 9.9, 2.9 Hz, 1H, H-3), 3.56 (dd, *J* = 9.9, 8.4 Hz, 1H, H-4), 2.44 (dd, *J* = 5.8, 2.4 Hz, 1H, H-1), 2.03 (q, *J* = 7.5 Hz, 1H, H-5), 1.97 (d, *J* = 5.8 Hz, 1H, H-7), 1.19 (d, *J* = 7.2 Hz, 3H, H-6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.2, 139.2, 128.4, 128.4, 128.2, 127.9, 127.7, 127.7, 127.5, 127.5 (Aromatic), 81.0 (C-3/C-4), 80.9 (C-3/C-4), 75.4 (C-2), 75.1 (CH_2-Bn), 73.5 (CH_2-Bn), 73.1 (CH_2-Bn), 37.6 (C-5), 35.1 (C-7), 34.9 (C-1), 18.8 (C-6). HRMS $[M+Na]^+$ calc. 430.2377 found 430.2374

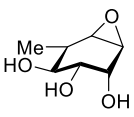
L-rhamnose-α-cyclophellitol-(N-azido-octane)-aziridine (43b)



8-azido-octanol (0.086 g, 0.5 mmol) was co-evaporated thrice with toluene and dissolved in dry DCM (2.5 mL, 0.2 M). This solution was added dropwise to a mixture of Tf_2O (0.1 mL, 0.6 mmol, 1.2 eq.) and pyridine (0.048 mL, 0.6 mmol, 1.2 eq.) in dry DCM (2.5 mL, 0.2 M, final concentration 0.1 M) at -20 °C under N_2. After stirring for 1 hour the reaction mixture was diluted with DCM and washed with cold H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. Compound **43a** (0.038 g, 0.089 mmol) was co-evaporated with toluene (3x), dissolved in dry DCM (1 mL, 0.089 M) under N_2, DIPEA (0.03 mL, 0.18 mmol, 2 eq.) and the freshly triflated 8-

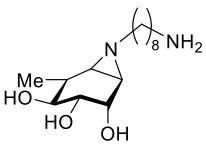
azido-octanol (0.18 mmol, 2 eq.) were added at 0 °C. The reaction was allowed to reach room temperature and after 24 hours the reaction was cooled to -20 °C. After stirring for an additional 24 hours, TLC-MS indicated complete conversion and the reaction was quenched with sat. aq. $NaHCO_3$, diluted with EtOAc, washed with H_2O, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Column chromatography (2% -> 30% EtOAc in pentane) yielded compound **43b** (0.033 g, 0.057 mmol) in 64%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 – 7.20 (m, 15H, Aromatic), 4.97 – 4.88 (m, 2H, CH_2-Bn), 4.77 – 4.68 (m, 2H, CH_2-Bn), 4.66 – 4.54 (m, 2H, CH_2-Bn), 4.19 (t, *J* = 2.6 Hz, 1H, H-2), 3.69 (dd, *J* = 10.0, 2.9 Hz, 1H, H-3), 3.48 (dd, *J* = 10.0, 8.7 Hz, 1H, H-4), 3.25 (t, *J* = 7.0 Hz, 2H, CH_2-linker), 2.21 (dt, *J* = 11.5, 7.0 Hz, 1H, CH_2-linker), 2.07 (dt, *J* = 11.5, 7.0 Hz, 1H, CH_2-linker), 1.98 (dt, *J* = 8.7, 7.1 Hz, 1H, H-5), 1.73 (dd, *J* = 5.9, 2.3 Hz, 1H, H-1), 1.63 – 1.55 (m, 2H, CH_2-linker), 1.44 (h, *J* = 6.9 Hz, 2H, CH_2-linker), 1.39 – 1.31 (m, 4H, CH_2-linker), 1.31 – 1.23 (m, 4H, CH_2-linker), 1.20 (d, *J* = 5.9 Hz, 1H, H-7), 1.17 (d, *J* = 7.2 Hz, 3H, H-6). ^{13}C NMR (126 MHz, $CDCl_3$) δ 139.4, 139.3, 128.4, 128.4, 128.4, 128.1, 127.8, 127.6, 127.5, 127.4 (Aromatic), 82.1 (C-3), 81.1 (C-4), 75.2 (C-2), 75.2 (CH_2-Bn), 73.4 (CH_2-Bn), 73.1 (CH_2-Bn), 60.9 (CH_2-linker), 51.6 (CH_2-linker), 44.0 (C-7), 43.3 (C-1), 37.8 (C-5), 29.8, 29.6, 29.2, 29.0, 27.3, 26.8 (CH_2-linker), 18.8 (C-6). HRMS $[M+H]^+$ calc. 583.3643 found 583.3636

