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Inhibitors and probes targeting endo-glycosidases

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INHIBITORS AND PROBES TARGETING ENDO-GLYCOSIDASES

PROEFSCHRIFT

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General introduction

The natural product *cyclophellitol* (**1**), discovered in 1989, is a mechanism-based, irreversible inhibitor of retaining β-glucosidases that reacts with the enzyme active site to form a stable covalent bond with the catalytic nucleophile.[1–3] Synthetic derivatives containing a reporter group have been synthesized having the configuration of numerous monosaccharides and are used as selective inhibitors and activity-based probes of exo-acting retaining glycosidases (Figure 1.1). In this dissertation glycosylated cyclophellitol derivatives are presented as inhibitors and activity-based probes of various retaining endo-glycosidases.

This chapter illustrates the value of cyclophellitol and its analogues, as exemplified by recent applications in the context of biomedicine and biotechnology. The mechanistic and structural characteristics of the glycosidase targets underlying the efficacy of the cyclophellitol scaffold are summarized and an overview of the activity-based protein profiling methodology is provided. The chapter is concluded with an outline of the rest of the dissertation.

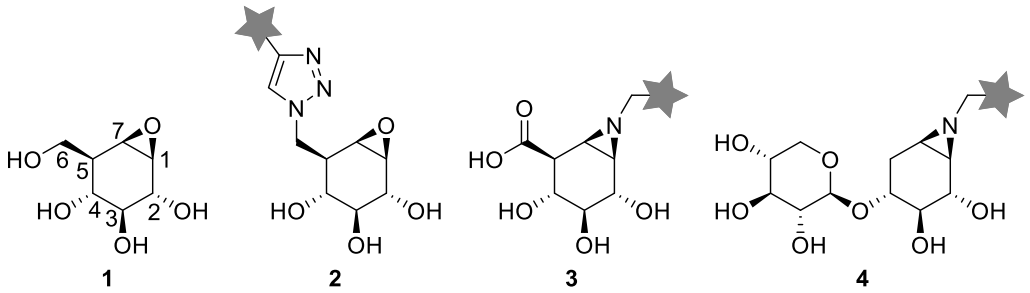

Figure 1.1| The structure of cyclophellitol and synthetic cyclophellitol derivatives. Stars denote various reporter moieties (biotin, fluorophore). For ease of comparison with parent glycosides carbon atoms in cyclophellitol and analogous compounds are numbered as if they were carbohydrates throughout this thesis.

Applications of cyclophellitol derivatives

Glycosyl hydrolases (Glycosidases/GHs) catalyze the hydrolysis of glycosidic linkages with incredible efficiency and enhance the reaction rate by a factor of up to 10^{17} compared to the uncatalyzed reaction at neutral pH.[4] Deficiency of a glycosidase activity can be detrimental to human health. A notable example is Gaucher disease in which a deficiency in glucocerebrosidase (acid glucosylceramidase, GBA) leads to harmful accumulation of glucosylceramide in the lysosome. Fluorescent cyclophellitol derivative **2** allows the visualization of active GBA in living cells and facilitates the diagnosis of the disease and the screening of molecules with potential use as molecular chaperones to regain GBA activity.[5] As well, derivatives of **2** carrying a non-fluorescent lipophilic group proved to be selective GBA inhibitors *in vivo* allowing the generation of a chemical GBA knockout model in zebrafish.[6]

Attachment of a reporter group to the C6 position – or any alcohol on a monosaccharide – is normally not tolerated by exo-glycosidases. The cyclophellitol aziridine analog, which is at least equally as potent as the epoxide variant, can accommodate the reporter group on the aziridine nitrogen leaving the alcohols available to interact with the enzyme. An additional advantage is the positioning of the reporter group in the space where the aglycon would naturally reside, resulting in minimal steric interactions between the reporter group and the enzyme. This makes cyclophellitol aziridine a more general scaffold that facilitated the successful development of many monosaccharide tagged cyclophellitol derivatives (Table 1.1). Selectivity for specific glycosidases is obtained by mimicking the configuration of the natural substrate.

Applying this design, fluorescent cyclophellitol aziridines having the glucuronic acid configuration (**3**) were synthesized that showed labeling of human and bacterial exo-β-

glucuronidases.[7] These molecules were recently employed to study bacterial glucuronidase-induced drug toxicity in the microbiome.[8] This monosaccharide mimic unexpectedly proved to label not only the intended target but the endo-glucuronidase, heparanase (HPSE) and the HPSE proenzyme as well. The labeling of endo-glycosidases by a monosaccharide cyclophellitol is not generally observed except in one other case where the hydrophobic spacer significantly contributes to binding.[9] HPSE in man is the main enzyme responsible for heparan sulfate degradation in the extracellular matrix and overexpression is associated with cancer metastasis and inflammation making the monitoring and inhibition of HPSE activity an area of intense research (Chapter 3).

Tools to discover and monitor endo-glycosidases, such as xylanases, cellulases and xyloglucanases, are also highly sought-after in the bio-based industry where renewable feedstocks, consisting mainly of polysaccharides, are transformed into useful products (Chapter 2). The first step in the generation of these products is often the hydrolysis of the different polysaccharides into fermentable monosaccharides. Cyclophellitol derivatives can aid in the discovery and characterization of suitable enzymes for this purpose. Cyclophellitol aziridine xylobiose mimics (**4**) were synthesized to study xylanases.[10] It was found that the disaccharide motif proved to be sufficient to target endo-xylanases, which normally process larger oligosaccharides. α-L-Arabinofuranose configured cyclophellitols have also been developed being the first furanose configured derivatives. They have been used to study retaining α-L-arabinofuranosidases secreted by basidiomycete fungi, important enzymes for the debranching and further processing of hemicellulose towards sustainable fuels.[11]

In summary, these examples show that properly designed cyclophellitol derivatives can function as diagnostic tools by monitoring known activities, discovery tools to find desired activities and *in vivo* active covalent glycosidase inhibitors. More examples of covalent glycosidase inhibitor design and application can be found in the reviews by Rempel and Wu.[12,13]

Glycosidase mechanism related to cyclophellitol inhibitors

Understanding of the enzymology of glycosidases is pivotal for the design of potent inhibitors. Sequence-based classification of the known and putative glycosyl hydrolases has over the years proven to be the most predictive and thus the most useful way to classify enzyme

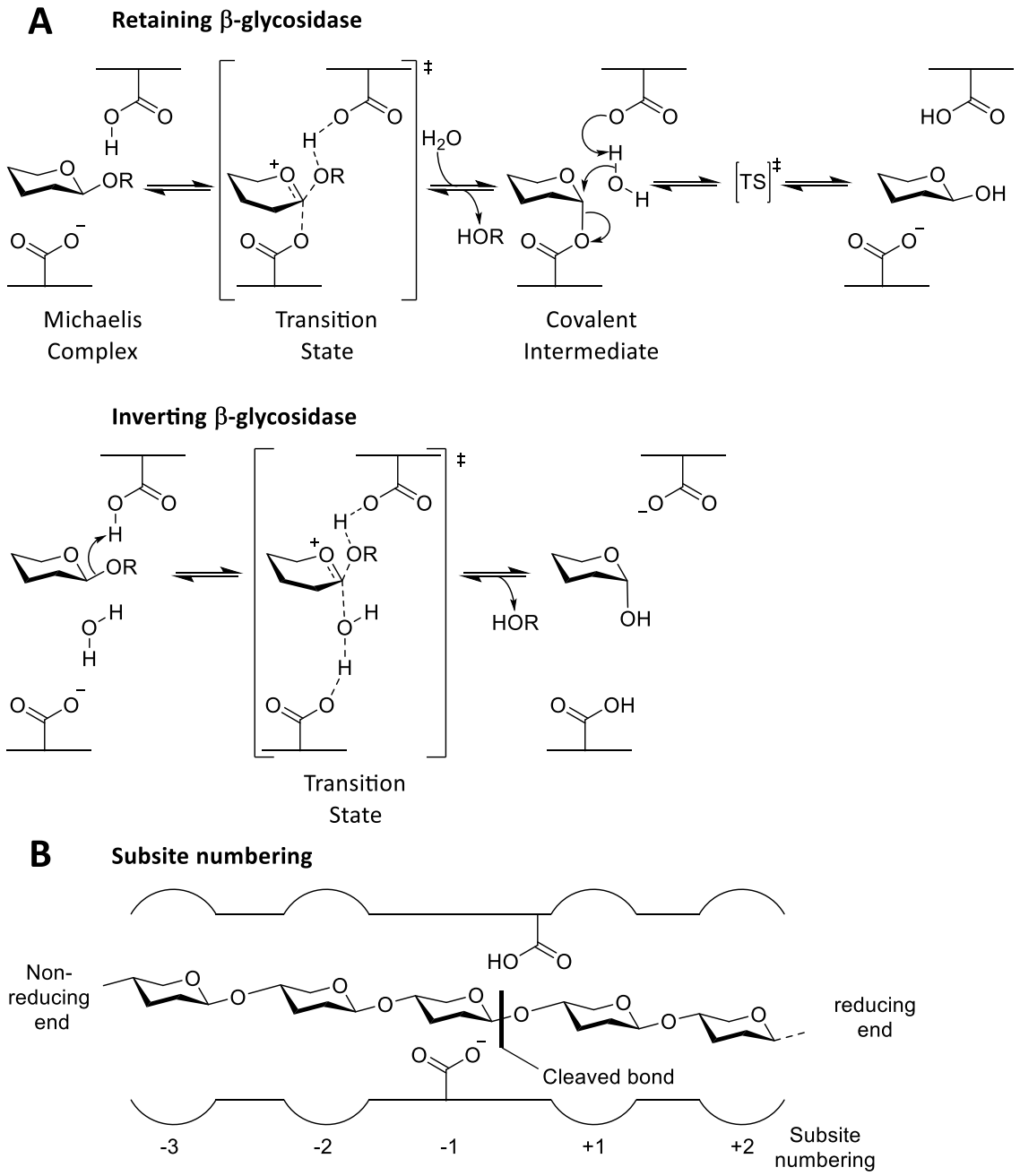

Figure 1.2 | A) The catalytic mechanism employed by most retaining and inverting glycosidases. **B**) The conventional numbering of glycosidase subsites.

families.[14] GHs classified as members of the same family display the same arrangement of the active site residues and protein fold,[15] also the vast majority of families only harbor members employing the same catalytic mechanism.[16] The assigned GHs are available in the carbohydrate active enzyme database (www.cazy.org).[17] This classification is not decisive in determining substrate specificity as many families contain diverse specificities and novel specificities are still discovered in established families.[18]

Enzyme specificity	General itinerary	Cyclophellitol derivative	Conformation	Ref
β-galactosidase	$^4H_3 \rightarrow [^4E]^{\ddagger} \rightarrow {}^4C_1$	Epoxide	$^4H_3$	27
		Cyclic sulfate	$^4C_1$	23
β-glucuronidase (GH79)[a]	$^1S_3 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$	Aziridine	$^4H_3$	7
β-glucosidase	$^1S_3 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$	Epoxide / Cyclopropane	$^4H_3$	25
		Cyclic sulfate	$^4C_1$	22
β-mannosidase	$^1S_5 \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^OS_2$	Epoxide	$^4H_3$	28
β-xylosidase GH10	$^1S_3 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$	Aziridine	$^4H_3$	10
β-xylosidase GH11	$^2S_O \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^5S_1$			
α-L-arabinofuranosidase	$^4E \rightarrow [E_3]^{\ddagger} \rightarrow {}^2E$	Epoxide/Aziridine	^3E	11
		Cyclic sulfate	E_3	
α-fucosidase	$^1C_4 \rightarrow [^3H_4]^{\ddagger} \rightarrow {}^3S_1$	Aziridine	-	29
α-galactosidase	$^4C_1 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^1S_3$	Epoxide	$^4H_3$	27
		Cyclic sulfate	$^4C_1$	23
α-glucosidase	$^4C_1 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^1S_3$	Aziridine	$^4H_3$	22
		Cyclic sulfate	$^4C_1$	
α-L-iduronidase	$^2S_O \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^5S_1$	Aziridine	$^4H_3$	30
α-mannosidase	$^OS_2 \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^1S_5$	Epoxide	$^4H_3$	28

Table 1.1| General itineraries of the first half of the mechanism (formation of the enzyme-substrate intermediate) of retaining glycosidases[31–33] and conformation of the cyclophellitol derived inhibitors with references for the experimental evidence for the inhibitor conformation. [a.] Based on partially unpublished crystal structure data, no QM/MM simulation done yet. (Wu, Davies, University of York, UK).

Glycosyl hydrolases employ either a retaining or inverting mechanism (Figure 1.2). In both mechanisms the substrate and enzyme first form a non-covalent *Michaelis complex*. The substrate binds in one or more subsites with catalysis taking place between the -1 and +1 subsite with the minus subsite(s) at the reducing end and the plus subsite(s) at the nonreducing end by convention.[19] The retaining mechanism cleaves the glycosidic linkage with net retention of stereochemistry by two consecutive inversions.[20] The anomeric position is attacked by a carboxylate residue (glutamate or aspartate) while the aglycon is protonated by

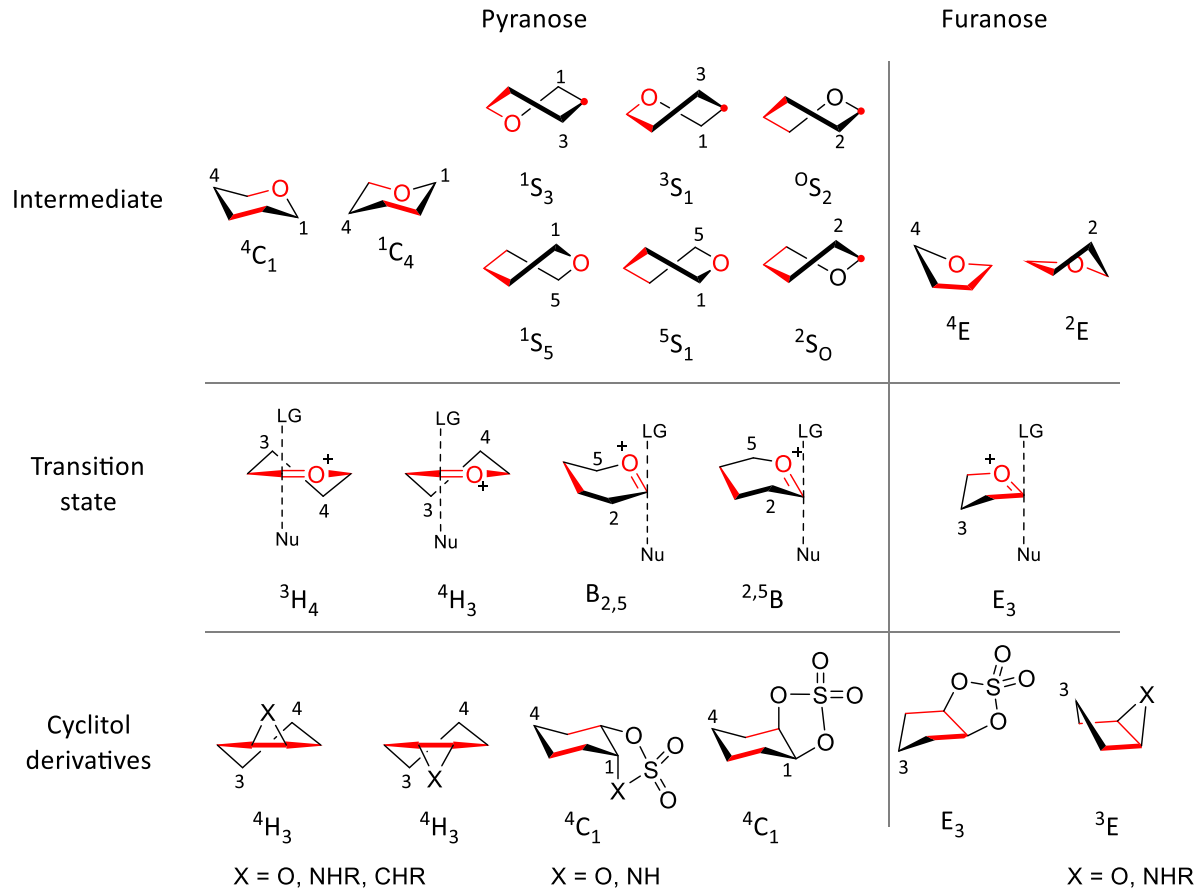

Figure 1.3| Conformations of pyranoses, furanose and cyclitol derivatives discussed in this chapter. Atoms and bonds in the same plane are red. Atoms above or below the plane are black and numbered. For transition states the general trajectory of the leaving group (LG) and catalytic nucleophile (Nu) is indicated.

a carboxylic acid side chain leading to an oxocarbenium ion like transition state. The result is a covalent enzyme-substrate intermediate. Via a second transition state water hydrolyses the intermediate acetal, liberating the enzyme and the product. In some cases instead of water another nucleophile, such as a carbohydrate or protein, is present in the second step leading to a trans-glycosylation product.[21] In the case of an inverting mechanism, water acts as the nucleophile directly, circumventing the covalent enzyme substrate intermediate.

Throughout the catalytic process, the conformation of the substrate in the -1 subsite is distorted away from its lowest energy conformation adopted in solution. X-ray snapshots of intermediates or inhibitors in complex with the enzyme combined with ab initio quantum mechanics/molecular mechanics dynamic simulations have given insight into the *catalytic itineraries* of several classes of glycosyl hydrolases (Table 1.1). Relevant conformations are shown in figure 1.3.

In the Michaelis complex the leaving group (the aglycon) is positioned pseudo-axially to allow attack of the side chain carboxylate in the σ* antibonding orbital of the anomeric carbon - aglycon oxygen bond. The conformation also minimizes steric interactions by placing the 2-OH pseudo equatorial. For α-D-glycosides having axial aglycons this can be low energy chair (C) conformations while for β-D-glycosides with equatorial aglycons the sugar in the Michaelis complex needs to adopt a higher energy skew boat (S) conformation. The anomeric carbon is sp^2 hybridized in the transition state to allow orbital overlap with the ring oxygen lone pairs to stabilize the developing positive charge. This is only possible in a conformation where four atoms surrounding the oxocarbenium ion are in the same plane. For pyranoses these are the half chair (H) and boat (B) conformations and for furanoses the envelope ones (E). Once the glycosylation of the enzyme is complete, β-glycosidases arrive at a chair conformation, while α-glycosidases follow the opposite trajectory and end up in a skew boat conformation.

Irreversible inhibition of retaining glycosidases by cyclophellitol derivatives follows a similar mechanism as substrate hydrolysis (Figure 1.4A). The cyclophellitol derivative binds to the enzyme and is attacked by the catalytic nucleophile at the 'anomeric carbon'. This leads to opening of the epoxide/aziridine forming a stabile ester (this in comparison to the acylal linkage formed during substrate glycoside hydrolysis). The enzyme is unable to hydrolyze the ester linked covalent product, due to the lack of the ring oxygen, resulting in irreversible inhibition of the enzyme. Replacing the electrophilic strained epoxide/aziridine for a cyclic sulfate also affords covalent inhibitors interacting with the enzyme in a similar fashion (Figure 1.4B).[22,23]

It is widely accepted that the conformations glycosidase inhibitors can adopt can have major influence on their potency.[24] For covalent and non-covalent cyclophellitol derivatives this has also proven to be an important factor. Although inhibitor potencies on different enzymes and under different assay conditions are hard to compare, the general trend is that inhibitors that match the conformation of either the Michaelis complex or the transition state preference of the enzyme are more potent inhibitors than the enzyme/inhibitor combinations where this is not the case. Epoxide/aziridine and cyclic sulfate inhibitors complement each other because their different conformational preference increases the conformational space accessible with cyclophellitol derivatives.

Compelling examples are the aziridine cyclophellitol xylanase inhibitors with a $^4H_3$ conformation interacting readily with a GH10 xylanase enforcing a $^4H_3$ transition state,

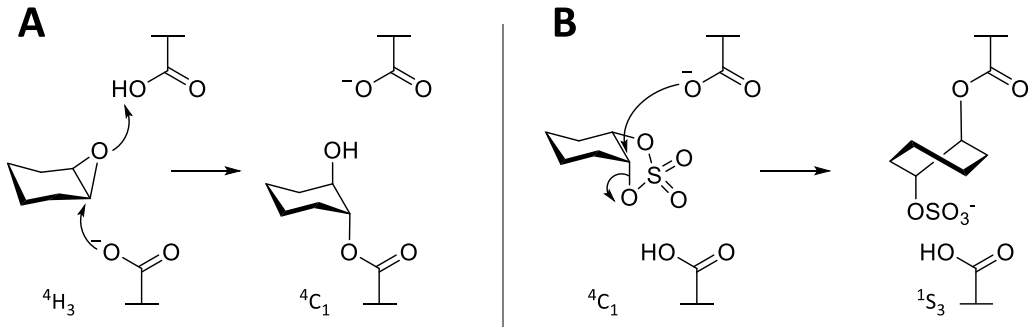

Figure 1.4| A) Mechanism of inactivation of a retaining β-glycosidase by a cyclitol epoxide derivative. **B**) Mechanism of inactivation of a retaining α-glycosidase by a cyclitol cyclic sulfate.

contrasting strongly with the apparent lack of interaction with a GH11 xylanase favoring a $^{2,5}B$ transition state.[10] The α- and β-glucose configured cyclophellitol epoxides and aziridines and the α-glucose configured cyclic sulfate are potent inhibitors for their respective enzymes because they adopt conformations that lie within the catalytic itinerary. The β-glucose configured cyclic sulfate adopting an unfavored $^4C_1$ conformation is inactive towards retaining β-glucosidases. A telling example of the complementarity of the epoxide/aziridine and cyclic sulfate inhibitors is the case of the α-L-arabinofuranosidase inhibitors where the cyclic sulfate adopts a favored E_3 conformation while the epoxide/aziridine are less potent because of their ^3E conformation.[11]

Stabilized analogs having the same conformational preferences such as cyclopropanes adopting half chair conformations and cyclic sulfamidates favoring chair conformations have also been synthesized as potent reversible inhibitors or small molecule chaperones.[23,25]

Even though the conformation of the molecules described in this thesis is an important factor in explaining their potency – or lack thereof – the structures are all drawn in a chair conformation to avoid ambiguity on the stereochemistry.

Activity-based protein profiling

Activity-based protein profiling (ABPP) is a technology in chemical biology that allows the selection of classes of active enzymes with specific activities from complex proteomes.[26] The technology relies on small molecule activity-based probes (ABPs) with a scaffold that harbors a reactive part, the warhead, and a fluorescent, affinity or bio-orthogonal reporter tag typically separated by a spacer (Figure 1.5A). As illustrated by the applications discussed at the beginning of this chapter, the mechanism-based retaining glycosidase inhibitors based on the cyclophellitol and cyclophellitol aziridine scaffold have proven to be suitable warheads

and placement of a tag on the C6 alcohol or, more generally, the aziridine nitrogen yields ABPs for various classes of retaining exo-glycosidases.

A general ABPP workflow is shown in figure 1.5B. First the probe is added to a proteome, *in vitro*, *in situ* or *in vivo* and in case of a fluorescent probe the labeled enzymes can be detected by resolving the mixture on an SDS-PAGE gel followed by fluorescence scanning or by microscopy to acquire information on cellular or subcellular localization of the active enzymes. In the case of a probe with an affinity label, usually biotin, the labeled enzymes can be separated from the rest of the proteome by affinity purification with streptavidin linked agarose beads. The labeled proteins are digested, usually by the serine protease trypsin, and the resulting peptides are analyzed by liquid chromatography-mass spectrometry. The labeled proteins are then identified by comparison of the obtained peptides to the predicted proteins from the genome of the analyzed organism(s).

Most ABPP experiments can be described as one of three types of experiments: Comparative ABPP, competitive ABPP and the study of protein substrate interactions and catalytic itineraries by X-ray crystallography of covalent enzyme/probe complexes.

In comparative ABPP different samples for instance, healthy and unhealthy individuals or microbial communities with different biomass degrading capabilities, are labeled under the same conditions allowing the detection of differences in enzyme activities (Figure 1.5C). The detection of differences in enzyme activities by ABPP in this way gives the first clue which enzyme activities are involved in a certain phenotype. In competitive ABPP samples are exposed to different conditions that can alter protein activity such as enzyme inhibitors (Figure 1.5D). The presence of a potent inhibitor would lead to diminished probe labeling. The selectivity of inhibitors can be assessed in case the probe applied labels multiple enzymes in the same sample. Also, the effectiveness of molecular chaperones can be assessed in this way, as they would lead to increased labeling compared to a misfolded enzyme. Other applications are the exposure of the sample to conditions that would otherwise alter activity such as different temperature or buffer conditions. This facilitates the assessment of the stability of the enzyme under different conditions which is relevant for quality control and enzyme catalysis in industry.

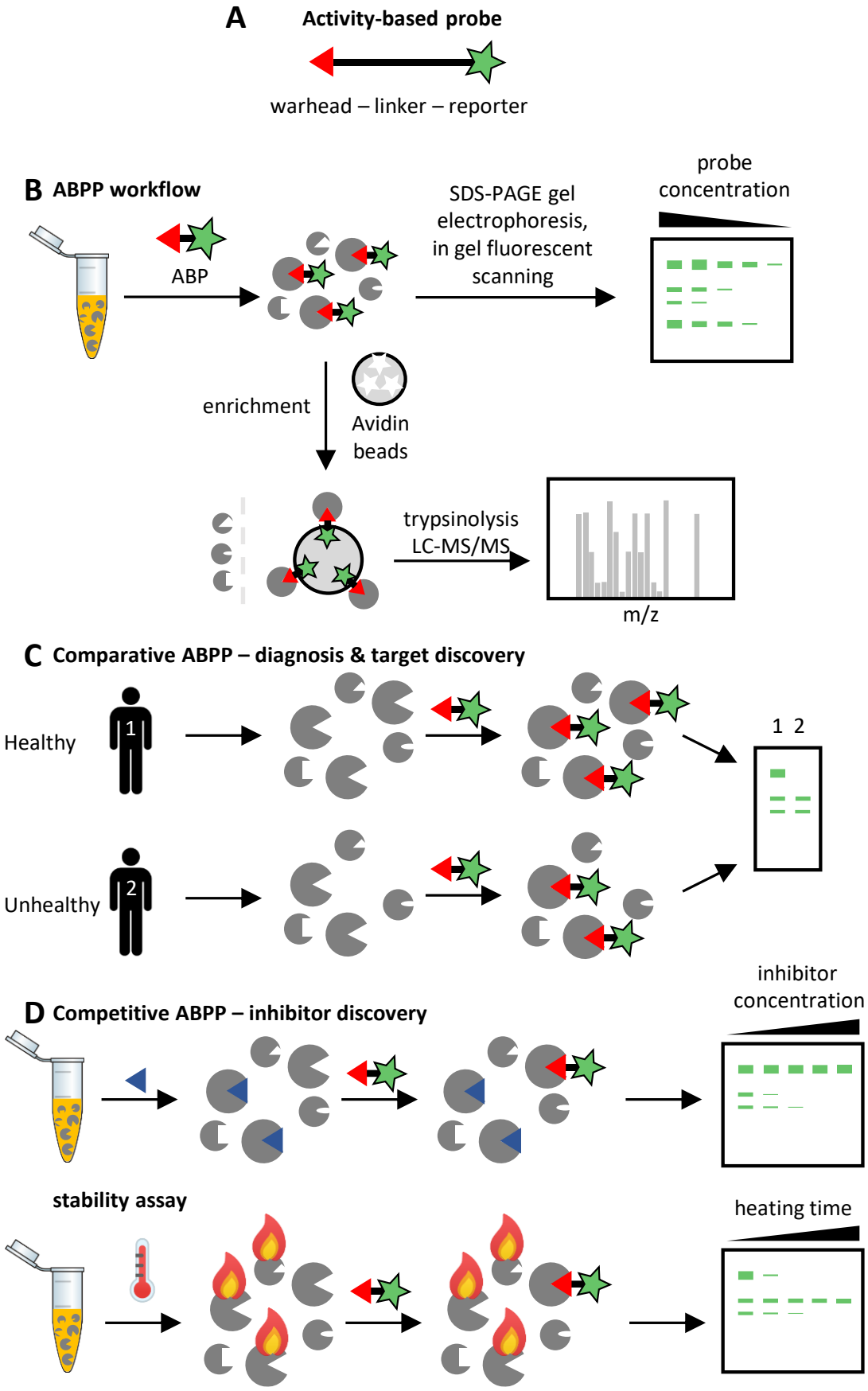

Figure 1.5| General structure of an activity-based probe and a schematic representation of several activity-based protein profiling experiments.

Thesis outline

In this thesis, the synthesis and biochemical evaluation of various cyclophellitol activity-based probes and inhibitors targeting endo-glycosidase is described. The probe design evolved from glycosylated cyclophellitol aziridines that labeled both exo- and endo-glycosidases. Exo-cleavage of the probe yields a monosaccharide probe followed by labeling of the exo-glycosidase (Chapter 2). To prevent this, the probes presented in this thesis have a tag separated from the warhead to ensure only labeling by an intact probe which may lead to higher selectivity (Figure 1.6).

Several probes targeting biomass degrading enzymes are described in *chapter 2*. Intended targets are cellulases, xyloglucanases and α-xylosidases. Glycosylation chemistry is described to perform β-glucosylations and α-xylosylations on cyclophellitol acceptors. The utility of the obtained probes is demonstrated by labeling in an *Aspergillus niger* secretome.

In *chapter 3,* glycosylated derivatives of β-glucuronic acid configured cyclophellitol are described as inhibitors of human heparanase. Selective α-glucosylation of glucose azide donors on cyclophellitol precursors is followed by elaboration into the epoxide. A set of inhibitors is synthesized to assess the importance of 6'O sulfation and 2'N acylation on HPSE inhibition. Also, the synthesis of a fluorescent HPSE probe is described.

In *chapter 4,* a putative probe for PslG, an endo-glycosidase acting on the polysaccharide Psl is described. Psl is part of the extracellular matrix generated by the pathogen *Pseudomonas Aeruginosa*. For the synthesis of this structure pre-activation glycosylation was employed on a mannose configured cyclophellitol alkene acceptor followed by late stage stereoselective introduction of the epoxide warhead on a fully deprotected trisaccharide in water.

In *chapter 5,* recommendations are provided for future work based on the projects described in the thesis.

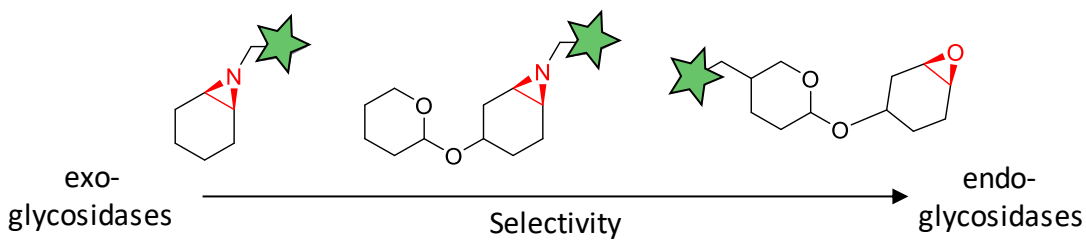

Figure 1.6| Design of selective endo-glycosidase inhibitors based on previous scaffolds.

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2

Activity-based probes targeting glycosidases acting on plant glycans

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2.1 Introduction

Plant glycans

Plant glycans are the most abundant and structurally diverse biopolymers on the planet and are a prominent source of energy and food. Depending on the plant species and the examined tissue the molecular structure of plant glycans can contain amylose and pectins, cellulose and hemicelluloses (xylan, arabinoxylan, glucuronoxylan, xyloglucans) and mixed linkage glucans (Figure 2.1).[1,2] The abundance of these glycans in many environments has prompted many organisms to evolve enzymes to modify or metabolize these structures. These enzymes are of great interest, as discussed below, as catalysts for biomass utilization or in the context of gut microbiomes and human health.

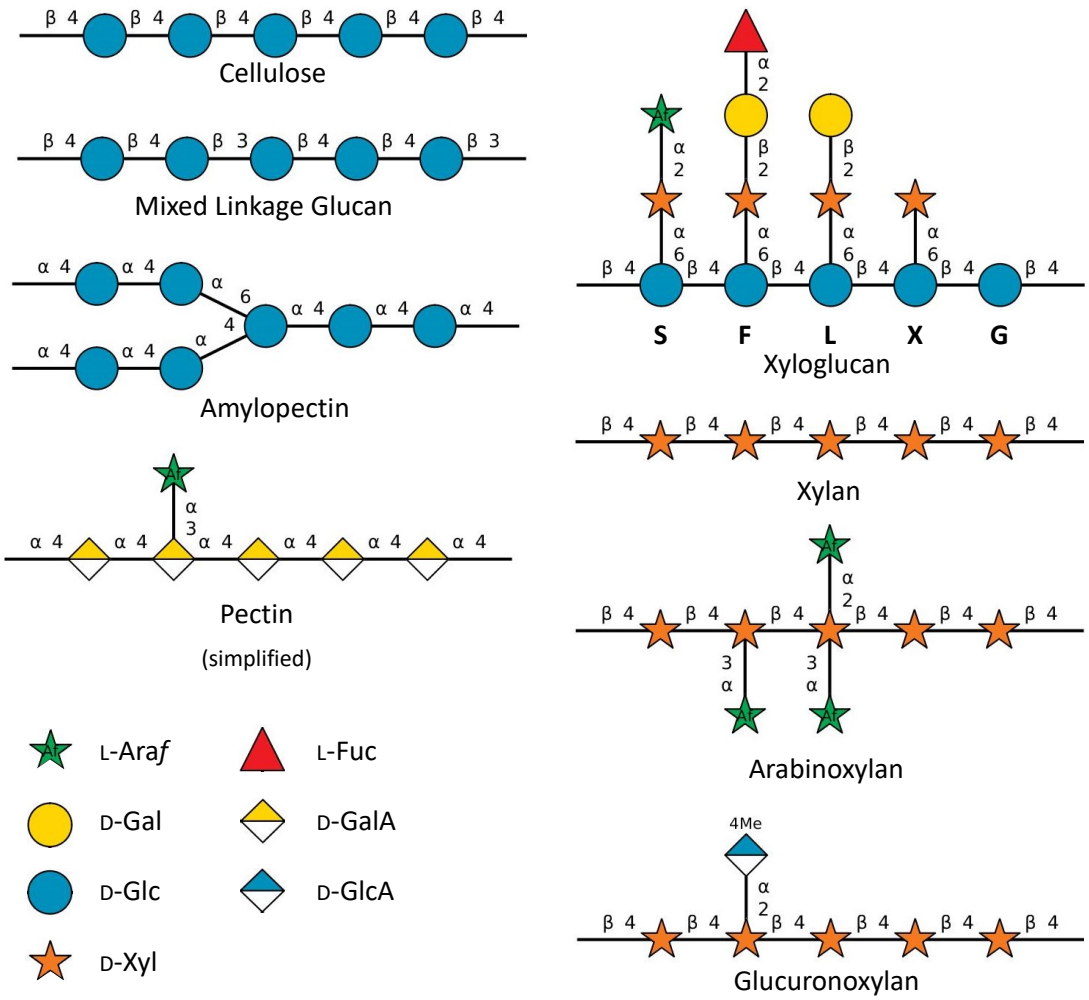


Figure 2.1| Structure of abundant plant polysaccharides. One letter abbreviation for the xyloglucan branches is shown under the structure.[3,4]

Glycoside hydrolases for biomass utilization

Efficient utilization of abundant plant biomass can provide a sustainable source of liquid fuel, platform chemicals and solvents.[5] Suitable biomass feedstocks such as wheat straw, wood and switchgrass contain between 35-48% cellulose, 22-32% hemicellulose and 12-22% polyaromatic lignin based on dry weight.[6] The recalcitrance and heterogeneity of the plant glycans is an obstacle for their efficient utilization.

Saprotrophs, organisms feeding on decaying matter, have evolved numerous glycosyl hydrolases (GHs) to degrade plant glycans.[7] Continuous efforts are made to discover highly active and stable enzymes for use in biotechnological catalysis. Many putative glycosidases have been found and documented based on genome analysis of these organisms and homology to known glycosidases (www.cazy.org).[8] The characterization of GH activity, specificity and stability based on sequence information alone, however, is complicated.

Common methodologies to properly characterize these glycosidases are laborious and require purified enzymes and therefore there is a need for new technologies to accelerate the characterization of (preferably) unpurified GHs.

Glycoside hydrolases in the human gut microbiome

An emerging field in relation to plant glycan active GHs is the study of the human gut microbiome. The human genome encodes for 97 GHs of which no more than 17 are associated with food digestion. Using our own gene products, humans are only able to enzymatically degrade the dietary glycans starch, sucrose and lactose while a healthy diet also contains many other glycans known as dietary fiber. The combined genome of the microbes living in the gut increases the digestive capability enormously, adding thousands of genes encoding GHs.[9] In this case, as well as in the previous paragraph, genetically derived primary sequence information alone does not provide sufficient information on the properties of the enzymes and, more importantly, the presence of a gene does not necessarily correlate with the expression of an active enzyme.

The importance of the constitution of an individual's microbiome is still poorly understood, but it is clear that it can be of profound influence on human health. For example, variations in microbial β-glucuronidase activity lead to variations in drug toxicity.[10] The microbial communities in the gut dynamically change their composition based on the available nutrients consisting mainly of complex glycans.[11] Several research groups have started to elucidate the metabolism of polysaccharides such as xyloglucans[12], xylans[13] and complex pectins[14] by glycosidases, secreted by gut symbionts. Monitoring these GH activities in the gut may lead to a better understanding of the significance of specific enzymatic activities in the microbiome.

Probes for plant glycan active GHs

Activity-based protein profiling (ABPP) is a powerful technique to discover and monitor glycosidases with plant polysaccharide degrading capability and can be used to increase the understanding of plant glycan degrading organisms in health an disease.[15,16] Activity-based probes (ABPs) are able to enrich low abundant enzymes in complex mixtures such as the gut microbiota or dilute secretomes, which would be difficult to detect by proteomic methods using unenriched samples.[17] Dedicated ABPs have been developed in the context of plant biomass polysaccharide processing.