L-rhamnose-α-cyclophellitol (42)



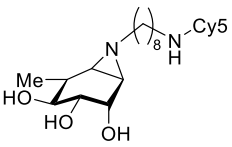
Compound **41b** (0.022 g, 0.050 mmol) was co-evaporated thrice with toluene and dissolved in THF (1 mL, 0.05 M) under N_2, and t-BuOH (0.17 mL, 1.8 mmol, 36 eq.) was added. Ammonia (±10 mL) was condensed at -60 °C while maintaining a dry atmosphere and Na (0.035 g, 1.5 mmol, 30 eq.) was added, while using a glass stirring bar. After stirring for 10-20 min the Na was completely dissolved and compound **41b** was added dropwise at -50 °C and the flask was washed with THF (0.5 mL), immediately thereafter the mixture was cooled to -70 °C. After stirring for 1 hour between -50 and -70°C, the reaction mixture was quenched with AcOH and the ammonia was allowed to evaporate at room temperature. C18 Column chromatography over a pre-packed C18 column (packed volume 1 mL, J.T. Bakerbond) (0% water) yielded compound **42** together with sodium salts. Silica gel column chromatography (5% -> 8% MeOH in DCM) yielded pure compound **42** (5.067 mg, 31.6 µmol) in 63%.

L-rhamnose-α-cyclophellitol-(N-amine-octane)-aziridine (44)



Compound **43b** (0.033 g, 0.057 mmol) was dissolved in MeCN (1.14 mL, 0.05 M) and PPh_3 on beads (0.038 g, 3 mmol/g, 2 eq.), H_2O (0.01 mL, 0.57 mmol, 10 eq.) were added. The reaction mixture was heated to 70 °C and after stirring for 8 hours TLC indicated complete conversion, the mixture was filtrated, concentrated *in vacuo* and the crude product was then directly used without further purification. Reduced compound **43b** (0.033 g, 0.057 mmol) was co-evaporated thrice with toluene and dissolved in THF (1.5 mL, 0.038 M) under N_2, and t-BuOH (0.16 mL, 1.7 mmol, 30 eq.) was added. Ammonia (±15 mL) was condensed at -60 °C while maintaining a dry atmosphere and Na (0.039 g, 1.7 mmol, 30 eq.) was added, while using a glass stirring bar. After stirring for 10-20 min the Na was completely dissolved and reduced compound **43b** was added dropwise at -50 °C and the flask was washed with THF (0.5 mL) and t-BuOH (0.4 mL), immediately thereafter the mixture was cooled to -70 °C. After stirring for 1 hour and 40 minutes between -50 and -70°C, the reaction mixture was quenched with NH_4Cl (0.11 g, 0.2 mmol, 36 eq.) and the ammonia was allowed to evaporate at room temperature. Silica gel column chromatography (2% -> 10% MeOH in DCM then 100% MeOH flush) yielded pure compound **44** together with salts. ^{1}H NMR (500 MHz, MeOD) δ 4.24 (dd, *J* = 3.6, 1.9 Hz, 1H, H-2), 3.39 (dd, *J* = 10.3, 3.5 Hz, 1H, H-3), 3.23 (dd, *J* = 10.3, 9.2 Hz, 1H, H-4), 2.97 – 2.89 (m, 2H, CH_2-linker), 2.33 – 2.23 (m, 2H, CH_2-linker), 1.84 (dd, *J* = 6.0, 1.9 Hz, 1H, H-1), 1.79 – 1.71 (m, 1H, H-5), 1.71 – 1.63 (m, 2H, CH_2-linker), 1.60 – 1.52 (m, 2H, CH_2-linker), 1.39 (qd, *J* = 10.5, 9.0, 3.1 Hz, 9H, H-7, CH_2-linker), 1.24 (d, *J* = 7.2 Hz, 3H, H-6). ^{13}C NMR (126 MHz, MeOD) δ 73.2 (C-4), 72.9 (C-3), 69.9 (C-2), 61.8 (CH_2-linker), 46.0 (C-1), 45.3 (C-7), 40.8 (CH_2-linker), 39.0 (C-5), 30.5, 30.4, 30.1, 28.6, 28.2, 27.4 (CH_2-linker), 18.7 (C-6).

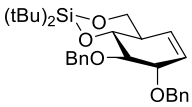
L-rhamnose-α-cyclophellitol-(N-amide-Cy5-octane)-aziridine (45a)



A pre-activated Cy5 acid solution was prepared by freeze drying Cy5 (0.059 g, 0.114 mmol, 1 eq.), subsequently dissolving it in dry DMF (0.57 mL, 0.2 M) and adding DIPEA (0.05 mL, 0.285 mmol, 2.5 eq.), pentafluoro-phenyl-2,2,2-trifluoroacetate (0.0241 mL, 0.1423 mmol, 1.25 eq.). After stirring for 2 hours, LC-MS indicated 50% conversion and additional DIPEA (0.05 mL, 0.285 mmol, 2.5 eq.), PFP-TFA (0.0241 mL, 0.1423 mmol, 1.25 eq.) were added. After stirring for an additional hour, the reaction was quenched with H_2O (0.05 mL, 0.85 mmol, 15 eq.) and additional DIPEA (0.05 mL, 0.285 mmol, 2.5 eq.). Subsequently, compound **44** (16 mg, 0.057 mmol) was dissolved in DMF (0.3 mL) and added to the reaction mixture, the flask was then washed with additional DMF (0.1 mL). After stirring for 1 hour, LC-MS indicated complete conversion and the reaction mixture

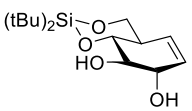
was diluted with MeOH and concentrated *in vacuo*. Column chromatography (0% -> 10% MeOH in DCM then MeOH flush) and subsequent size exclusion chromatography yielded compound **45a** (8.5 mg, 10.5 µmol) in 18% over 3 steps. ^{1}H NMR (600 MHz, MeOD) δ 8.31 – 8.19 (m, 2H), 7.50 (d, *J* = 7.4 Hz, 2H), 7.41 (tdd, *J* = 7.7, 5.3, 1.2 Hz, 2H), 7.34 – 7.24 (m, 4H), 6.63 (t, *J* = 12.4 Hz, 1H), 6.28 (dd, *J* = 13.7, 7.2 Hz, 2H), 4.20 (dd, *J* = 3.6, 1.9 Hz, 1H, H-2), 4.11 (t, *J* = 7.5 Hz, 2H), 3.63 (s, 3H), 3.36 (dd, *J* = 10.3, 3.6 Hz, 2H, H-3), 3.19 (dd, *J* = 10.2, 9.3 Hz, 1H, H-4), 3.12 (t, *J* = 7.2 Hz, 2H), 2.21 (dt, *J* = 18.2, 7.3 Hz, 4H), 1.90 (s, 10H), 1.86 – 1.81 (m, 2H), 1.79 (dd, *J* = 6.0, 1.9 Hz, 1H, H-1), 1.73 (s, 10H),1.72 – 1.70 (m, 1H, H-5), 1.70 – 1.66 (m, 2H), 1.54 – 1.49 (m, 2H), 1.49 – 1.43 (m, 4H), 1.39 – 1.26 (m, 18H, H-7), 1.21 (d, *J* = 7.2 Hz, 3H, H-6). Due to lack of signal only selected peaks are shown: ^{13}C NMR (151 MHz, MeOD) δ 73.1 (C-3/C-4), 72.8 (C-3/C-4), 69.9 (C-2), 61.8, 49.4, 49.3, 49.3, 49.1, 46.0 (C-1), 45.3 (C-7), 18.8 (C-6).