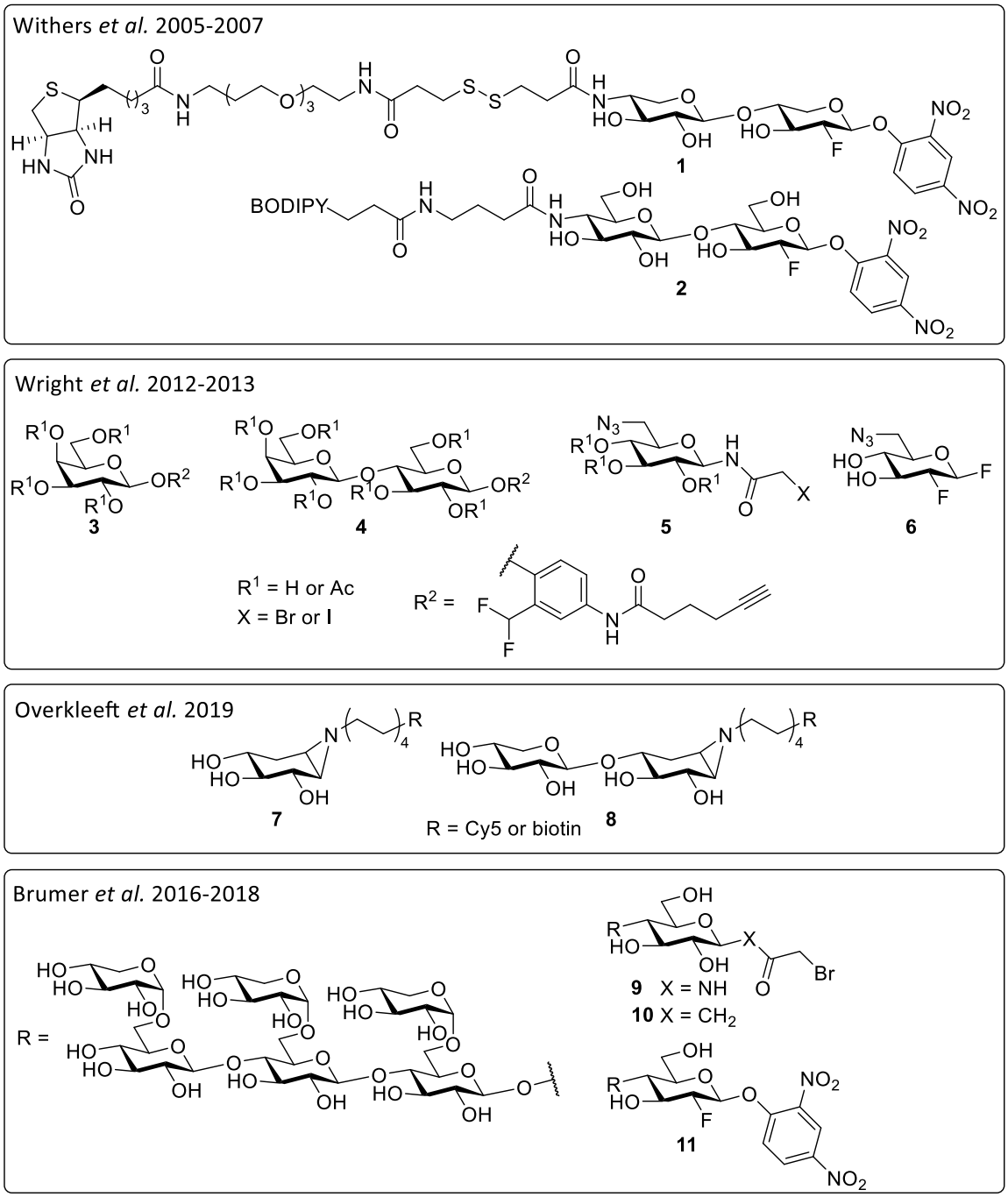


Figure 2.2| Affinity- and activity-based probes used to study biomass active enzymes.

The Withers group synthesized 2-deoxy-2-fluoro xylobiose (**1**) and cellobiose (**2**) derivatives with biotin and fluorescent reporter groups to profile endo-xylanase and cellulase activity (Figure 2.2).[18–20] This design affords selective probes for these enzymes and facilitated the discovery of a novel β-1,4-glycanase in a *Cellulomonas fimi* secretome. They also showed that the introduction of the linker with the reporter groups at the non-reducing end does not significantly change the inactivation kinetics for the two examined endo-xylanases.

The Wright group used a set of mono- and disaccharide probes with different warheads to study secretomes of *Clostridium thermocellum* and *Trichoderma reesei* (Figure 2.2).[21,22] In their protocols, the quinone methide activity-based probes (**3** and **4**) enrich carbohydrate active enzymes and other proteins associated with the cellulosome, a multi enzyme complex excreted by cellulase degrading organisms. This is probably due to diffusion of the tag away from the GH before covalent attachment. The α-halo acetamide affinity-based probes (**5**) enrich retaining and inverting GHs but do not discriminate between GHs and other carbohydrate active enzymes such as glycosyltransferases and carbohydrate kinases. The more selective 2-deoxy-2-fluoroglucose mechanism-based probe (**6**) does not show labeling in this setting, possibly due to the turnover of the covalent enzyme-probe adduct before analysis.

Overkleeft *et al.* reported the synthesis and application of β-xylosidase (**7**) and β-1,4-xylanase (**8**) probes based on the cyclitol aziridine warhead (Figure 2.2).[23] These probes react selectively with GH3 and GH10 xylan degrading enzymes in *Aspergillus niger* secretomes. The monosaccharide probe reacts selectively with exo-acting β-xylosidases. The larger recognition element of the disaccharide probe facilitates labeling of endo-acting enzymes. The exo-activity of β-xylosidases on a portion of the (excess) disaccharide probe also generates the β-xylosidase probe *in situ*. As a consequence, application of this probe shows labelling of both endo- and exo-acting retaining β-xylosidases (Figure 2.3). Curiously the retaining GH11 xylanase that is also present in the secretome did not appear to react with the probe.

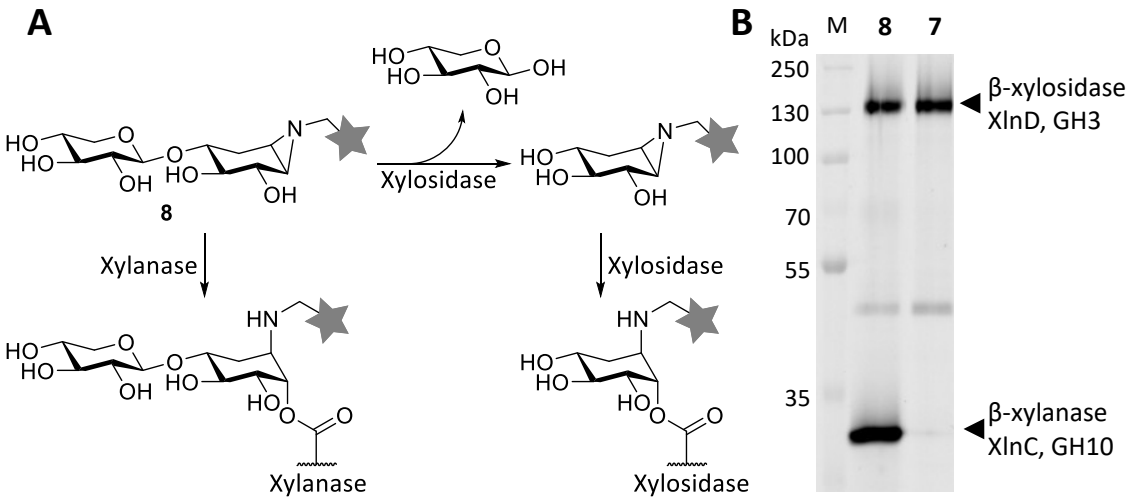


Figure 2.3|A) ABP **8** is enzymatically hydrolyzed resulting in labeling of both xylanases and xylosidases. **B**) SDS-PAGE gel showing Cy5 fluorescence of an *Aspergillus niger* secretome labeled with probe **8** or **7**.

Although mainly used as probes for crystallographic enzymology purposes, the inhibitors designed and synthesized by the Brumer group are noteworthy for containing the most extensive recognition element for xyloglucanases to date. The elaborate scaffold is accessible because the *N*-bromoacetylglycosylamine **9** and bromoketone *C*-glycoside **10** are conveniently synthesized in two steps from the oligosaccharide lactol. Xyloglucan oligosaccharides are readily available by hydrolysis of the appropriate plant material with a suitable glycosyl hydrolase.[24] The large recognition element considerably increases the affinity for xyloglucanases compared to smaller inhibitors and the inhibitors (**9** and **10**) have been used to observe enzyme substrate interactions by X-ray crystallography. As commonly observed with α-halo ketone inhibitors retaining glycosidases are covalently attached via the general acid/base residue instead of the naturally more reactive catalytic nucleophile.[25,26] To access the natural binding mode and increase selectivity a synthesis starting from the same lactol towards the mechanism-based 2-deoxy-2-fluoro inhibitor **11** was developed.[27]

Cyclitol epoxide-based ABPs for xyloglucan active glycosyl hydrolases

In this chapter a set of ABPs needed to monitor and discover cellulose and xyloglucan active retaining glycosidases is presented (Figure 2.4A). The set consists of GG, GX and XG configured cyclophellitols potentially active on retaining cellulases and xyloglucanases grouped in CAZY families 5, 7, 10, 12, 16, 44 and 51. The tag is positioned at the non-reducing end C4' position where the oligomer would naturally be elongated. This position is most likely to be large enough to accommodate the tag in the active site. Moreover, the bulky non-reducing end tag protects against cleavage by exo-glycosidases of the probe and separation of the tag and the warhead ensures labeling is only observed with the intact probe. Untagged GG and GGG configured cyclophellitols were chosen as initial targets to develop the glycosylation chemistry of this class of molecules.

α-Xylose cyclophellitol aziridine potentially targeting exo-α-xylosidases present in CAZY family 31 completes this set. Together with previously developed ABPs for β-glucosidases (**12**)[28,29], β-galactosidases (**13**)[30], α-fucosidases (**14**)[31] and α-L-arabinofuranosidases (**15**)[32] (Figure 2.4B) these probes may target most of the retaining endo- and exo-acting glycosidases active on the glycosidic linkages in xyloglucan.

For the synthesis of GX and XG configured probes **16** and **17** a strategy based on the use of three building blocks was proposed (Scheme 2.1). Selective attachment of the tag at the non-

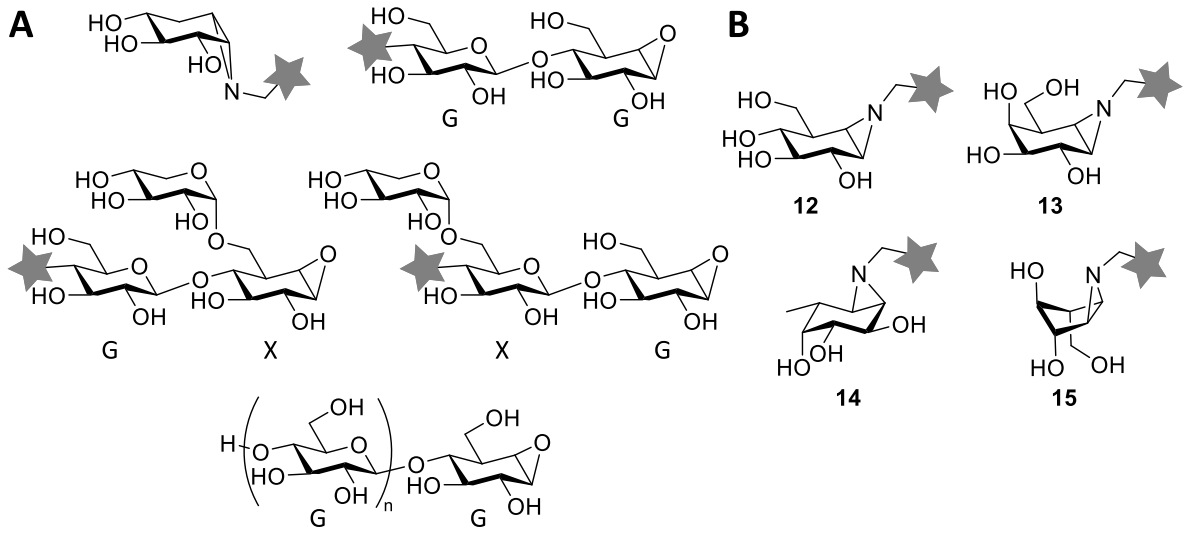
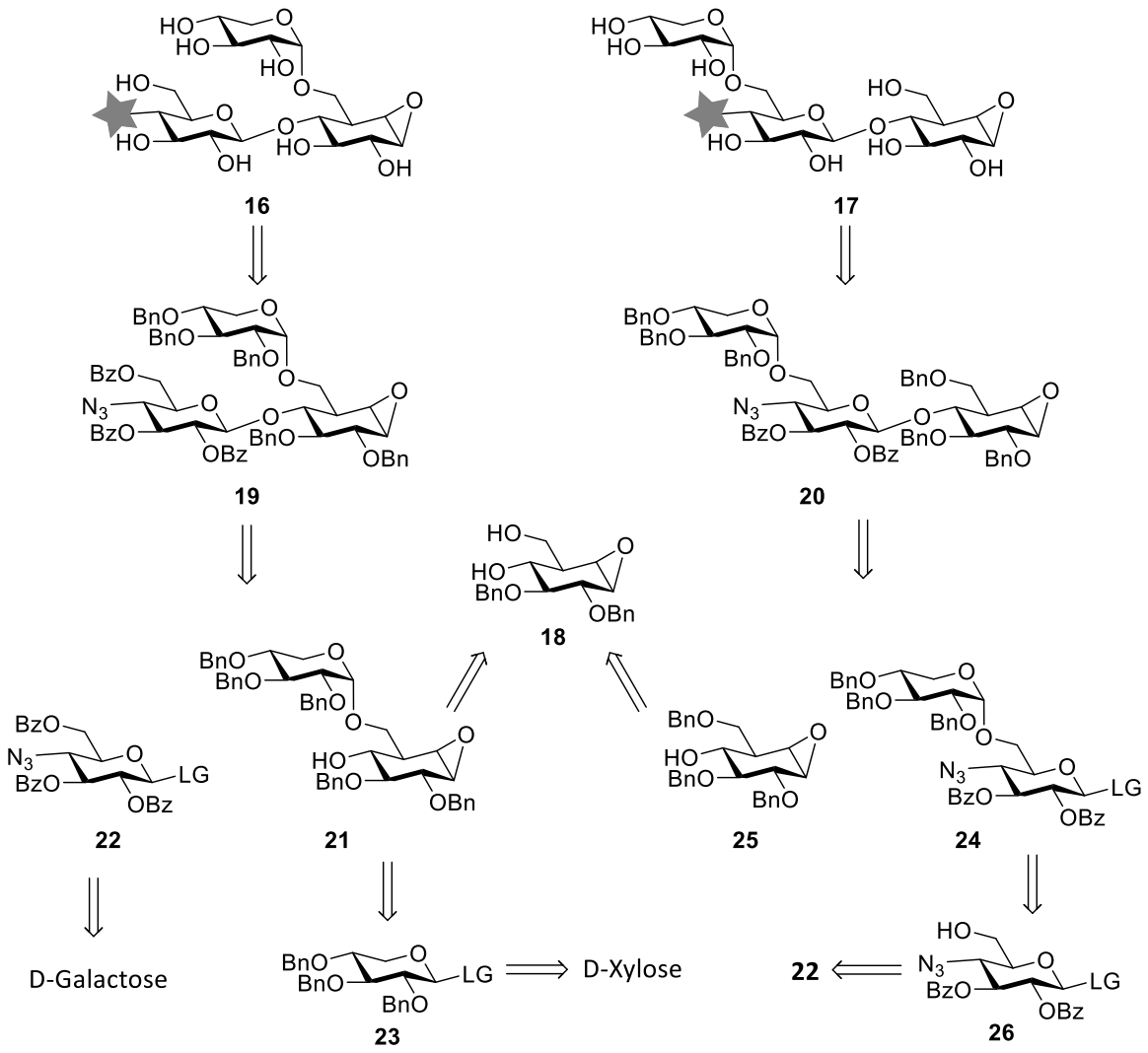


Figure 2.4| A) Structures of activity-based probes mimicking parts of the xyloglucan structure. **B**) Previously synthesized probes that target β-glucosidases (**12**), β-galactosidases (**13**), α-fucosidases (**14**) and α-L-arabinofuranosidases (**15**) relevant for xyloglucan hydrolysis. Stars denote various reporter groups.

reducing end was envisioned via amide bond formation on a C4' amine which could be masked as an azide during the synthesis. For the 1,2-trans glucosylation participating benzoyl esters were chosen. The convenient cyclophellitol synthesis first developed by Madsen *et al.*[33] yields partially benzyl ether protected building block **18**, therefore these non-participating protecting groups were selected for the 1,2-cis xylosylation. This would allow two step deprotection of the complete construct. This strategy would result in fully protected trisaccharides **19** and **20**.

The glucose moiety in **19**, attached to the least reactive 4' secondary alcohol, is first disconnected leading to pseudo-disaccharide **21** and 4-deoxy-4-azido-glucoside **22**. **22** can be accessed from galactose by S_N2 displacement of the activated axial alcohol with an azide nucleophile. **21** is accessible from cyclophellitol building block **18** by regio- and stereoselective xylosylation of the primary alcohol with xylosyl donor **23**.

Trisaccharide **20** is first disconnected into disaccharide **24** and cyclophellitol building block **25** to minimize the number of steps after introduction of the valuable cyclophellitol building block. **25** can be obtained from **18** by regioselective benzylation. **24** can be obtained by 1,2-cis xylosylation with **23** and acceptor **26** which is accessible from **22** by protecting group manipulations.



Scheme 2.1| Retrosynthetic analysis of XG and GX branched probes.

2.2 Results and discussion

The synthesis of the α-xylose cyclitol aziridine probes, cellulose cyclophellitol inhibitors and probes and xyloglucan cyclophellitol probes is described in the following sections. The chapter concludes with a section in which the utility of the endo-glycosidase probes is demonstrated in preliminary labeling studies on an *Aspergillus niger* secretome.

α-Xylosidase activity-based probes

α-Xylosidase ABPs were synthesized starting from aziridine **27** (Scheme 2.2) which was prepared from D-xylose in 11 steps following literature procedures.[34] Selective alkylation of the aziridine nitrogen over the secondary alcohol was achieved using the alkyl triflate and *N,N*-diisopropylethylamine (DIPEA). Subsequently **28** was deprotected in a two-step procedure: Staudinger reduction of the azide to the amine followed by dissolving metal

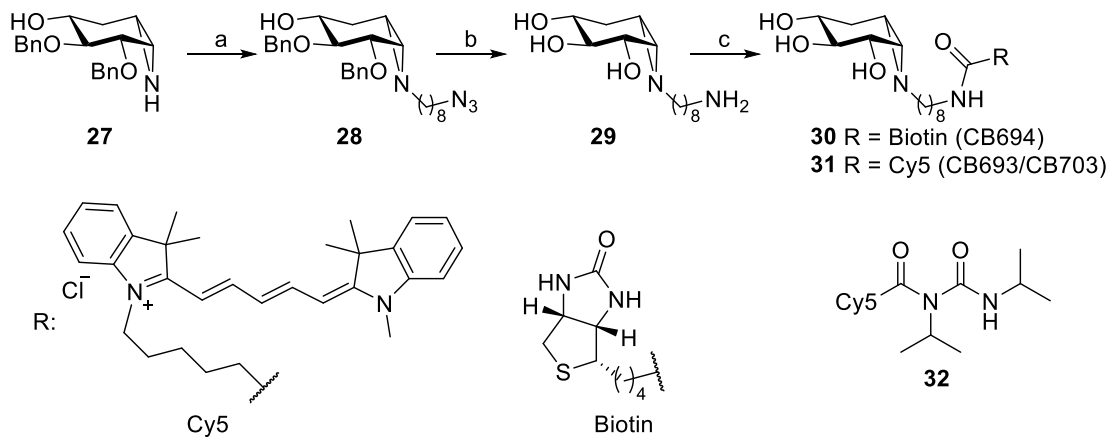
hydrogenolysis of the benzyl groups leading to **29**. The two step sequence is preferred over direct hydrogenolysis of **28** because, although sparsely reported in literature, deamination is a common side product under these conditions.[23,35]

Amide coupling of amine **29** with biotin mediated by *N,N'*-diisopropylcarbodiimide (DIC) afforded **30** after HPLC purification. Attempts to synthesize fluorescent **31** using the same conditions yielded the product contaminated with the rearranged DIC-Cy5 adduct **32**. PyBOB (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) mediated coupling of **29** with Cy5 avoided the formation of such byproducts and the desired product was obtained after HPLC purification.