2,3-*O*-benzyl-4,6-*O*-ditertbutylsilyl-D-glucose-cyclohexene (59)



Diol **58** (1.46 mmol, 0.50 g, 1 eq.) and imidazole (5.82 mmol, 0.40, 4 eq.) were co-evaporated three times with toluene under N_2, thereafter it was dissolved in dry DCM (14.6 mL, 0.1 M). $(tBu)_2Si(OTf)_2$ (3.64 mmol, 1.19 mL, 2.5 eq) was added dropwise at 0°C and the mixture was warmed to rt. After 15 min of stirring at room temperature TLC showed full conversion and the reaction was quenched with methanol. The solution was diluted with diethyl ether and washed with HCl (1M), sat. aq. $NaHCO_3$ and brine. The combined water layers were back extracted (1x) with diethyl ether and the combined organic phases were dried over Na_2SO_4, filtrated and concentrated *in vacuo*. Purifying via silica gel column (2% -> 10%, EtOAc in pentane) yielded product **59** (1.49 mmol, 0.714 g) with minor impurities as a colorless oil 102%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 – 7.26 (m, 10H, aromatic), 5.68 (dt, J = 10.0, 2.8 Hz, 1H, H-1/H-7), 5.23 (dt, *J* = 10.1, 1.9 Hz, 1H, H-1/H-7), 5.11 (d, *J* = 11.0 Hz, 1H, CH_2Bn), 4.82 – 4.63 (m, 3H, CH_2Bn), 4.19 – 3.99 (m, 3H, H-6a, H-2, H-3/H-4), 3.82 – 3.68 (m, 2H, H-6a, H-3/H-4), 2.75 – 2.58 (m, 1H, H-5), 1.06 (dd, *J* = 11.0, 1.8 Hz, 18H, (*t*-Bu)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 138.7, 128.7 (C7/C1), 128.4, 128.3, 127.9, 127.6 (Aromatic), 125.1 (C1/C7), 84.7 (C3/C4), 80.1 (C2/C3/C4), 78.7 (C2/C3/C4), 75.3 (CH_2Bn), 72.6 (CH_2Bn), 68.4 (C6), 43.6 (C5), 27.5 (*t*-Bu), 27.2 (*t*-Bu), 22.0 (*t*-Bu), 20.0 (*t*-Bu)

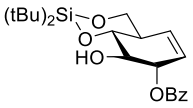
4,6-*O*-ditertbutylsilyl-D-glucose-cyclohexene (60)



Compound **59** (0.2 mmol, 0.096 g, 1 eq.) was co-evaporated with toluene three times under N_2 and subsequently dissolved in dry DCM (2 mL, 0.1 M), thereafter $TiCl_4$ (0.4 mmol, 0.4 mL, 1M in toluene, 2 eq.) was added dropwise at -20°C. TLC showed full

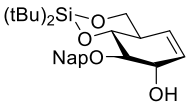
conversion after 45 min and the reaction mixture was quenched with sat. aq. $NaHCO_3$ and filtrated over Celite®. The solution was diluted with EtOAc and washed with sat. aq. $NaHCO_3$ and brine. The combined water layers were back extracted (1x) with EtOAc. The combined organic layers were dried over Na_2SO_4, filtrated and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (10% -> 40% EtOAc in pentane) and yielded pure product **60** (0,187 mmol, 0.056 g) as a colorless oil 94%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.65 (dt, *J* = 10.1, 2.7 Hz, 1H, H-7), 5.26 (dt, *J* = 10.1, 2.0 Hz, 1H, H-1), 4.35 – 4.26 (m, 1H, H-2), 4.12 (dd, *J* = 10.4, 4.7 Hz, 1H, H-6a), 3.84 – 3.72 (m, 2H, H-6b, H-4), 3.67 (dd, *J* = 10.0, 7.5 Hz, 1H, H-3), 3.24 (s, 2H, OH), 2.68 – 2.55 (m, 1H, H-5), 1.03 (d, *J* = 16.1 Hz, 18-H, (*t*-Bu)$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 130.1 (C7), 124.5 (C1), 78.0 (C3), 76.8 (C4), 72.9 (C2), 68.2 (C6), 43.1 (C5), 27.5 (*t*-Bu), 27.1 (*t*-Bu), 22.9 (*t*-Bu), 20.0 (*t*-Bu).

2-*O*-benzoyl-4,6-*O*-ditertbutylsilyl-D-glucose-cyclohexene (61)



Diol **60** (0.1 mmol, 0.03 g, 1 eq.) was co-evaporated three times with toluene and dissolved in dry MeCN (0.5 mL, 0.2 M) under N_2. Afterwards DIPEA (0.5 mmol, 0.09 mL, 5 eq.), 2-aminoethyl diphenylborinate (0.01 mmol, 2.24 mg, 0.1 eq.) and benzoyl chloride (0.3 mmol, 0.042 g, 3 eq.) were added. After stirring for 17 hours at room temperature, TLC showed full conversion and the reaction mixture was diluted with diethyl ether, washed with HCl (1M), sat. aq. $NaHCO_3$ and brine. The combined water layers were back extracted 1x with diethyl ether and the combined organic phases were dried over Na_2SO_4, filtrated and concentrated *in vacuo.* The crude product was purified by silica gel column chromatography (2% -> 5% EtOAc in pentane) and yielded pure product **61** (0.075 mmol, 0.03 g) in 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 – 8.06 (m, 2H, aromatic), 7.59 – 7.53 (m, 1H, aromatic), 7.47 – 7.41 (m, 2H, aromatic), 5.75 – 5.71 (m, 1H, H-7/H-1), 5.71 – 5.67 (m, 1H, H-2), 5.39 (dq, *J* = 9.6, 2.0 Hz, 1H, H-1/H-7), 4.19 – 4.14 (m, 1H, H-6a), 4.03 (dd, *J* = 10.0, 7.5 Hz, 1H, H-3), 3.93 (t, *J* = 9.7 Hz, 1H, H-4), 3.81 (dd, *J* = 12.0, 10.4 Hz, 1H, H-6b), 3.04 (s, 1H, OH), 2.75 – 2.63 (m, 1H, H-5), 1.05 (d, *J* = 15.2 Hz, 18H, (*t*-Bu)$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3 (Ph_2COO), 134.7, 133.1 (aromatic), 130.1 (aromatic), 129.8, 128.3 (aromatic), 126.9 (C7), 126.5 (C1), 77.1 (C4), 75.7 (C2), 74.7 (C3), 67.9 (C6), 42.6 (C5), 27.4 (*t*-Bu), 27.1 (*t*-Bu), 22.8 (*t*-Bu), 19.9 (*t*-Bu).