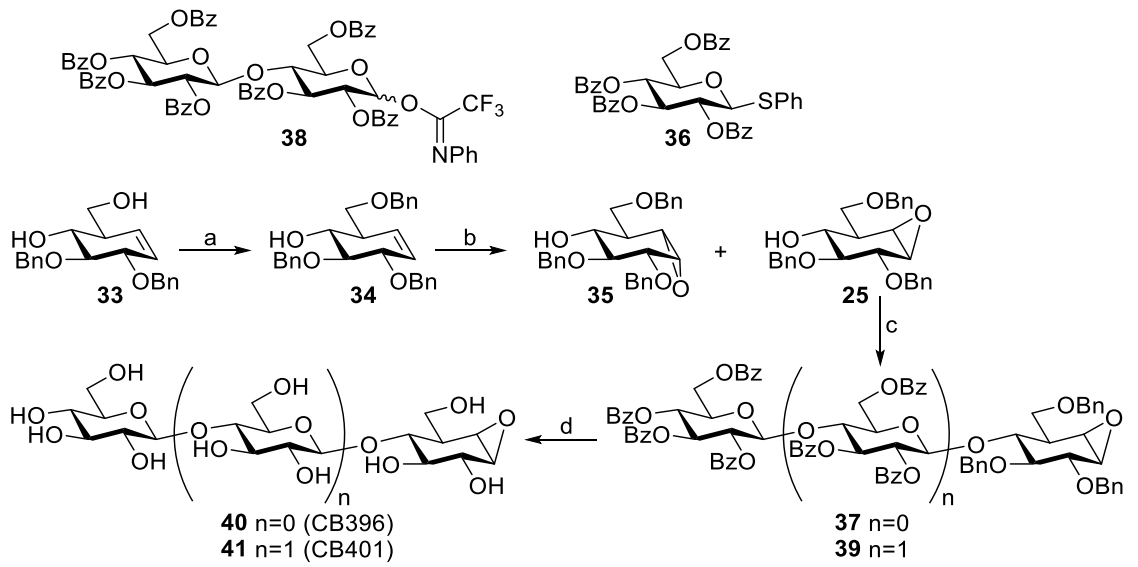
Covalent cellulase inhibitors

Alkene **33** obtained by published methods[33,36] was selectively benzylated at the primary alcohol using borinate catalysis (Scheme 2.3).[37] Epoxidation of **34** using *in situ* generated methyl(trifluoromethyl)dioxirane afforded epoxides **35** and **25** that were separated by silica column chromatography.

Glycosylation of **25** with thioglycoside donor **36**[38] afforded pseudo-disaccharide product **37**. Glycosylation with cellobiose derived *N*-phenyl trifluoroacetimidate **38** afforded pseudo-trisaccharide **39** in comparable yield. Attempts to access the trisaccharide from the thioglycoside donor employing similar conditions as for **37** were sluggish and afforded the product in low yield. This was mainly due to the poor reactivity of the fully benzoylated cellobiose donor. Attempts to increase the yield increasing the equivalents of donor were unsuccessful presumably because activated glycoside are able to react with epoxides.[39]



Scheme 2.2| Reagents and conditions: **a**) 8-azidooctyl trifluoromethanesulfonate, DIPEA, DCM, 86%. **b**) i. PPh_3 polymer bound, H_2O, MeCN, 70°C, 95%; ii. Na(s), *t*-BuOH, THF, NH_3, -60°C, quant. **c**) Biotin, DIC, DMAP, DIPEA, DMF, 18% or Cy5COOH, PyBOB, DIPEA, DMF, 19%.

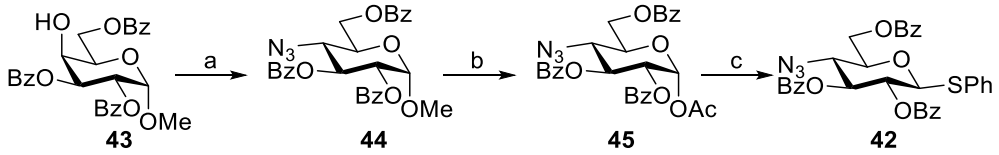


Scheme 2.3| Reagents and conditions: **a**) 2-aminoethyl diphenylborinate, KI, K_2CO_3, BnBr, MeCN, 60°C, 91%. **b**) 1,1,1 trifluoroacetone, oxone, EDTA, $NaHCO_3$, H_2O, MeCN, 0°C, 52% (**25**) and 46% (**35**). **c**) for **37**: **36**, NIS, TMSOTf, DCM, -30°C to -10°C, 53%. For **39**: **38**, TSMOTf, DCM, -15°C to 0°C, 45%. **d**) i. NaOMe, MeOH; ii. H_2, $Pd(OH)_2/C$, H_2O, MeOH, dioxane, for **40** quant., for **41** 45%.

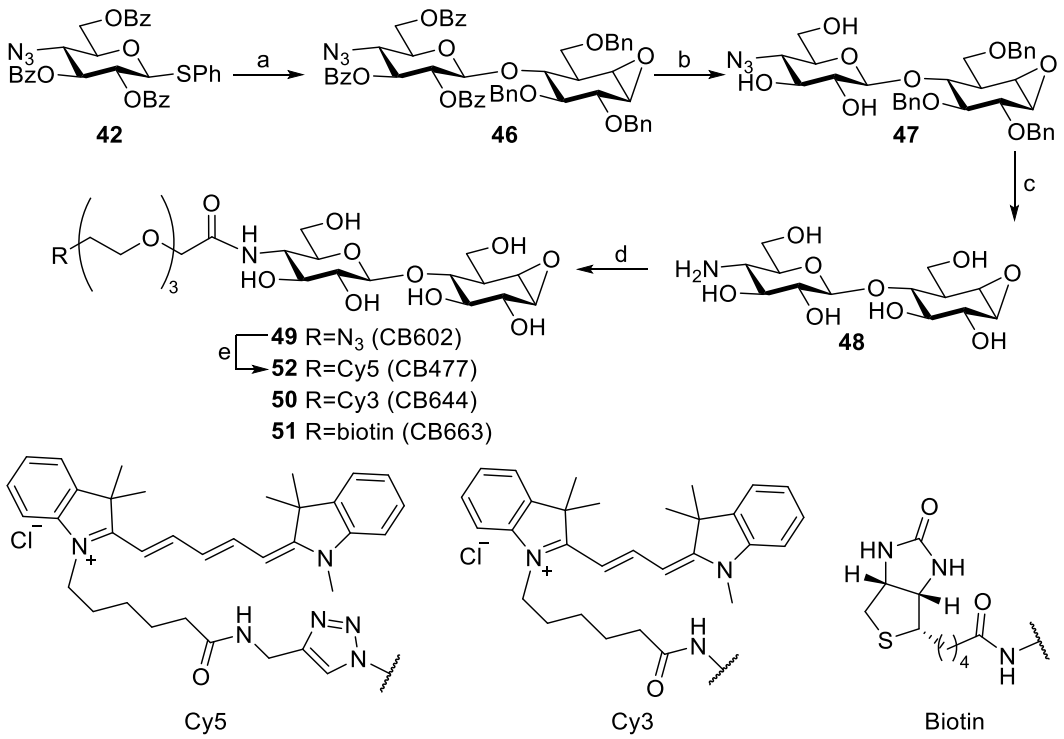
The deprotection sequence comprised of benzoyl removal by NaOMe in MeOH followed by short hydrogenation over a high loading of Pearlman's catalyst[34], provided inhibitor **40** in good yield. Trisaccharide inhibitor **41** was obtained in moderate yield mainly due to the poor solubility of the partially protected trisaccharide. To remove the methyl benzoate formed during the debenzoylation step the mixture was triturated in cold ether, lowering the yield considerably compared to the chromatography procedure used for the disaccharide.

Cellulase (GG) activity-based probes

To gain access to cellobiose configured ABPs 4-deoxy-4-azido-thioglucoside donor **42** was synthesized. The methods are similar to a published synthesis of 4-deoxy-4-fluoro-thioglucoside donors (Scheme 2.4).[40] Regioselective benzoylation of methyl α-D-galactopyranose afforded partially protected **43** of which the axial 4-OH was activated as a triflate and substituted with sodium azide leading to **44**. Acid catalyzed displacement of the



Scheme 2.4| Reagents and conditions: **a**) i. Tf_2O, pyr, DCM, -55°C to rt; ii. NaN_3, DMF, 80°C, 90%. **b**) Ac_2O, AcOH, H_2SO_4. **c**) HSPh, $BF_3{\cdot}Et_2O$, DCM, 46% over 2 steps.



Scheme 2.5| Reagents and conditions: **a**) **25**, Ph_2SO, Tf_2O, TTBP, DCM, -70°C to rt, 64%. **b**) NaOMe, MeOH, DCM, 60%. **c**) Na (s), *t*-BuOH, NH_3, -60°C, 53%. **d**) for **49**: N_3TEGCOOPFP (**S5**), DIPEA, DMF, 27%; for **50**: Cy3TEGCOOH (**S10**), DIC, PFP, DIPEA, DMF, 69%; for **51**: BiotinTEGCOOH (**S8**), DIC, PFP, DIPEA, DMF, 44%. **e**) Cy5 alkyne, THPTA, CuI, DIPEA, DMSO, 46%.

anomeric methoxy group afforded anomeric acetate **45**. Introduction of the anomeric thiophenol yielded donor **42**.

Donor **42** was reacted with acceptor **25** (Scheme 2.5). The yield of the glycosylation reaction was improved compared to the moderate yields obtained for the cellobiose and cellotriose inhibitors **37** and **39** in the previous section. This was achieved by adoption of a Tf_2O/ Ph_2SO pre-activation protocol in combination with the sterically hindered base 2,4,6-tri-*tert*-butylpyrimidine (TTBP). This way relatively high temperatures and long reaction times to activated this type of donors by N-iodosuccinimide (NIS)/triflic acid (TfOH), in the presence of the acid labile epoxide, were avoided. Disaccharide **46** is obtained in 74% yield without the use of a large excess of donor. Unreacted acceptor was recovered indicating the stability of the epoxide functionality under these conditions. Increasing the amount of donor led to diminished yield and complex mixtures presumably by reaction of the epoxide in the product with the excess activated donor.[41]

Subsequently, the benzoyl esters were removed with NaOMe (**47**) followed by reduction of the azide to avoid migration of the esters to the liberated amine. The reduction was

performed in two steps: a Staudinger reduction was performed to reduce the azide followed by benzyl removal under Birch conditions (**48**). Sodium hydroxide, formed while quenching the Birch reaction, was neutralized with NH_4Cl. Omission of this neutralization step leads to hydrolysis of the epoxide during concentration of the reaction mixture.

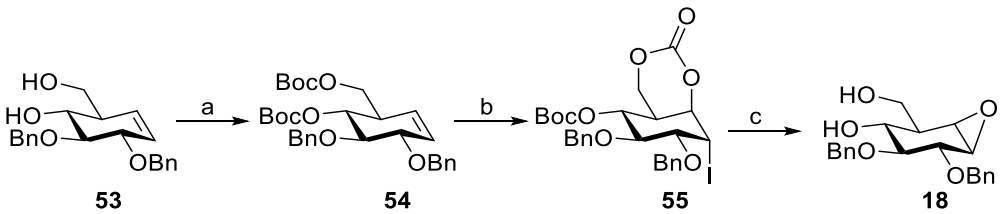
Fully deprotected disaccharide **48** was reacted with the pentafluorophenol activated ester of an azide (**S5**), Cy3 (**S10**) or biotin (**S12**) terminated triethylene glycol (TEG) spacer, yielding the azide (**49**), Cy3 (**50**) and biotin (**51**) equipped probes after semi preparative HPLC purification. Cy5 labeled probe **52** was obtained after copper catalyzed click reaction of **49** with Cy5 alkyne. Synthetic procedures towards the spacers are given in the experimental section (Scheme 2.9).

Xyloglucanase (GX) activity-based probes

To gain access to the GX motif a convenient synthesis of β-configured epoxide **18** was developed (Scheme 2.6). Epoxidation of diol **53** with *meta*-Chloroperoxybenzoic acid (mCPBA) is sluggish on this diol[33] so a diastereoselective iodocarbonylation approach to the β-epoxide was explored instead. [42–47]

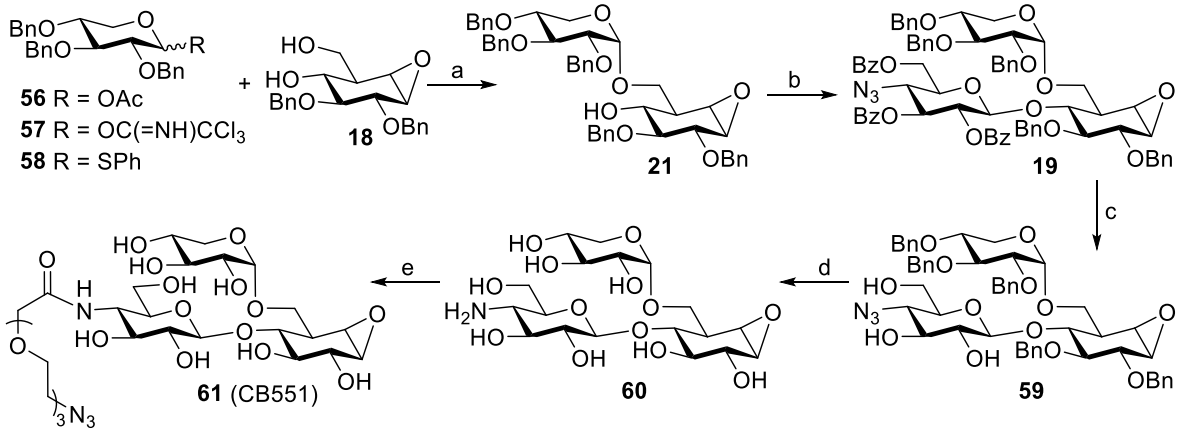
The three-step sequence started with *t*-butyloxycarbonyl (Boc) protection of diol **53** yielding fully protected **54**. Subsequent NIS induced iodocarbonylation afforded iodocarbonate **55**. The obtained iodocarbonate was treated with base in methanol generating the epoxide with concomitant solvolysis of the remaining Boc group leading to epoxide **18** in one step.

With acceptor **18** in hand access to the GX motif was gained by two subsequent glycosylations under basic or mildly acidic conditions to take the acid sensitivity of the epoxide warhead into account (Scheme 2.7). The first glycosylation on the primary alcohol was achieved by reacting xylosyl acetate donor **56**[48] with trimethylsilyl iodide (TMSI), which resulted in the formation of the xylosyl iodide and trimethylsilyl acetate (TMSOAc). The TMSOAc was removed by evaporation before addition of acceptor **18**. $OPPh_3$ was added as an



53 54 55 18

Scheme 2.6| Reagents and conditions: **a**) Boc_2O, DMAP, THF. **b**) NIS, AcOH. **c**) K_2CO_3, MeOH, 75% over three steps.



Scheme 2.7| Reagents and conditions: **a**) i. **56**, TMSI, DCM; ii. **18**, $OPPh_3$, DIPEA, DCM, 46%. **b**) **42**, Tf_2O, Ph_2SO, TTBP, DCM -70°C to rt. **c**) NaOMe, MeOH/DCM, 73% over 2 steps. **d**) i. PPh_3 polymer bound, H_2O, MeCN, 55°C; ii. Na (s), *t*-BuOH, THF, NH_3, 91%. **e**) N_3TEGCOOPFP (**S5**), Et_3N, DMF, 14%.

α-directing catalyst which led to the regio- and stereoselective generation of **21**.[49] Omission of the evaporation step led to significant regeneration of the acetate donor over the course of the reaction consistent with previous reports.[50]

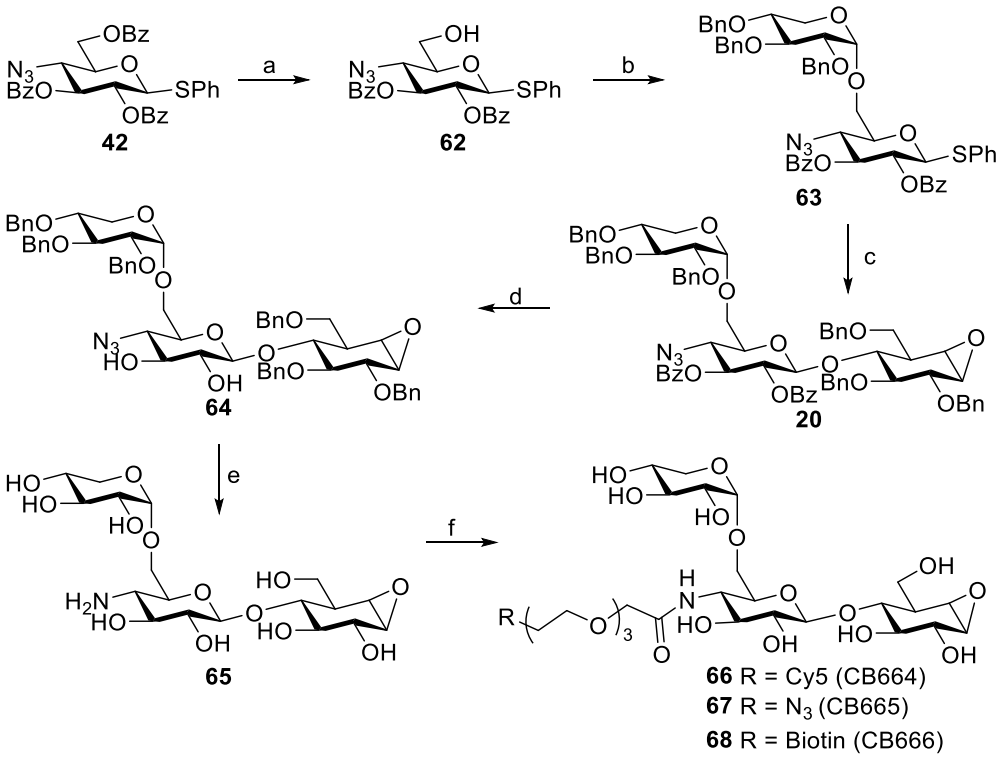
Several alternative leaving groups on the donor were examined. The reaction with trichloroacetimidate donor **57** had an equal yield to the previous method, without the need to co-evaporate the intermediate anomeric iodide. However due to the instability of the armed perbenzylated trichloroacetimidate donor the anomeric acetate was preferred. Pre-activation of thioglycoside donor **58** with tetrabutylammonium iodide (TBAI) as an α-directing additive and *N*-ethylmaleimide as a thiophenol scavenger was unsuccesfull.[51] This may be due to the reagent combination thiophenol and *in situ* generated iodo species that has been shown to react readily with aziridines and epoxides.[52]

Elaboration into the trisaccharide **19** was accomplished with the same pre-activation protocol and donor (**42**) as for the unbranched acceptor (**25**) which led to GX configured **19** in comparable yield to GG configured **46**. Deprotection of the benzoyl groups afforded **59**, which was then completely deprotected under Birch conditions to afford **60** after desalting by HW-40 size exclusion chromatography. The amine on **60** was reacted with a slight excess of the pentafluorophenol activated N_3 TEG spacer **S5** yielding azide tagged activity-based probe **61** after HPLC purification.

Xyloglucanase (XG) activity-based probes

The XG configured probes were synthesized using the conditions developed in the previous sections for the GG and GX configured probes. To this end the central 4-deoxy-4-azido-glucose building block **42** was turned into primary acceptor **62** by complete debenzoylation and subsequent silyl ether protection of the primary alcohol, benzoylation of the two secondary alcohols followed by removal of the silyl ether (Scheme 2.8).

Acceptor **62** was glycosylated α-selectively with armed donor **56** in a TMSI/$OPPh_3$ mediated reaction. Resulting disaccharide thioglycoside **63** was used as a donor in the subsequent glycosylation reaction with cyclophellitol acceptor **25** employing the Tf_2O/Ph_2SO pre-activation conditions completing the XG motif (**20**). The deprotection was accomplished by first removing the benzoyl esters under basic conditions (**64**) followed by dissolving metal hydrogenation to remove the benzyl ethers and reduce the azide to the amine, which, in this case, goes in satisfying yield to fully deprotected amine **65**. The amine was reacted with *in situ* generated pentafluorophenol activated esters of the tagged TEG spacers to yield the Cy5 (**66**), azide (**67**) and biotin (**68**) tagged XG configured ABPs after HPLC purification.