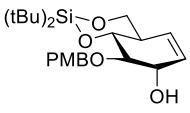
4,6-*O*-ditertbutylsilyl-3-*O*-naphthyl-D-glucose-cyclohexene (62a)



Diol **60** (0.1 mmol, 0.03 g, 1 eq.) was co-evaporated three times with toluene and dissolved in dry MeCN (0.47 mL, 0.22 M) under N_2. Afterwards KI (0.1 mmol, 0.017 g, 1 eq.), K_2CO_3 (0.12 mmol, 0.017 g, 1.2 eq.), 2-aminoethyl diphenylborinate (0.03 mmol, 0.007 g, 0.3 eq.) and 2-(Bromomethyl)-naphthalene (0.12 mmol, 0.027 g, 1.2 eq.)

were added to the flask and the mixture was stirred at 60°C for 24 hours until TLC showed conversion. The reaction was quenched with H_2O and the reaction mixture was diluted with ethyl acetate and washed with sat. aq. $NaHCO_3$ and brine. The combined water phases were back extracted (1x) with ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtrated and concentrated *in vacuo.* The resulting crude product was purified using silica gel column chromatography (10% -> 30% EtOAc in pentane) and product **62a** (0.04 mmol. 0.023 g) was obtained in 40% together with 48% of starting material **60**. ^{1}H NMR (400 MHz, CDCl3) δ 7.87 – 7.82 (m, 5H, aromatic), 7.60 (dd, *J* = 8.4, 1.7 Hz, 1H, aromatic), 7.53 – 7.47 (m, 3H, aromatic), 5.65 (ddd, *J* = 10.0, 3.0, 2.3 Hz, 1H, H-7), 5.33 (d, *J* = 11.4 Hz, 1H, CH_2Nap), 5.25 (dt, *J* = 10.0, 2.0 Hz, 1H, H-1), 4.96 – 4.86 (m, 2H, CH_2Nap), 4.32 (dp, *J* = 6.1, 2.3 Hz, 1H, H-2), 4.14 (q, *J* = 7.1 Hz, 2H, H-6, H-4), 3.82 (dd, *J* = 12.0, 10.4 Hz, 1H,), 3.61 (dd, *J* = 10.0, 7.7 Hz, 1H, H-3), 2.75 (tdt, *J* = 9.0, 6.9, 4.5 Hz, 1H, H-5), 2.38 (m, 1H, OH), 1.11 (d, *J* = 12.4 Hz, 18H, (*t*-Bu)$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 136.4, 133.4, 133.2, 129.8 (C7), 128.5, 128.4, 128.0 (aromatic), 128.0 (aromatic), 127.8, (aromatic), 127.0, 126.3 (aromatic), 126.2 (aromatic), 126.2 (aromatic), 126.0 (aromatic) 124.7 (C1), 124.5, 85.1 (C3), 78.5 (C4), 75.2 (CH_2Nap), 72.6 (C2), 68.5 (C6), 65.6, 60.5, 44.5 (C5), 31.6, 30.2, 27.6 (*t*-Bu), 27.3 (*t*-Bu), 22.9 (*t*-Bu), 20.0 (*t*-Bu).

4,6-*O*-ditertbutylsilyl-3-*O*-methoxybenzyl-D-glucose-cyclohexene (62b)



Diol **60** (0.1 mmol, 0.03 g, 1 eq.) was co-evaporated three times with toluene and dissolved dry MeCN (0.47 mL, 0.22 M) under N_2. Afterwards KI (0.1 mmol, 0.017 g, 1 eq.), K_2CO_3 (0.12 mmol, 0.017 g, 1.2 eq.), 2-aminoethyl diphenylborinate (0.03 mmol, 0.007 g, 0.3 eq.) and PMB-Cl (or PMB-Br) (0.12 mmol, 0.027 g, 1.2 eq.) were added to the flask and the mixture was stirred at 60°C. After 24 hours 0.1 mmol of PMB-Cl (1 eq.) was added. After another 7 hours 0.1 mmol of PMB-Cl (1 eq.), 0.1 mmol K_2CO_3 (1 eq.) and 0.03 mmol 2-aminoethyl diphenylborinate (0.3 eq.) were added and the reaction mixture was stirred for an additional 17 hours, but TLC still indicated the presence of starting material. The reaction was quenched with H_2O and the reaction mixture was diluted with ethyl acetate and washed with sat. aq. $NaHCO_3$ and brine. The combined water phases were back extracted (1x) with ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtrated and concentrated *in vacuo*. The resulting crude product was purified using silica gel column chromatography (15% -> 30%, EtOAc in pentane) to obtain product **62b** (0.04 mmol. 0.023 g) with minor impurities (hydrolyzed PMB-Cl) in 53%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.28 (m, 2H, aromatic), 6.92 – 6.87 (m, 2H, aromatic), 5.65 – 5.59 (m, 1Hm H-7), 5.22 (dt, *J* = 10.1, 2.1 Hz, 1H, H-1), 5.09 (d, *J* = 10.8 Hz, 1H, CH_2-PMB), 4.66 (d, *J* = 10.9 Hz, 1H, CH_2PMB), 4.27 – 4.19 (m, 1H, H-2), 4.15 – 4.09 (m, 1H, H-6), 4.06 (t, *J* = 9.7 Hz, 1H, H-4), 3.81 (d, *J* = 2.7 Hz, 3H, CH_3), 3.79 – 3.73 (m, 1H, H-6), 3.51 (dd, *J* =