Scheme 2.8| Reagents and conditions: **a**) i. NaOMe, MeOH, DCM, 81%; ii. TBSCl, imidazole, DMF; iii. BzCl, pyr, DCM. iv) TBAF, THF, 79% over 4 steps. **b**) i. **56**, TMSI, DCM; ii. **62**, $OPPh_3$, DIPEA, DCM, rt, 70%. **c**) i. **63**, Tf_2O, Ph_2SO, TTBP, DCM, -70°C to -40°C; ii. **25**, -70°C to rt, 63%; **d**) NaOMe, MeOH, DCM, 81%. **e**) Na (s), *t*-BuOH, THF, NH_3, 76%. **f**) Cy5TEGCOOH (**S12**) or N_3TEGCOOH (**S4**) or BiotinTEGCOOH (**S8**), DIC, PFP, DIPEA, DMF, 23% **66**; 27% **67** and 26% **68**.

Profiling of an Aspergillus niger secretome

Initial assessment of the biological activity of the probes (Figure 2.5A) was made by analyzing the labeling in *Aspergillus niger* U1 mutant secretomes. The secretomes were obtained after growth in fructose containing liquid culture for four days and these secretomes are rich in many different GHs.[53,54]

Labeling with GG configured probe **52** gave, after SDS PAGE resolution of the protein content and in-gel fluorescence scanning, five mayor bands (Figure 2.5B). Comparing this band pattern with that obtained with previously developed β-glucosidase probe **12** and xylanase and xylosidase active probe **8** suggests that these bands do not correspond to exo-glucosidases or xylanases. XG branched probe **66** shows labeling of three of the five bands labeled with **52** with similar intensity indicating that these three labeled proteins may be retaining endo-xyloglucanases acting on an unbranched glucose in a xyloglucan oligomer, as do most known xyloglucanases.

GX branched probe **61** does not show labeling of any of these bands, after azide alkyne ligation of Alexa 488 to the probe, but shows labeling of a lower molecular weight protein.

Dependence of the Alexa 488 signal on probe labeling was confirmed by incubation of the secretome with various concentrations of GX probe followed by ligation to the fluorophore. This might indicate that this protein has xyloglucanase activity with activity on the branched glucose. Although this activity was historically believed to be less prevalent, enzymes have recently been characterized with dominant hydrolytic activity on the substituted glucose (Figure 2.5C).[55–57]

Labeling with the GG probe (**52**) in the secretome at different buffer pH showed different optima for the different bands (Figure 2.5D). Competition with the GGG configured inhibitor (**41**) shows inhibition of most of the bands with much higher potency for most of the bands than the disaccharide inhibitor (**40**) indicating the preference of the anticipated endo-glucanases for polysaccharide substrates (Figure 2.5E).

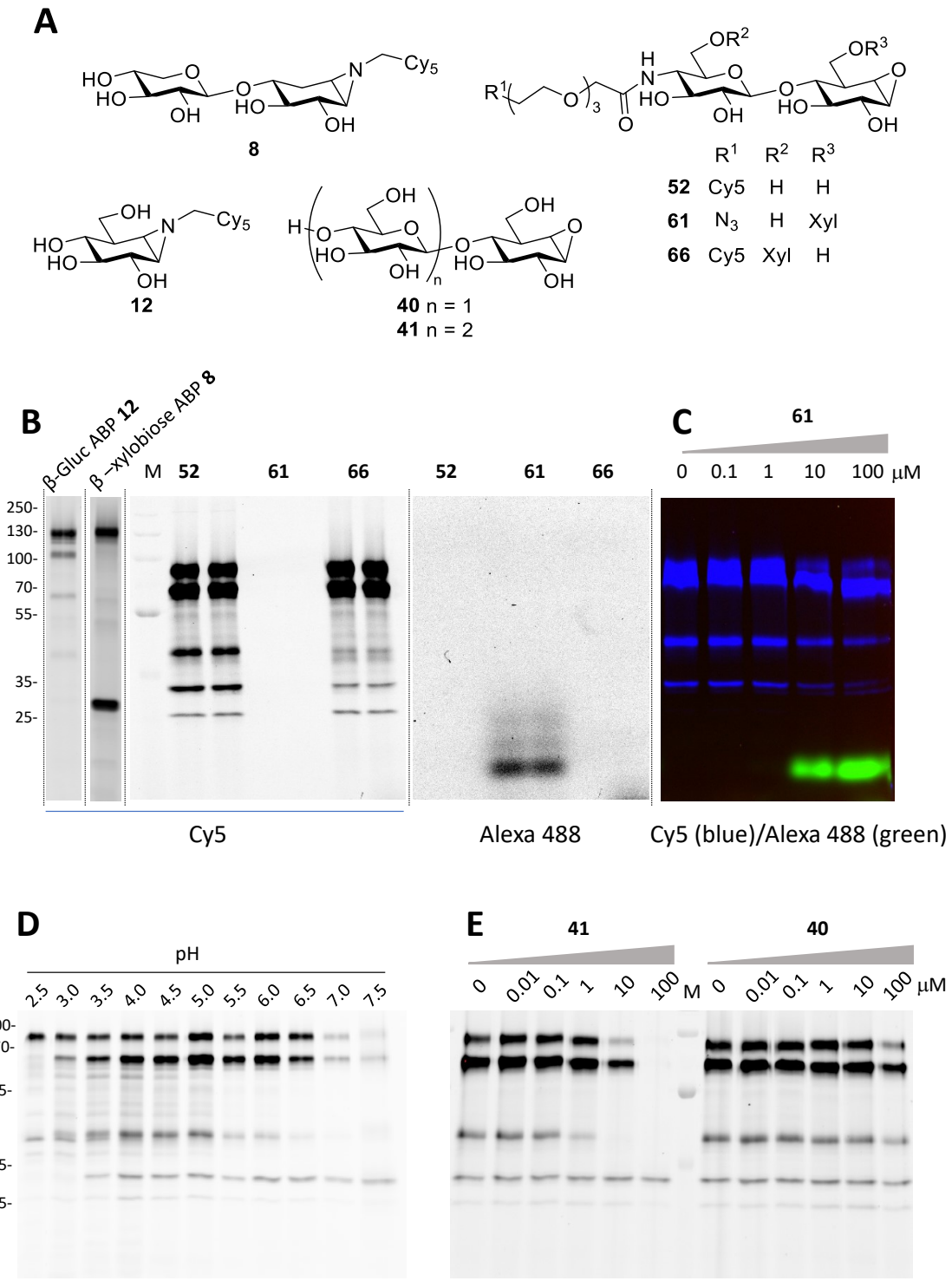


Figure 2.5| Labeling of *Aspergillus niger* secretomes in McIlvaine buffer pH 5.0 or the indicated pH. **A**) Structures of probes used in this figure. **B**) Labeling with probes with different recognition elements shows a different pattern. **C**) Incubation with various concentrations of GX **61** followed by labeling with GG **52** and ligation with Alexa 488 alkyne shows Alexa 488 labeling is dependent on concentration of **61**. **D**) Labeling with GG **52** is pH dependent. **E**) Incubation with GGG (**41**) and GG (**40**) configured inhibitors impairs labeling with GG configured probe **52**.

2.3 Conclusion

In this chapter the synthesis of a set of cellulase and xyloglucanase active ABPs is presented. Tf_2O/Ph_2SO mediated pre-activation glycosylations of thioglucoside donors and $OPPh_3$ mediated 1,2-cis xylosylations with anomeric iodide donors on cyclophellitol acceptors afforded access to the desired structural motifs.

Biological applicability of the set of endo-glucanase probes was revealed by showing distinct labeling in *Aspergillus niger* secretomes. The labeled proteins should be further characterized by use of the biotin tagged probe followed by pull-down and proteomic identification.

In the future the suite of dedicated xyloglucan ABPs can be used for the discovery of unknown enzymes from species or environments with beneficial characteristics.

2.4 Acknowledgements

Aspergillus niger secretomes were kindly provided by Jos Reijngoud, Mark Arenthorst, Gilles van Wezel and Arthur Ram from the Institute of Biology Leiden.

2.5 Experimental

ABPP procedures

Micron filtered *Aspergillus niger* secretome (2 µl) was added to McIlvaine buffer (150 mM), pH 5.0 or indicated in the figure, containing the indicated inhibitor in the indicated concentration and was shaken at 40°C for 30 minutes. The appropriate ABP (final ABP concentration 10 µM for **52**, **61** and **66**, 1 µM for **12** and 5 µM **8**) was added and the sample was shaken at 40°C for another 30 minutes (total volume 10 µl).
To samples with fluorophore containing probes loading buffer (3.5 µl) was added and the samples were boiled for 5 min and stored on ice. To the azide containing samples 2 µl 10% SDS was added followed by click mix (2 µl). The samples were left shaking for 1 hour at 40°C. 4 µl loading buffer was added and the samples were run on a 10% SDS-PAGE gel.
Fluorescently labeled bands were visualized on a ChemiDoc MP imager (BioRad) using Cy3, Cy5 and Alexa 488 multichannel settings and processed using ImageLab 6.0.1 (BioRad). PageRuler Plus Prestained protein ladder (Thermo Fisher Scientific) was used as marker.
Click mix: Alexafluor 488 (90 mM in DMSO, 0.5 µl) CuSO4 (18 mM in water, 0.5 µl), sodium ascorbate (150 mM in water, 0.5 µl), THPTA (18mM in DMSO, 0.5 µl).

General chemical synthesis procedures

All reactions were carried out in oven-dried glassware. Trace amounts of water were removed by co-evaporation with toluene. Reactions were carried out under an atmosphere of nitrogen unless stated otherwise. Tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF) dichloromethane (DCM) and toluene were of reagent grade and were stored over molecular sieves before use. Pentane, petroleum ether and diethyl ether used for workup and column chromatography were of technical grade and used as received. Ethyl acetate (EtOAc) was distilled under reduced pressure before use. Unless stated

otherwise, solvents were removed by rotary evaporation under reduced pressure at 40°C. Triflic acid anhydride (Tf_2O, Fluorochem Ltd) was distilled over P_2O_5 and stored at -20°C for no more than 3 months before use. All other chemicals (Acros, Sigma-Aldrich, TCI, Carbosynth, Merck, Boom, Honeywell & Biosolve) were used as received. Reactions were monitored by TLC analysis using Merck aluminum sheets (Silica gel 60 F254) with detection by UV absorption (254 nm) and 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. Silica gel column chromatography was performed on Screening Devices silica gel 60 (particle size of 40 – 63 μm, pore diameter of 60 Å). Gel filtration was performed on an ÄKTA explorer (GE Healthcare) using a 1.6x60 cm Toyopearl HW-40S resin eluting with a solution of NH_4HCO_3 (150mM), NH_4OAc (150mM) or AcOH (1%) in MilliQ. Fraction monitoring was performed using refractive index. For reversed-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. ^1H and ^{13}C NMR spectra were recorded on a 300/75, 400/100, 500/125, 600/150 or 800/200 MHz spectrometer. Chemical shifts (δ) are given in ppm relative to tetramethylsilane or the residual solvent. Coupling constants are given in Hz. High-resolution mass spectrometry (HRMS) analysis was performed with a LTQ Orbitrap mass spectrometer (Thermo Finnigan), equipped with an electronspray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL/min, capillary temperature 250°C) with 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 mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

General procedure A| Cy TEG amide couplings

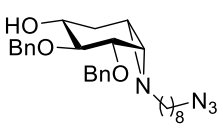
Cy-carboxylic acid was dissolved in dry DCM (0.2 M) and cooled to 0°C. Amine **S6** (1 eq), DMAP (0.05 eq) and DIC (1.2 eq) were added and the mixture was stirred overnight at rt. The mixture was loaded directly on a silica column and purification by flash chromatography.

General procedure B| Amide coupling reporter tag to warhead PFP method

The appropriate carboxylic acid (25 μmol) was dissolved in DMF (0.5 ml), 2,3,4,5,6-pentafluorophenol (23 mg, 0.13 μmol), Et_3N (10 μl, 0.13 mmol) and DIC (3.9 μl,25 μmol) were added and the mixture was stirred for 90 minutes. Part of the stock solution (1.2 eq acid compared to amine) was added to the amine and stirred overnight. LC-MS indicated full conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50 mM AcOH in H_2O). The fractions were concentrated under reduced pressure, co-evaporated with water, diluted with water and lyophilized to yield the product.

α-Xylose activity-based probes

***N*-8-azidooctyl-2,3-di-*O*-benzyl-D-xylose-cyclophellitol aziridine (28)**

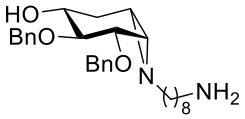


2,3-di-*O*-benzyl-D-xylose-cyclophellitol aziridine[34] **27** (0.15 g, 0.46 mmol) was dissolved in DCM (1.8 ml). The solution was cooled to 0°C and DIPEA (0.12 ml, 0.69 mmol) and freshly prepared 8-azidooctyl trifluoromethanesulfonate[29] (1M in DCM, 0.55 ml, 0.55 mmol) were added. The reaction was slowly warmed to room temperature and stirred for 21 hours. MeOH (2 ml) was added and the mixture was stirred for 2 hours. Toluene was added and the mixture was evaporated to dryness. Column chromatography (DCM/MeOH, 1/0 -> 99/1, v/v) afforded the product as an oil (0.19 g, 0.40 mmol, 86%).

^1H NMR (500 MHz, $CDCl_3$) δ 7.40 – 7.22 (m, 10H, benzyl), 4.85 (br, 1H, OH), 4.70 (d, *J* = 12.2 Hz, 1H, CH_2Bn), 4.65 (d, *J* = 12.2 Hz, 1H, CH_2Bn), 4.62 (d, *J* = 11.7 Hz, 1H, CH_2Bn), 4.50 (d, *J* = 11.7 Hz, 1H, CH_2Bn), 3.83 (dd, *J* = 4.7, 3.4 Hz, 1H, H2), 3.73 (br, 1H, H4), 3.55 (dd, *J* = 5.6, 3.4 Hz, 1H, H3), 3.21 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 2.39 (dt, *J* = 11.5, 6.8 Hz, 1H, CH_2N aziridine), 2.14 – 2.05 (m, 2H, H5a/CH_2N aziridine), 1.97 (ddd, *J* = 14.3, 5.3, 1.8 Hz, 1H, H5b), 1.88 (dd, *J* = 6.3, 4.7 Hz, 1H, aziridine), 1.82 – 1.76 (m, 1H, aziridine),

1.60 – 1.50 (m, 4H, CH_2 spacer), 1.41 – 1.23 (m, 8H, CH_2 spacer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.5, 138.3, 128.4, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.6, 79.7 (C3), 76.6 (C2), 72.6 (CH_2Bn), 70.7 (CH_2Bn), 68.3 (H4), 60.4 (CH_2N aziridine), 51.4 (CH_2N_3), 39.7 (aziridine), 38.2 (aziridine), 29.5, 29.4, 29.0, 28.8, 27.1, 26.8 (C5), 26.6 (spacer). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{28}H_{39}N_4O_3$ 479.3014, found 479.3017.

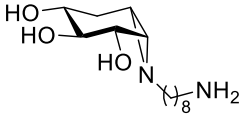
_N_-8-aminooctyl-2,3-di-_O_-benzyl-D-xylose-cyclophellitol aziridine (S1)



Azide **28** (0.189 g, 0.396 mmol) was dissolved in MeCN (7.9 ml). H_2O (71 μl, 3.96 mmol) and PPh3 polymer bound (3 mmol/g, 0.264 g, 0.792 mmol) were added and the mixture was stirred at 70°C for 13 hours. H_2O (0.5 ml) was added and the mixture was stirred for 4.5 hours at the same temperature. The solids removed by filtration, volatiles were removed under reduced pressure and the product was used and analyzed without further purification (0.171 mg, 0.378 mmol, 95%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 – 7.24 (m, 10H), 4.71 (d, *J* = 12.2 Hz, 1H, CH_2Bn), 4.66 (d, *J* = 12.2 Hz, 1H, CH_2Bn), 4.63 (d, *J* = 11.7 Hz, 1H, CH_2Bn), 4.51 (d, *J* = 11.7 Hz, 1H, CH_2Bn), 3.84 (dd, *J* = 4.7, 3.5 Hz, 1H, H2), 3.73 (q, *J* = 5.2 Hz, 1H, H4), 3.55 (dd, *J* = 5.6, 3.5 Hz, 1H, H3), 2.65 (t, *J* = 7.0 Hz, 2H, C**H$_2$**NH$_2$), 2.41 (dt, *J* = 11.5, 6.9 Hz, 1H, CH_2N aziridine), 2.13 – 2.05 (m, 2H, CH_2N aziridine/H5a), 2.02 – 1.94 (m, 1H, H5b), 1.88 (dd, J = 6.3, 4.7 Hz, 1H, aziridine), 1.83 – 1.77 (m, 1H, aziridine), 1.60 – 1.52 (m, 2H), 1.46 – 1.22 (m, 10H).^{13}C NMR (126 MHz, CDCl3) δ 138.6, 138.4, 128.5, 128.4, 127.8, 127.7, 127.6, 79.9 (C3), 76.7 (C2), 72.8 (CH2Bn), 70.9 (CH2Bn), 68.4 (C4), 60.6 (CH2N aziridine), 42.3 (CH2NH2), 39.9 (aziridine), 38.3 (aziridine), 33.8, 29.6, 29.6, 29.4, 27.3, 27.0, 26.9. HRMS (ESI) m/z: [M+H]+ calculated for C28H41N2O3 453.3112, found 453.3112.

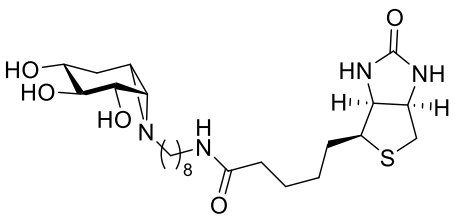
_N_-8-aminooctyl-D-xylose-cyclophellitol aziridine (29)



Ammonia (20 ml) was condensed and kept at -60°C. Sodium (0.26 g, 11.5 mmol) was added and stirred for 10 minutes. Benzyl protected **S1** (0.171 g, 0.382 mmol) dissolved in *t*-BuOH (0.35 ml, 3.68 mmol) and THF (5 ml) was slowly added to the blue solution. The color disappeared immediately so more sodium (85 mg, 3.7 mmol) was added. The blue solution was stirred for 35 minutes. Water was slowly added and the mixture was evaporated. The residue was dissolved in water and eluted over a short column of amberlite CG50 (NH_4^+) with 0.5 M NH_4OH. The combined fractions were concentrated under reduced pressure providing the product as an oil (105 mg, 0.386 mmol quant.).

^{1}H NMR (500 MHz, MeOD) δ 3.70 (dd, *J* = 7.5, 4.0 Hz, 1H, H2), 3.35 – 3.25 (m, 2H, H3/H4), 2.81 – 2.72 (m, 2H, C**H$_2$**NH$_2$), 2.35 – 2.24 (m, 2H, H5a/CH_2N aziridine), 2.15 (ddd, *J* = 11.7, 8.5, 6.4 Hz, 1H, CH_2N aziridine), 1.84 (dd, *J* = 6.5, 4.0 Hz, 1H, aziridine), 1.69 (td, *J* = 6.6, 1.2 Hz, 1H, aziridine), 1.67 – 1.53 (m, 5H, H5b/CH_2 spacer), 1.42 – 1.31 (m, 8H, CH_2 spacer). ^{13}C NMR (126 MHz, MeOD) δ 76.2 (H3), 73.6 (H2), 71.3 (H4), 61.9 (CH_2N aziridine), 45.7 (aziridine), 41.6 (CH_2NH_2), 38.3 (aziridine), 32.6 (C5), 31.0, 30.5, 30.5, 30.3, 28.3, 27.6. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{14}H_{29}N_2O_3$ 273.2172, found 273.2173.

Biotin-D-xylose-cyclophellitol aziridine (30)

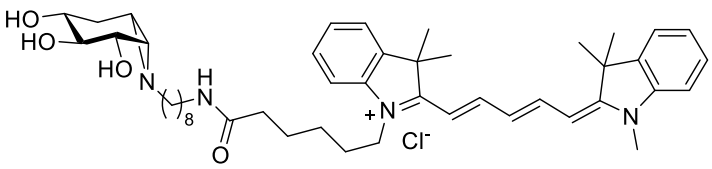


Amine **29** (6.5 mg, 24 μmol) was dissolved in DMF (0.24 ml) and to the solution was added Biotin (6.5 mg , 26 μmol). DIPEA (6.3 μl, 36 μmol), DMAP (cat.) and DIC (6.8 μl ,43 μmol) were added and the mixture was stirred overnight. LC-MS indicated conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM NH_4HCO_3 in H_2O). The fractions were concentrated under reduced pressure, co-evaporated with water, diluted with water and lyophilized to yield the product as a white solid (2.20 mg, 4.40 μmol, 18%).

^{1}H NMR (500 MHz, MeOD) δ 4.49 (dd, *J* = 7.8, 5.0 Hz, 1H), 4.30 (dd, *J* = 7.9, 4.4 Hz, 1H), 3.70 (dd, *J* = 7.5, 4.0 Hz, 1H), 3.29 – 3.25 (m, 2H), 3.23 – 3.18 (m, 1H), 3.16 (td, *J* = 7.0, 1.9 Hz, 2H), 2.93 (dd, *J* = 12.8, 5.0

Hz, 1H), 2.71 (d, *J* = 12.7 Hz, 1H), 2.33 – 2.25 (m, 2H), 2.22 – 2.12 (m, 3H), 1.84 (dd, *J* = 6.5, 4.0 Hz, 1H), 1.65 (tddd, *J* = 26.9, 21.2, 13.2, 6.4 Hz, 8H), 1.46 (dp, *J* = 23.2, 7.8, 7.4 Hz, 4H), 1.34 (s, 8H). ^{13}C NMR (126 MHz, MeOD) δ 176.0, 76.2, 73.6, 71.3, 63.4, 62.0, 61.6, 57.0, 45.7, 41.1, 40.4, 38.3, 36.8, 32.6, 30.6, 30.5, 30.4, 30.4, 29.8, 29.5, 28.4, 28.0, 27.0. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{24}H_{43}N_4O_5S$ 499.2947, found 499.2949.

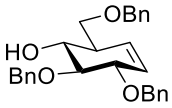
Cy5-D-xylose-cyclophellitol aziridine (31)



Amine **29** (49 mg, 0.18 mmol) was dissolved in DMF (0.25 ml). DIPEA (76 µl, 0.44 mmol), Cy5COOH (94 mg, 0.18 mmol) and PyBOB (0.10 g, 0.20 mmol) were added and the mixture was stirred overnight. LC-MS indicated conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM NH_4HCO_3 in H_2O). The fractions were concentrated under reduced pressure, co-evaporated with water, diluted with water and lyophilized to yield the product as a blue solid (17.4 mg, 0.034 mmol, 19%).
^{1}H NMR (500 MHz, CD_3CN) δ 8.08 (t, *J* = 13.1 Hz, 2H), 7.47 (d, *J* = 7.4 Hz, 2H), 7.43 – 7.37 (m, 2H), 7.28 – 7.21 (m, 4H), 6.65 (d, *J* = 5.8 Hz, 1H), 6.55 (t, *J* = 12.4 Hz, 1H), 6.21 (dd, *J* = 21.1, 13.8 Hz, 1H), 4.00 (t, *J* = 7.5 Hz, 2H), 3.58 (dd, *J* = 7.4, 3.8 Hz, 1H), 3.54 (s, 3H), 3.24 (td, *J* = 9.4, 6.7 Hz, 1H), 3.18 – 3.13 (m, 1H), 3.07 (q, *J* = 6.6 Hz, 2H), 2.20 – 2.07 (m, 4H), 1.77 (p, *J* = 7.4 Hz, 3H), 1.67 (s, 16H), 1.56 – 1.49 (m, 2H), 1.48 – 1.37 (m, 6H), 1.26 (s, 9H).^{13}C NMR (126 MHz, CD_3CN) δ 174.9, 174.3, 173.4, 154.9, 154.8, 144.1, 143.3, 142.4, 142.3, 129.5, 129.5, 126.0, 125.9, 125.6, 123.2, 123.1, 112.0, 111.8, 104.1, 76.1, 73.3, 70.9, 61.4, 50.2, 50.1, 45.1, 44.9, 39.8, 37.8, 36.6, 32.2, 32.0, 30.4, 30.2, 30.1, 29.8, 27.9, 27.8, 27.8, 27.6, 27.5, 27.0, 26.1. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{46}H_{65}N_4O_4$, 737.4996 found 737.5000.

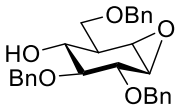
Cyclophellitol acceptor

2,3,6-tri-*O*-benzyl-cyclophellitol alkene (34)



Dibenzyl cyclohexene **33**[33,36] (2.15 g, 6.31 mmol) was co-evaporated with toluene and subsequently dissolved in acetonitrile (32 ml). K_2CO_3 (0.96 g, 6.94 mmol), KI (1.05 g, 6.31 mmol), 2-aminoethyl diphenylborinate (0.14 g, 0.63 mmol) and benzyl bromide (1.13 ml, 9.47 mmol) were added and the mixture was stirred for 20 hours at 60°C. The reaction was quenched with $NaHCO_3$ (250 ml aq. sat.) and the mixture was extracted with EtOAc (2x). The combine organic layers were washed with brine, dried with $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the mixture was purified by column chromatography (pentane/Et_2O, 8/2 -> 7/3, v/v) to afford the product as a white solid (2.45 g, 5.74 mmol, 91%).
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.41 – 7.30 (m, 15H), 5.78 (ddd, *J*=10.2, 2.7, 2.0, 1H, alkene), 5.67 (dt, *J*=10.3, 2.0, 1H, alkene), 5.03 (d, *J*=11.3, 1H, CH_2Bn), 4.82 (d, *J*=11.3, 1H, CH_2Bn), 4.71 (apparent q, *J*=11.5, 2H, CH_2Bn), 4.57 (d, *J*=1.3, 2H, CH_2Bn), 4.23 (ddd, *J*=7.3, 3.5, 1.8, 1H, H2), 3.75 (ddd, *J*=10.2, 8.9, 1.4, 1H, H6A), 3.71 – 3.58 (m, 3H, H3/H4/H6B), 2.58 (m, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.4, 128.7, 128.6, 128.5, 128.3, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 126.8, 84.0 (C3), 80.3 (C2), 75.1 (CH_2Bn), 73.5 (CH_2Bn), 71.7 (CH_2Bn), 71.22 (C4), 71.15 (C6), 44.1 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{28}H_{30}O_4Na$ 453.2042, found 453.2039.

2,3,6-tri-*O*-benzyl-cyclophellitol (25)

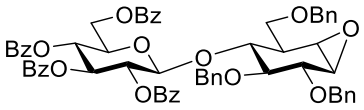


Cyclohexene **34** (1.18 g, 2.74 mmol) was dissolved in acetonitrile (18 ml). EDTA in water was added (9.0 ml, 0.4M) and the mixture was cooled to 0°C. 1,1,1 trifluoroacetone (3.7 ml, 41.13 mmol) was added followed by portion wise addition of a solid mixture of oxone (8.43 g, 13.71 mmol) and $NaHCO_3$ (1.61 g, 19.19 mmol) The reaction was stirred for 2 hours and was then diluted with water and extracted with EtOAc (3x). The combine organic layers were washed with brine and dried with $MgSO_4$. Solids were filtered of and

the solvent was evaporated under reduced pressure. Column chromatography eluting with pentane/Et_2O (8/2 -> 7/3, v/v) afforded first the product (640 mg, 1.42 mmol, 52%) and the epimeric epoxide (560 mg, 1.26 mmol, 46%) as white solids.
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.41 – 7.25 (m, 15H), 4.93 (d, *J*=11.3, 1H, CH_2Bn), 4.81 (d, *J*=11.3, 1H, CH_2Bn), 4.69 (apparent dd, *J*=11.3, 1.5, 2H, CH_2Bn (2x)), 4.58 (d, *J*=1.4, 2H, CH_2Bn), 3.90 (dd, *J*=8.9, 5.0, 1H, H6a), 3.81 (d, J = 7.2, 1H, H2), 3.69 (t, *J*=8.7, 1H, H6b), 3.45 – 3.42 (m, 1H, H7), 3.42 – 3.30 (m, 2H, H3/H4), 3.20 (d, *J*=3.7, 1H, H1), 2.32 – 2.18 (m, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.5, 138.2, 137.6, 128.7, 128.7, 128.6, 128.2, 128.1, 128.0, 127.8, 83.9 (C3), 79.5 (C2), 75.1 (CH_2Bn), 73.6 (CH_2Bn), 72.8 (CH_2Bn), 70.1 (C6), 67.5 (C4), 54.9 (C7), 54.0 (C1), 42.2 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{28}H_{30}O_5Na$ 469.1991, found 469.1985.
Epimeric epoxide **35**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.39 (m, 2H), 7.38 – 7.27 (m, 13H), 4.99 (d, *J* = 11.2 Hz, 1H, CH_2Bn), 4.86 – 4.76 (m, 2H, CH_2Bn), 4.66 (d, *J* = 11.2 Hz, 1H, CH_2Bn), 4.53 (s, 2H, CH_2Bn), 3.86 (dd, *J* = 8.0, 1.8 Hz, 1H, H2), 3.68 (dd, *J* = 4.4, 1.1 Hz, 2H, H6 (2x)), 3.59 – 3.45 (m, 2H, H3/H4), 3.36 (dd, *J* = 4.0, 1.8 Hz, 1H, epoxide), 3.18 (d, *J* = 4.0 Hz, 1H, epoxide), 2.57 (s, 1H, -OH), 2.24 – 2.16 (m, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 138.5, 138.2, 138.2, 128.7, 128.6, 128.6, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 81.3 (C3), 79.7 (C2), 75.7 (CH_2Bn), 73.5 (CH_2Bn), 72.3 (CH_2Bn), 69.5 (C4), 69.1 (C6), 54.8 (epoxide), 54.7 (epoxide), 42.5 (C5).

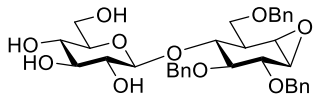
GG inhibitor

4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (37)



Tribenzyl cyclophellitol **25** (45 mg, 0.10 mmol) and phenyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-glucopyranoside (**36**) (0.172 mg, 0.25 mmol)[38] were co-evaporated with toluene (3x). DCM (0.5 ml) and 4Å molecular sieves were added and the mixture was stirred for 30 minutes. NIS (56 mg, 0.25 mmol) was added and the mixture was cooled to −30°C. TMSOTf (5.4 µl, 0.03mmol) was added and the mixture was warmed to −10°C during 2 hours. The reaction was quenched with triethylamine, diluted with DCM and washed with $NaHCO_3$ (aq. sat.) and brine. $MgSO_4$ was added, solids were removed by filtration and the mixture was concentrated under reduced pressure. Column chromatography eluting with pentane/EtOAc (8/2, v/v) yielded the product as a colorless oil (53 mg, 0.053 mmol, 53%).
^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.00 – 7.88 (m, 4H), 7.82 (m, 4H), 7.58 – 7.15 (m, 27H), 5.75 (t, *J*=9.7, 1H, H3'), 5.57 (t, *J*=9.7, 1H, H4'), 5.50 (dd, *J*=9.8, 8.0, 1H, H2'), 5.03 (d, *J*=11.8, 1H, CH_2Bn), 4.94 (d, *J*=8.0, 1H, H1'), 4.81 (d, *J*=11.8, 1H, CH_2Bn), 4.72 – 4.60 (m, 2H, CH_2Bn), 4.38 (d, *J*=11.9, 1H, CH_2Bn), 4.32 (dd, *J*=12.2, 3.4, 1H, H6a'), 4.23 (d, *J*=12.0, 1H, CH_2Bn), 4.17 – 4.06 (m, 1H, 6b'), 3.83 (d, *J*=7.4, 1H, H2), 3.79 – 3.69 (m, 2H, H4/H5'), 3.63 (dd, *J*=8.9, 3.4, 1H, H6a), 3.54 (dd, *J*=9.4, 7.4, 2H, H6b/H3), 3.32 (d, *J*=3.7, 1H, epoxide), 3.12 (d, *J*=3.7, 1H, epoxide), 2.21 (tt, *J*=8.9, 3.3, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 165.9, 165.2, 139.3, 138.2, 137.7, 133.6, 133.5, 133.3, 133.1, 129.9, 129.9, 129.8, 129.7, 129.2, 128.9, 128.9, 128.6, 128.6, 128.6, 128.5, 128.4, 128.4, 128.3, 128.0, 127.9, 127.7, 127.3, 127.1, 101.6 (C1'), 83.1 (C3), 79.5 (C2), 76.5 (C5'), 74.6 (CH_2Bn), 73.3 (C3'), 73.22 (CH_2Bn), 73.19 (CH_2Bn), 72.8 (C2'), 72.1 (C4), 69.8 (C4'), 68.3 (C6), 63.0 (C6'), 55.8 (epoxide), 53.3 (epoxide), 42.1 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{62}H_{57}O_{14}$ 1025.37428, found 1025.37453.

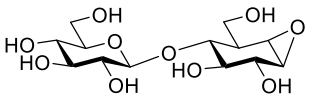
4-*O*-(β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (S2)



Disaccharide **37** (53 mg, 0.052 mmol) was dissolved in DCM/MeOH (0.5 ml, 1/1, v/v) NaOMe (5µl, 5.4M in MeOH) was added and the mixture was stirred overnight. Et_3N·HCl (4.3 mg, 0.031 mmol) was added and the solvent was removed under reduced pressure. Column chromatography eluting with DCM/MeOH (3% -> 5% MeOH) afforded the product as a colorless oil (32 mg, 0.053 mmol, quant.).

^{1}H NMR (500 MHz, MeOD) δ = 7.35 (m, 15H), 5.00 (d, *J*=10.5, 1H, CH_2Bn), 4.82 (d, *J*=11.6, 1H, CH_2Bn), 4.72 – 4.48 (m, 4H, CH_2Bn), 4.34 (d, *J*=7.3, 1H, H1'), 4.04 – 3.87 (m, 2H, H6ab), 3.83 – 3.69 (m, 3H, H2/H4/H6a'), 3.62 – 3.52 (m, 1H, H3), 3.52 – 3.39 (m, 2H, H6b'/epoxide), 3.40 – 3.26 (m, 2H, H3'/H2'), 3.26 – 3.18 (m, 2H, epoxide/H4'), 3.18 – 3.11 (m, 1H, H5'), 2.46 – 2.35 (m, 1H, H5). ^{13}C NMR (126 MHz, MeOD) δ 139.7, 139.5, 139.3, 129.8, 129.5, 129.4, 129.2, 129.1, 128.9, 128.9, 128.7, 128.6, 104.4 (H1'), 84.7 (H3), 80.2 (H2), 78.5 (H5'), 77.9 (H3'), 77.1 (CH_2Bn), 75.9 (C2'), 75.1 (C4), 74.1 (CH_2Bn), 73.8 (CH_2Bn), 72.0 (C4'), 69.6 (C6), 63.0 (C6'), 57.2 (epoxide), 53.8 (epoxide), 44.2 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{34}H_{40}O_{10}$ 609.26942, found 609.26941.

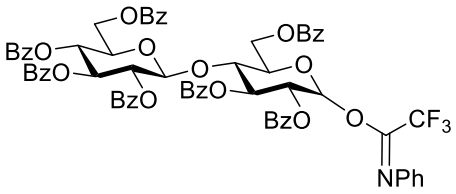
4-*O*-(β-D-glucopyranosyl)-cyclophellitol (40)



Disaccharide **S2** (18 mg, 0.030 mmol) was dissolved in H_2O/MeOH/dioxane (0.6 ml, 1/1/1 v/v) The solution was purged with nitrogen and $Pd(OH)_2$/C (18 mg) was added. The flask was purged with hydrogen and the reaction was stirred under hydrogen atmosphere 2.5 hours. The flask was purged with nitrogen solids were removed by filtration over celite and the solvent was removed in vacuo yielding the product as a white solid (10 mg, 0.030 mmol, quant.).
^{1}H NMR (400 MHz, D_2O) δ = 4.35 (d, *J*=7.9, 1H, H1'), 3.96 (dd, *J* = 11.0, 2.7 Hz, 1H, H6a), 3.88 – 3.70 (m, 3H, H6b/H6a'/H2), 3.61 (dd, *J*=12.4, 4.8, 1H, H6b'), 3.51 – 3.26 (m, 7H), 3.21 (t, *J*=8.9, 1H, H2'), 3.12 (d, *J*=2.9, 1H, epoxide), 2.25 – 2.12 (m, 1H, H5). ^{13}C NMR (101 MHz, D_2O) δ 103.0 (H1'), 77.7, 76.0 (H5'), 75.5, 74.7, 73.3 (C2'), 70.8 (C2), 69.3 (C4'), 60.4 (C6'), 59.7 (C6), 56.6 (epoxide), 55.2 (epoxide), 42.7 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{13}H_{23}O_{10}$ 339.1287, found 339.1286.

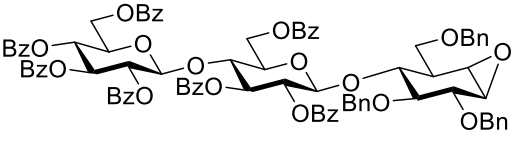
GGG inhibitor

4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl 1-(N-phenyl)-2,2,2-trifluoroacetimidate (38)



4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranose[58] (2.14 g, 2.00 mmol) was dissolved in acetone (13 ml). Cs_2CO_3 (0.977 g, 3.0 mmol) and 2,2,2-trifluoro-*N*-phenyl-acetimidoyl chloride (0.39 ml, 2.4 mmol) were added and the reaction was stirred overnight. The mixture was diluted with EtOAc and washed with $NaHCO_3$ (aq. sat.) and brine. $MgSO_4$ was added, solids were removed by filtration and the mixture was concentrated under reduced pressure. Column chromatography eluting with pentane/EtOAc (8/2, v/v) yielded the product as a white solid as an E/Z mixture (2.50 g, 1.98 mmol, 99%).
^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.17 – 7.88 (m, 18H), 7.84 – 7.71 (m, 7H), 7.67 – 7.48 (m, 6H), 7.48 – 7.12 (m, 33H), 7.12 – 7.00 (m, 3H), 6.95 (t, *J*=7.3, 1H), 6.65 (d, *J*=8.1, 1H), 6.36 (s, 2H), 6.13 (t, *J*=9.1, 1H), 5.78 (td, *J*=9.7, 4.1, 2H), 5.64 – 5.37 (m, 5H), 5.01 (m, 2H), 4.75 – 4.43 (m, 3H), 4.42 – 4.24 (m, 3H), 3.99 – 3.75 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8, 165.5, 165.2, 165.0, 164.9, 142.9, 133.8, 133.5, 133.3, 130.0, 129.9, 129.9, 129.8, 129.8, 129.7, 129.5, 129.4, 128.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 101.2, 76.0, 73.0, 72.7, 72.5, 72.0, 71.5, 70.5, 69.9, 69.4, 62.7, 61.9, 60.5.