10.0, 7.7 Hz, 1H, H-3), 2.76 – 2.63 (m, 1H, H-5), 2.27 (s, 1H, OH), 1.09 (s, 9H, *t*-Bu), 1.05 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.4 (aromatic), 131.2 (aromatic), 129.9, 129.7, 128.8 (aromatic), 124.7 (C1), 114.1 (aromatic), 84.7 (C3), 78.4 (C4), 74.7 (CH_2-PMB), 72.5 (C2), 68.5 (C6), 55.4 (CH_3-PMB), 44.5 (C5), 27.5 (*t*-Bu), 27.3 (*t*-Bu).

ABPP labelling experiment with different secretomes

Secretomes (30 µL) from *A. niger* mutants MW2.1#112, MW3.8#1, EA21.6, MW3.1, grown on fructose and *A. niger* (strain N402) grown on fructose, pectin from apple, rhamnogalacturonan, L-rhamnose monohydrate or guar gum were incubated with probe **45a** (3 µL, 10 µM final concentration) for 1h15min at 37 °C, while shaking at 400 rpm. The reaction was quenched with 4x Laemmli buffer (7.5 µl) and boiled for 5 minutes at 95 °C. The denatured enzyme was then loaded on gel. Gel-analysis was done using a biorad chemidoc imager with Cy5 excitation.

Competitive ABPP labelling experiment

Secretomes (30 µL) from MW3.8#1, pectin apple and guar gum, were incubated with only probe **45a** (3 µL, 10 µM final concentration or first inhibitor **42** (1 mM final concentration). Secretomes with inhibitor were then incubated for 2h20min at 37 °C, while shaking at 400 rpm. Then the probe (10 µM, final concentration) was added and after shaking for an additional 60 minutes at 37 °C, the reaction was quenched with 4x Laemli buffer (7.5 µl) and boiled for 5 minutes at 95 °C. The control experiments were done as follows; secretome (30 µl) was heated to 95 °C for 5 min and then shaken for 2h20 min at 37 °C. Then the secretome was incubated with the probe (3 µL, 10 µM final concentration) for 60 minutes at 37 °C, the reaction was quenched with 4x Laemmli buffer (7.5 µl) and boiled for 5 minutes at 95 °C. The denatured enzyme was then loaded on gel. Gel-analysis was done using a biorad chemidoc imager with Cy5 excitation.

pH dependent ABPP labelling experiment

Secretome (30 µL, pH 6) from pectin apple was adjusted to pH 2/3/4/5 using 1 M HCl or pH 7/8/9 using 1 M NaOH. Subsequently, the secretomes were incubated with probe **45a** (3 µL, 10 µM final concentration) for 1 hour at 37 °C, while shaking at 400 rpm. The reaction was quenched with 4x Laemli buffer (7.5 µl) and boiled for 5 minutes at 95 °C. The denatured enzyme was then loaded on gel. Gel-analysis was done using a biorad chemidoc imager with Cy5 excitation.

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