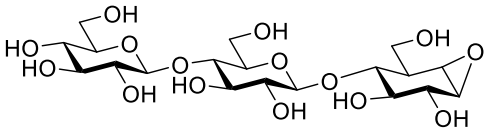
4-*O*-(4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (39)



Donor **38** (0.311 g, 0.25 mmol) and acceptor **25** (45 mg, 0.10 mmol) were co-evaporated with toluene (3x). DCM (1.0 ml) and 4Å molecular sieves were added and the mixture was stirred for 30 minutes. The mixture was cooled to −15°C. TMSOTf (5.4 µl, 0.03 mmol) was added and the mixture was warmed to 0 °C and stirred for 4.5 hours. The reaction was quenched with triethylamine, diluted with DCM and washed with $NaHCO_3$ (aq. sat.) and brine. $MgSO_4$ was added,

solids were removed by filtration and the mixture was concentrated under reduced pressure. Column chromatography eluting with pentane/EtOAc (8/2 -> 7.5/2.5, v/v) yielded the product as a white solid. (67 mg, 0.045 mmol, 45%).
^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.01 – 7.89 (m, 10H), 7.79 – 7.69 (m, 4H), 7.60 – 7.13 (m, 36H), 7.01 (t, *J*=7.6, 2H), 6.93 – 6.77 (m, 1H), 5.62 (m, 2H, H3'/H3"), 5.47 (dd, *J*=9.8, 7.9, 1H, H2"), 5.39 (dd, *J*=9.9, 8.0, 1H, H2'), 5.31 (t, *J*=9.5, 1H, H4"), 4.90 (d, *J*=12.0, 1H, CH_2Bn), 4.82 – 4.74 (m, 3H, H1'/H1"/CH_2Bn), 4.68 – 4.53 (m, 2H, CH_2Bn), 4.33 (d, *J*=12.0, 1H, CH_2Bn), 4.25-4.12 (m, 3H, H6a'/H4'/CH_2Bn), 4.08 – 3.96 (m, 2H, H6b'/H6a"), 3.78 (d, *J*=7.4, 1H, H2), 3.73 – 3.53 (m, 4H, H6b"/H4/H5"/H6b), 3.53 – 3.40 (m, 2H, H6a/H3), 3.30 (m, 2H, H5'/epoxide), 3.09 (d, *J*=3.7, 1H, epoxide), 2.26 – 2.09 (m, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.8, 165.7, 165.6, 165.5, 165.3, 165.1, 164.8, 139.2, 138.1, 137.7, 133.6, 133.5, 133.5, 133.3, 133.3, 133.2, 123.0, 129.8, 129.8, 129.7, 129.7, 129.7, 129.6, 129.6, 129.3, 128.8, 128.7, 128.7, 128.6, 128.6, 128.6, 128.5, 128.4, 128.4, 128.3, 128.1, 128.0, 127.9, 127.9, 127.7, 127.0, 126.6, 101.4 (C1'), 100.8 (C1"), 83.2 (C3), 79.5 (C2), 76.4 (C4), 76.0 (C4'), 74.3 (CH_2Bn), 73.2 (CH_2Bn), 73.2 (CH_2Bn), 73.1 (C5'), 73.0, 72.9, 72.9 (C3"/C3'/C2'), 72.4 (C5"), 71.9 (C2"), 69.6 (C4"), 68.4 (C6), 62.7 (C6"), 62.3 (C6'), 55.6 (epoxide), 53.3 (epoxide), 41.9 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{89}H_{79}O_{22}$ 1499.5058, found 1499.5058.

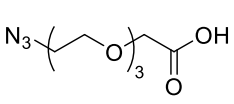
4-*O*-(4-*O*-[β-D-glucopyranosyl]-β-D-glucopyranosyl)-cyclophellitol (41)



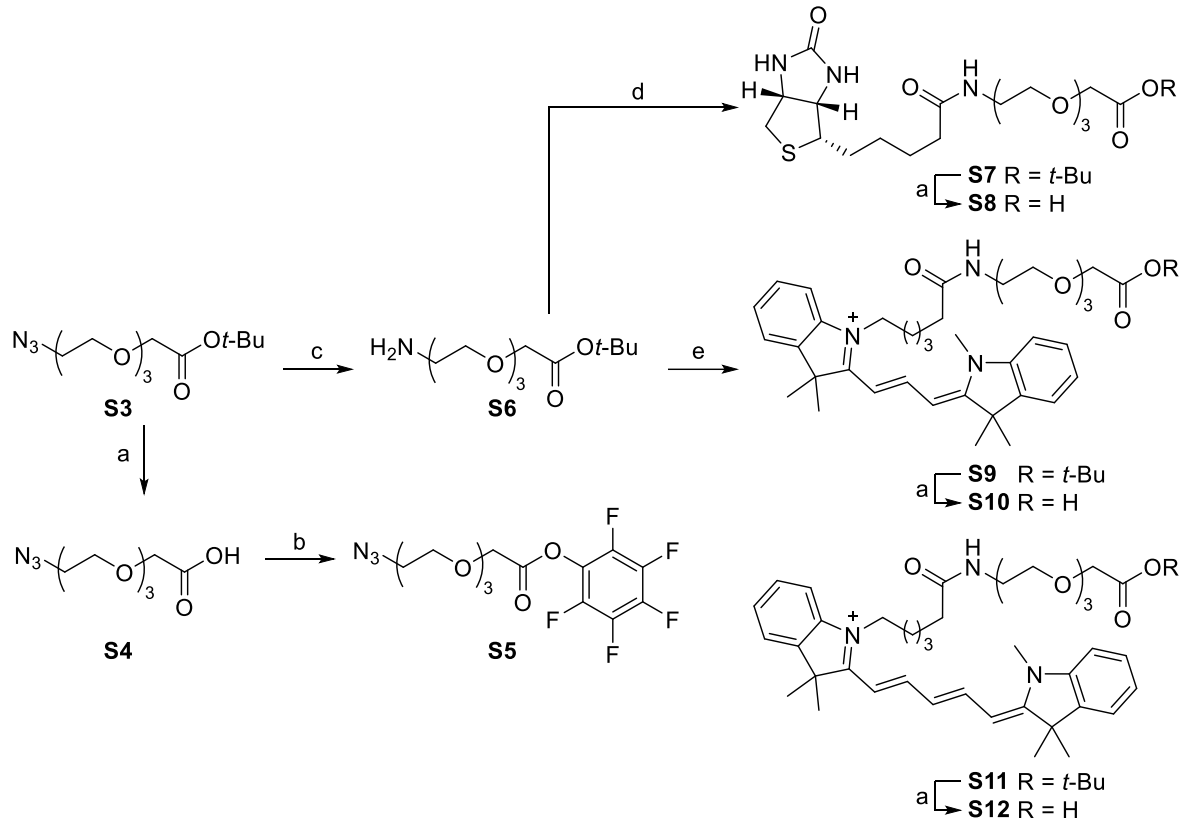
Trisaccharide **39** (64 mg, 0.043 mmol) was dissolved in DCM/MeOH (0.85 ml, 1/1, v/v) NaOMe (4 µl, 5.4M in MeOH) was added and the mixture was stirred overnight. Amberlite CG-50 (NH_4^+) was added until the mixture was no longer strongly alkaline. The resin was filtered of and the solvent was removed under reduced pressure. The residue was dissolved in MeOH (1 ml) and added to cold Et_2O (10ml). The suspension was centrifuged and the solvent was decanted. Subsequently the residue was dissolved in H_2O/MeOH/dioxane (0.4 ml, 1/1/1 v/v). The solution was purged with nitrogen and $Pd(OH)_2$/C (10 mg) was added. The flask was purged with hydrogen and the reaction was stirred under hydrogen atmosphere 2.5 hours. The flask was purged with nitrogen solids were removed by filtration over celite and the mixture was concentrated in vacuo. Water was added and the sample was lyophilized yielding the product as a white powder (8.5 mg, 0.017 mmol, 40%).
^{1}H NMR (500 MHz, D_2O) δ = 4.55 – 4.48 (m, 2H, H1 2x), 4.09 (dd, *J*=11.3, 3.6, 1H, 6a), 4.00 – 3.91 (m, 3H, 6b/6a(2x)), 3.90 – 3.81 (m, 2H, 6b), 3.79 – 3.72 (m, 1H, 6b), 3.72 – 3.64 (m, 2H, H3), 3.62 – 3.55 (m, 1H, epoxide), 3.55 – 3.48 (m, 4H, H3 (2x)/H4/H5), 3.44 (m, 1H), 3.42 – 3.36 (m, 1H, H2), 3.33 (dd, *J*=9.3, 7.9, 1H, H2), 3.25 (d, *J*=3.9, 1H, epoxide), 2.37 – 2.29 (m, 1H, H5). ^{13}C NMR (126 MHz, D_2O) δ 102.9 (C1), 102.6 (C1), 78.3, 77.7, 76.0, 75.5, 74.9, 74.8, 74.2, 73.2 (H2 2x), 70.9, 69.5, 60.6 (C6), 59.8 (C6 2x), 56.7 (epoxide), 55.3 (epoxide), 42.8 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{19}H_{33}O_{15}$ 501.1814, found 501.1818.

Tags with TEG spacers

COOH-TEG-N_3 (S4)

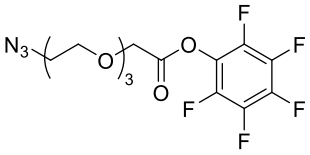


Ester **S3** (100 mg, 0.346 mmol) was dissolved in DCM/TFA (7 ml, 1/1, v/v, 0.05 M) and stirred for 30 minutes. The mixture was repeatedly co-evaporated with toluene and used and analyzed without further purification.
^{1}H NMR (400 MHz, CD_3CN) δ = 4.08 (s, 2H, OC**H_2**COOH), 3.67 – 3.57 (m, 10H), 3.37 (t, *J*=4.9, 2H, CH_2N_3). ^{13}C NMR (101 MHz, CD_3CN) δ 172.2 (COOH), 71.5, 71.1, 71.0, 71.0, 70.5, 68.8, 51.5 (CH_2N_3). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_8H_{15}N_3O_5Na$ 256.0904, found 256.0902.



Scheme 2.9| Reagents and conditions: **a**) TFA, DCM; **b**) PFPOH, DIC, DMAP, DCM, 67% **c**) PPh_3, H_2O, THF, 88%; **d**) Biotin-NHS, DIPEA, DMF, 83%; **e**) DIC, DMAP, DCM, Cy5 acid, 73% or Cy3 acid, 66%.

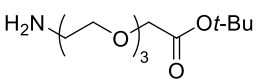
PFP-TEG-N_3 (S5)



Crude acid **S4** (0.346 mmol) was dissolved in DCM (1.9 ml, 0.2 M). 2,3,4,5,6-pentafluorophenol (70 mg, 0.381 mmol), DIC (0.059 ml, 0.381 mmol) and DMAP (cat) were added and the mixture was stirred overnight. Volatiles were evaporated under reduced pressure and the mixture was separated by column chromatography (pentane/EtOAc, 9/1 -> 8/2, v/v) providing the product as a colorless oil (93 mg, 0.23 mmol, 67 % over 2 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.56 (s, 2H, OCH_2C=O), 3.86 – 3.81 (m, 2H), 3.78 – 3.74 (m, 2H), 3.71 – 3.66 (m, 6H), 3.40 (t, *J* = 5.1 Hz, 2H, CH_2N_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.8, 71.4, 70.9, 70.8, 70.8, 70.2, 68.0 (, O**C**H_2C=O), 50.8 (**C**H_2N_3). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{14}H_{18}F_5N_4O_5$ 417.1192 found 417.1190.

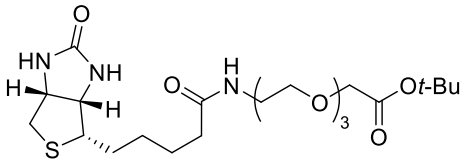
***t*-Bu-TEG-NH_2 (S6)**



Azide **S3**[59] (1.0 g, 3.46 mmol) was dissolved in THF (11.5 ml, 0.3 M), PPh_3 (1.81 g, 6.91 mmol) and H_2O (1.5 ml, 83 mmol) were added and the mixture was stirred 72 hours at rt. The mixture was diluted with H_2O (140 ml) and washed with toluene (3 x 50 ml). The combined organic layers were extracted with H_2O (4 x 20 ml), and then the water layers were combined and evaporated. The residual oil was co-evaporated with dioxane (3x) to give the title compound as an oil (803 mg, 3.04 mmol, 88%).

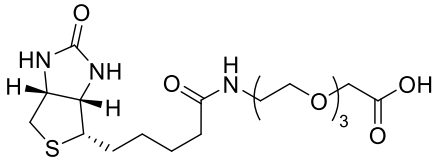
^{1}H NMR (400 MHz, $CDCl_3$) δ 4f.03 (s, 2H), 3.79 – 3.61 (m, 8H), 3.52 (t, *J* = 5.2 Hz, 2H), 2.87 (t, *J* = 5.2 Hz, 2H), 1.82 (br s, 2H, NH2), 1.48 (s, 9H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.6, 81.5, 73.3, 70.6, 70.5, 70.5, 70.2, 68.9, 41.7, 28.0 ppm HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{12}H_{26}NO_5$ 264.1806 found 264.1803.

_t_-Bu-TEG-biotin (S7)



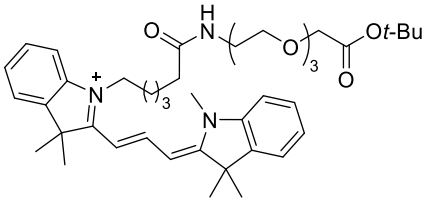
Biotin-NHS[60] (171 mg, 0.5 mmol) was dissolved in dry DMF (1.0 ml, 0.5 M), then DIPEA (105 µl, 0.6 mmol) and amine **S6** (145 mg, 0.55 mmol) were added and the mixture was stirred 16 hours at rt. The mixture was evaporated at 60°C and purification by silica column chromatography (DCM/MeOH, 49/1 -> 9/1, v/v) afforded the title compound as a white solid (202 mg, 0.42 mmol, 83%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 (t, *J* = 5.2 Hz, 1H, NH), 6.94 (s, 1H, NH), 6.24 (s, 1H, NH), 4.59 – 4.43 (m, 1H), 4.36 – 4.18 (m, 1H), 4.02 (s, 2H), 3.81 – 3.61 (m, 8H), 3.57 (t, *J* = 4.8 Hz, 2H), 3.51 – 3.33 (m, 2H), 3.14 (q, *J* = 7.0 Hz, 1H), 2.90 (dd, *J* = 12.7, 4.6 Hz, 1H), 2.84 – 2.64 (m, 1H), 2.23 (t, *J* = 7.4 Hz, 2H), 1.80 – 1.60 (m, 4H), 1.48 (s, 11H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4, 169.5, 164.4, 81.5, 70.5, 70.3, 70.3, 69.9, 69.9, 68.8, 61.7, 60.2, 55.7, 40.4, 39.0, 35.9, 28.3, 28.0, 25.6 ppm. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{22}H_{40}N_3O_7S$ 490.2582 found 490.2571.

COOH-TEG-biotin (S8)



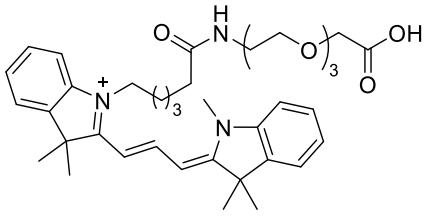
Ester **S7** (195 mg, 0.398 mmol) was dissolved in DCM/TFA (4.0 ml, 0.1 M, 20%). The mixture was stirred for 16 hours at rt, subsequently diluted with toluene (20 ml) and evaporated (3x) to furnish the title product as a white solid (173 mg, 0.399 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$ + 3 drops of CD_3OD) δ 7.13 (s, 1H, NH), 7.00 (s, 1H, NH), 4.55 (dd, *J* = 7.6, 4.8 Hz, 1H), 4.37 (dd, *J* = 7.6, 4.5 Hz, 1H), 4.32 – 4.05 (m, 2H), 3.83 – 3.60 (m, 8H), 3.57 (t, *J* = 4.7 Hz, 2H), 3.52 – 3.33 (m, 2H), 3.18 (q, *J* = 7.3 Hz, 1H), 2.93 (dd, *J* = 13.0, 4.8 Hz, 1H), 2.84 – 2.67 (m, 1H), 2.27 (t, *J* = 7.7 Hz, 2H), 1.80 – 1.58 (m, 4H), 1.51 – 1.39 (m, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$ + CD_3OD) δ 174.7, 174.0, 165.2, 71.0, 70.6, 70.5, 70.1, 69.7, 68.8, 62.2, 60.7, 55.4, 40.5, 39.6, 35.6, 28.1, 27.9, 25.7 ppm. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{18}H_{32}N_3O_7S$ 434.19555 found 434.19565.

_t_-Bu-TEG-Cy3 (S9)



Reaction of **S6** (132 mg, 0.50 mmol) with Cy3-carboxylic acid[61] (229 mg, 0.5 mmol) according to general procedure A followed by flash chromatography (DCM/MeOH, 1/0 -> 94.5/5.5 v/v) afforded the title compound as a red solid (0.24 g, 0.33 mmol 66%).
^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.45 (t, *J*=13.5, 1H), 7.57 – 7.36 (m, 5H), 7.34 – 7.06 (m, 5H), 6.98 (d, *J*=13.4, 1H), 4.17 (t, *J*=7.7, 2H), 4.02 (d, *J*=1.4, 2H), 3.81 (d, *J*=1.5, 3H), 3.76 – 3.55 (m, 10H), 3.46 (dd, *J*=7.8, 3.8, 2H), 2.38 (t, *J*=7.1, 2H), 1.90 (q, *J*=9.3, 8.5, 2H), 1.75 (d, *J*=2.2, 14H), 1.71 – 1.58 (m, 2H), 1.47 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1, 173.5, 173.3, 150.4, 142.3, 141.5, 140.3, 140.1, 128.6, 128.6, 125.2, 122.0, 121.9, 110.7, 110.5, 104.2, 103.7, 70.3, 70.1, 70.1, 69.8, 69.3, 68.6, 48.8, 48.7, 46.1, 44.4, 38.6, 35.8, 31.9, 27.8, 27.8, 26.8, 26.0, 24.9. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{42}H_{60}N_3O_6$ 702.4477, found 702.4473.

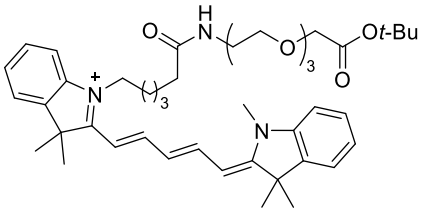
COOH-TEG-Cy3 (S10)



Tert-butyl ester **S9** (121 mg, 0.165 mmol) was dissolved in TFA/DCM (2.42 ml, 0.1 M, 17%) and stirred for 4 hours at rt. The mixture was diluted with toluene (20 ml) and evaporated (3x) to furnish the product as a red solid (112 mg, 0.164 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.41 (t, *J*=13.4, 1H), 7.83 (t, *J*=5.6, 1H), 7.48 – 7.34 (m, 4H), 7.34 – 7.23 (m, 3H), 7.16 (dd, *J*=8.0, 6.0, 2H), 6.49 (dd, *J*=19.3, 13.5, 2H), 4.20 (s, 2H), 4.06 (t, *J*=7.8, 2H), 3.80 – 3.72 (m, 2H), 3.73 – 3.55 (m, 11H), 3.52 – 3.43 (m, 2H), 2.42 (t, *J*=7.5, 2H), 1.89 – 1.64 (m, 16H), 1.60 – 1.49 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.0, 174.6, 174.2, 172.4, 150.6, 142.6, 141.8, 140.6, 140.4, 129.2, 129.0, 125.8, 125.7, 122.3, 122.2, 111.2, 110.9, 103.6, 103.4, 71.1, 70.5, 70.4, 70.0, 69.4,

68.9, 49.4, 49.2, 46.3, 44.5, 39.8, 35.5, 31.5, 28.1, 28.1, 27.1, 26.3, 25.6. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{38}H_{52}N_3O_6$ 646.38506 found 646.38514.

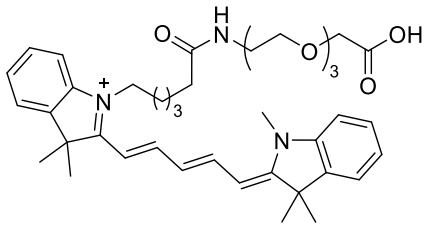
_t_-Bu-TEG-Cy5 (S11)



Reaction of **S6** (66 mg, 0.25 mmol) with Cy5-carboxylic acid[61] (130 mg, 0.25 mmol) according to general procedure A followed by flash chromatography (DCM/MeOH, 98/2 -> 95/5, v/v) afforded the title compound as a blue solid (139 mg, 0.183 mmol, 73%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (t, *J* = 13.0 Hz, 2H), 7.42 – 7.35 (m, 6H), 7.23 (dt, *J* = 10.6, 5.4 Hz, 2H), 7.14 (t, *J* = 6.8 Hz, 3H), 6.79 (t, *J* = 12.4 Hz, 1H), 6.29 (t, *J* = 14.6 Hz, 2H), 4.10 – 4.05 (m, 2H), 4.02 (s, 2H), 3.78 – 3.62 (m, 12H), 3.60 (t, *J* = 5.6 Hz, 2H), 3.51 – 3.40 (m, 2H), 2.34 (t, *J* = 7.1 Hz, 2H), 1.87 – 1.79 (m, 2H), 1.78 (s, 6H), 1.76 (s, 6H), 1.64 – 1.50 (m, 2H), 1.47 (s, 9H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.2, 173.1, 169.6, 154.0, 153.7, 142.6, 141.8, 141.2, 140.8, 128.5, 128.5, 126.2, 125.1, 124.9, 122.2, 122.1, 110.6, 110.3, 103.7, 103.5, 81.5, 77.5, 70.5, 70.4, 70.4, 70.0, 69.6, 68.9, 49.4, 49.2, 44.2, 38.9, 35.9, 31.8, 28.0, 28.0, 27.9, 27.0, 26.4, 25.1 ppm. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{44}H_{62}N_3O_6$ 728.4633 found 728.4628.

COOH-TEG-Cy5 (S12)

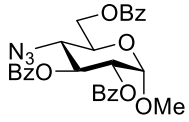


Ester **S11** (139 mg, 0.18 mmol) was dissolved in TFA/DCM (1.8 ml, 0.1 M, 50%) and stirred for 30 minutes at rt. The mixture was diluted with toluene (20 ml) and evaporated (3x) to furnish the title product as a blue solid (128 mg, 0.18 mmol, quant.).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.46 (br s, 2H, COOH), 7.93 (td, *J* = 12.9, 6.4 Hz, 2H), 7.56 (m, 1H), 7.42 – 7.31 (m, 4H), 7.31 – 7.19 (m, 3H), 7.12 (dd, *J* = 16.3, 7.9 Hz, 2H), 6.72 (t, *J* = 12.4 Hz, 1H), 6.30 (d, *J* = 13.6 Hz, 1H), 6.21 (d, *J* = 13.5 Hz, 1H), 4.22 (s, 2H), 4.04 (t, *J* = 7.3 Hz, 2H), 3.79 – 3.72 (m, 2H), 3.71 – 3.56 (m, 11H), 3.53 – 3.30 (m, 2H), 2.36 (t, *J* = 7.3 Hz, 2H), 1.87 – 1.59 (m, 16H), 1.59 – 1.44 (m, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 173.3, 172.9, 172.3, 153.7, 153.0, 142.8, 141.9, 141.2, 140.8, 128.9, 128.7, 126.1, 125.5, 125.1, 122.3, 122.2, 111.0, 110.4, 104.1, 103.5, 70.9, 70.5, 70.0, 69.8, 69.0, 49.5, 49.2, 44.4, 39.4, 35.9, 31.5, 31.3, 28.1, 27.1, 26.4, 25.4 ppm. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{40}H_{54}N_3O_6$ 672.4007 found 672.4003.

GG probes

Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-azido-α-D-glucopyranoside (44)

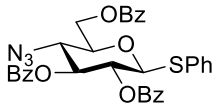


Alcohol **43**[40] (19.0 g, 37.5 mmol) was dissolved in DCM (150 ml, 0.25 M). Pyridine (16.6 ml, 206 mmol) was added and the mixture was cooled to -55°C. Tf_2O (8.84 ml, 52.5 mmol) was added and the mixture was slowly warmed to room temperature. When TLC (8/2, v/v, Pentane/EtOAc) indicated complete consumption of the starting material water and DCM were added and the organic layer was washed twice with brine, dried over $MgSO_4$ and filtered. The volatiles were removed under reduced pressure and the crude triflate was dissolved in DMF (125 ml, 0.3 M). NaN_3 (4.88 g, 75.1 mmol) was added and the mixture was stirred overnight at 80°C. The mixture was allowed to cool to room temperature and was poured over $NaHCO_3$ (aq, sat.). The water layer was extracted trice with EtOAc. The combined organic layers were washed subsequently with $NaHCO_3$ (aq, sat.) and brine, dried with $MgSO_4$ and filtered. Volatiles were removed under reduced pressure and the product was isolated after column chromatography (pentane/EtOAc, 9/1, v/v,) as a colorless oil (17.9 g, 33.8 mmol, 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.16 – 8.09 (m, 2H), 8.05 – 7.96 (m, 4H), 7.62 – 7.53 (m, 1H), 7.52 – 7.42 (m, 4H), 7.39 – 7.29 (m, 4H), 6.06 (t, *J* = 9.9 Hz, 1H, H3), 5.27 (dd, *J* = 10.1, 3.6 Hz, 1H, H2), 5.20 (d, *J* = 3.6 Hz, 1H, H1), 4.74 (dd, *J* = 12.2, 2.4 Hz, 1H, H6a), 4.66 (dd, *J* = 12.2, 4.7 Hz, 1H, H6b), 4.15 – 4.04 (m,

1H, H5), 3.95 (t, *J* = 10.1 Hz, 1H, H4), 3.42 (s, 3H, OMe). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 166.1, 165.8, 165.5, 133.4, 133.4, 133.3, 129.9, 129.7, 129.7, 129.0, 128.8, 128.5, 128.4, 97.1 (C1), 71.9 (C2), 71.1 (C3), 68.0 (5), 63.3 (C6), 61.0 (C4), 55.6 (OMe). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{28}H_{25}N_3O_8Na$ 554.1539, found 554.1542.

Phenyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-azido-1-thio-β-D-glucopyranoside (42)



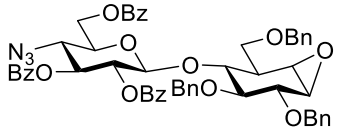
44 (17.88 g, 33.64 mmol) was dissolved in Ac_2O (63.5 ml, 673 mmol). The mixture was cooled to 0°C and AcOH (9.24 ml, 161 mmol) and H_2SO_4 (1.79 ml, 33.6 mmol) were added slowly. The mixture was allowed to warm to rt overnight. TLC (9/1, v/v, pentane/EtOAc) showed full conversion to a lower running spot. $NaHCO_3$ (aq. sat.) was added slowly and the water layer was extracted with toluene three times. The combined organic layers were washed with $NaHCO_3$ (aq. sat.) and brine, dried with $MgSO_4$, filtered and the volatiles were removed under reduced pressure.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 – 8.08 (m, 2H), 8.04 – 7.98 (m, 2H), 7.94 – 7.87 (m, 2H), 7.64 – 7.46 (m, 5H), 7.43 – 7.33 (m, 4H), 6.57 (d, *J* = 3.7 Hz, 1H, H1), 6.00 (dd, *J* = 10.8, 9.2 Hz, 1H, H3), 5.43 (dd, *J* = 10.2, 3.7 Hz, 1H, H2), 4.67 (d, *J* = 3.0 Hz, 2H, H6ab), 4.16 (dt, *J* = 10.5, 3.0 Hz, 1H, H5), 4.00 (t, *J* = 10.1 Hz, 1H, H4), 2.18 (s, 3H, OAc). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 168.7, 166.2, 165.7, 165.5, 133.7, 133.7, 133.5, 129.9, 129.9, 129.6, 128.8, 128.7, 128.6, 128.6, 89.4 (C1), 70.9 (C3), 70.7 (C5), 70.3 (C2), 62.9 (C6), 60.5 (C4), 20.9 (OAc).

The crude product **45** was dissolved in DCM (85ml, 0.4 M) and thiophenol (4.12 ml, 40.4 mmol) and $BF_3{\cdot}Et_2O$ (4.98 ml, 40.4 mmol) were added and the reaction was stirred for 40 hours. Thiophenol (2.05 ml, 20 mmol) and $BF_3.Et_2O$ (4.98 ml, 40.4 mmol) were added and the reaction was stirred for another 2 hours. The reaction was quenched with $NaHCO_3$ (aq. sat.) and the organic layer was washed with $NaHCO_3$ (aq. sat.) and brine and was subsequently dried over $MgSO_4$ and filtered. The volatiles were removed under reduced pressure the product was crystalized out of Et_2O and pentane as a white solid. (9.43 g, 15.47 mmol, 46%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.11 – 8.07 (m, 2H), 7.95 (m, 4H), 7.68 – 7.59 (m, 1H), 7.57 – 7.33 (m, 10H), 7.29 – 7.22 (m, 1H), 7.19 – 7.10 (m, 2H), 5.70 (t, *J* = 9.5 Hz, 1H, H3), 5.36 (dd, *J* = 10.0, 9.4 Hz, 1H, H2), 4.95 (d, *J* = 10.0 Hz, 1H, H1), 4.81 (dd, *J* = 12.1, 1.9 Hz, 1H, H6a), 4.59 (dd, *J* = 12.1, 4.8 Hz, 1H, H6b), 3.92 – 3.76 (m, 2H, H4/H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 166.2, 165.7, 165.3, 133.7, 133.5, 133.5, 131.5, 130.0, 130.0, 130.0, 129.7, 129.1, 129.0, 128.8, 128.7, 128.6, 128.6, 128.5, 86.2 (C1), 76.6 (C5), 75.1 (C3), 70.4 (C2), 63.6 (C6), 60.8 (C4). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{33}H_{27}N_3O_7SNa$ 632.1467, found 632.1467.

4-*O*-(2,3,6-tri-*O*-benzoyl-4-deoxy-4-azido-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (46)

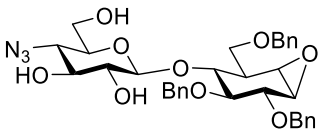


A mixture of **42** (150 mg, 0.246 mmol), Ph_2SO (68 mg, 0.336 mmol) and TTBP (303 mg, 1.22 mmol) was co-evaporated twice with dry toluene and dissolved in dry DCM (2.5 ml). Crushed 3Å molecular sieves were added and the mixture was stirred for 45 min at room temperature. The mixture was cooled to -60°C and freshly distilled Tf_2O (49 µl, 0.291 mmol) was added. The mixture was allowed to warm to -40°C within 30 minutes and was subsequently cooled back to -70°C. **25** (100 mg, 0.224 mmol, co-evaporated trice with dry toluene) was added in DCM (1.0 ml). The mixture was slowly warmed to room temperature overnight. Pyridine (0.05 ml) was added and the mixture was poured over brine. DCM was added and the layers were separated. The organic layer was washed with brine, dried with $MgSO_4$ and filtered. The volatiles were evaporated under reduced pressure and the product was isolated column chromatography (pentane/EtOAc, 9/1 -> 8/2, v/v) provided the product (136 mg, 0.143 mmol, 64%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.04 – 8.00 (m, 2H), 7.92 (m, 4H), 7.61 – 7.12 (m, 24H), 5.50 (t, J=9.8, 1H, H3'), 5.35 (dd, J=9.8, 8.0, 1H, H2'), 4.97 (d, J=11.9, 1H, CH_2Bn), 4.86 – 4.79 (m, 2H, CH_2Bn/H1'), 4.66 (d, J=11.5, 1H, CH_2Bn), 4.61 (d, J=11.5, 1H, CH_2Bn), 4.40 – 4.27 (m, 2H, CH_2Bn/H6a'), 4.24 (dd, J=12.2, 4.2, 1H, H6b'), 4.17 (d, J=11.9, 1H, CH_2Bn), 3.82 (d, J=7.4, 1H, H2), 3.78 (t, J=10.0, 1H, H4'), 3.68 (t, J=9.8, 1H, H4), 3.60 (dd, J=8.8, 3.4, 1H, H6a), 3.52 (dd, J=9.6, 7.4, 1H, H3), 3.46 (t, J=8.5, 1H, H6b), 3.34 – 3.29

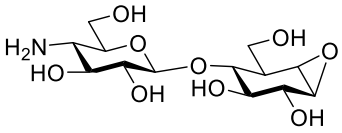
(m, 2H, H5'/epoxide), 3.11 (d, J=3.7, 1H/epoxide), 2.22 – 2.14 (m, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.1, 165.7, 165.3, 139.3, 138.2, 137.7, 133.6, 133.3, 129.9, 129.9, 129.7, 129.1, 128.9, 128.7, 128.6, 128.3, 128.0, 127.9, 127.7, 127.3, 126.8, 101.5 (C1'), 83.2 (C3), 79.5 (C2), 76.5 (C4), 74.4 (CH_2Bn), 74.0 (C3'), 73.2 (CH_2Bn), 73.1 (CH_2Bn), 72.9 (C2'), 72.6 (C5'), 68.4 (C6), 63.2 (C6'), 60.9 (C4'), 55.6 (epoxide), 53.3 (epoxide), 41.9 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{55}H_{51}N_3O_{12}Na$ 968.3365, found 968.3387.

4-*O*-(4-deoxy-4-azido-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (47)



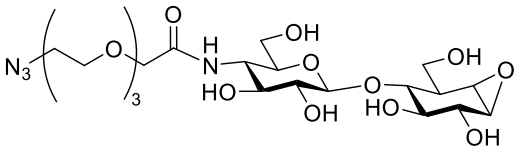
46 (62 mg, 0.098 mmol) was dissolved in a mixture of DCM and methanol (1.3 ml, 1/1, v/v). NaOMe (12 µl of a 5.4 M solution, 0.065 mmol) was added and the mixture was stirred overnight. The reaction was quenched by adding solid CO_2. Volatiles were removed under reduced pressure and column chromatography (EtOAc/DCM, 1/4, v/v) provided the product (25 mg, 0.058 mmol, 60%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 – 7.24 (m, 15H), 4.87 – 4.79 (m, 2H, CH_2Bn (2x)), 4.77 (d, *J* = 11.4 Hz, 1H, CH_2Bn), 4.69 (d, *J* = 11.3 Hz, 1H, CH_2Bn), 4.65 – 4.56 (m, 2H, CH_2Bn), 4.37 (d, *J* = 7.8 Hz, 1H, H1'), 3.90 – 3.80 (m, 3H, H2/H6ab), 3.78 – 3.71 (m, 2H,H4/OH), 3.56 – 3.43 (m, 3H, H3/H3'/H6a'), 3.37 – 3.29 (m, 3H, H4'/H6b'/epoxide), 3.26 (m, 1H, H2'), 3.17 (d, *J* = 3.7 Hz, 1H, epoxide), 2.91 (m, 1H, H5'), 2.79 (d, *J* = 2.9 Hz, 1H, OH), 2.38 – 2.27 (m, 1H, H5), 1.87 (s, 1H, OH). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.7, 137.5, 137.4, 128.7, 128.2, 128.2, 128.2, 128.1, 127.8, 127.0, 102.4 (C1'), 82.9 (C3), 79.5 (C2), 75.8 (C3'), 75.1 (C4), 74.9 (C5'), 74.6 (C2'), 74.6 (CH_2Bn), 73.6 (CH_2Bn), 73.2 (CH_2Bn), 69.1 (C6), 61.9 (C6'), 61.1 (C4'), 56.2 (epoxide), 53.2 (epoxide), 42.3 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{34}H_{39}N_3O_9Na$ 656.2579, found 656.2594.

4-*O*-(4-deoxy-4-amino-β-D-glucopyranosyl)-cyclophellitol (48)



Ammonia (3 ml) was condensed at -50°C. Sodium (36 mg, 1.57 mmol) was added. **47** (33 mg, 0.052 mmol) was dissolved in THF (3 ml) and *t*-BuOH (0.15 ml, 1.57 mmol) and the solution was added dropwise at -60°C. After 45 minutes the reaction was quenched with NH_4Cl (97 mg, 1.82 mmol). The mixture was warmed to room temperature and the ammonia was evaporated.
The crude product was purified by size exclusion chromatography over HW-40 eluting with 150 mM NH_4HCO_3 in H_2O yielding the product as a white solid (9.3 mg, 0.028 mmol, 53%).
^{1}H NMR (400 MHz, D_2O) δ 4.42 (d, *J* = 7.8 Hz, 1H, H1'), 4.04 (dd, *J* = 11.3, 3.6 Hz, 1H, H6a), 3.94 – 3.78 (m, 3H, H6b/H6a'/H2), 3.70 (t, *J* = 12.5, 5.3 Hz, 1H, H6b'), 3.52 (m, 1H, epoxide), 3.49 – 3.45 (m, 2H, H4/H3), 3.44 – 3.34 (m, 2H, H5'/H3'), 3.29 (dd, *J* = 9.3, 7.7 Hz, 1H, H2'), 3.20 (d, *J* = 3.8 Hz, 1H, epoxide), 2.76 (t, *J* = 9.8 Hz, 1H, H4'), 2.28 (m, 1H, H5). ^{13}C NMR (101 MHz, D_2O) δ 103.1 (C1'), 77.7 (C4), 76.0 (C5'), 75.0 (C3'), 74.7 (C3), 73.7 (C2'), 70.8 (C2), 60.6 (C6'), 59.7 (C6), 56.5 (epoxide), 55.2 (epoxide), 52.1 (C4'), 42.6 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{13}H_{24}NO_9$ 338.1446, found 338.1453.

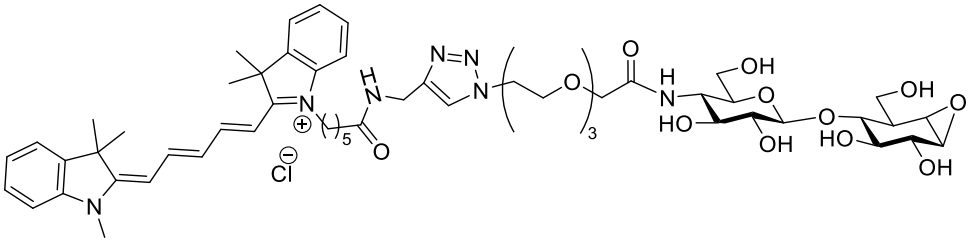
GG azide probe (49)



48 (38 mg, 0.113 mmol) was dissolved in DMF (0.4 ml, 0.3 M) and added to PFP ester **S5** (59 mg, 0.147 mmol). DIPEA (20 µl, 0.136 mmol) was added and the reaction was stirred overnight. LCMS indicated full conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50 mM AcOH in H_2O). The fractions were concentrated under reduced pressure, diluted with water and lyophilized to yield the product as a white solid (16.6 mg, 0.031 mmol, 27%).
^{1}H NMR (500 MHz, D_2O) δ 4.45 (d, *J* = 7.9 Hz, 1H, H1'), 4.11 (s, 2H, OCH_2C=O), 4.06 (dd, *J* = 11.3, 3.5 Hz, 1H, H6a), 3.91 (dd, *J* = 11.3, 6.7 Hz, 1H, H6b), 3.87 – 3.81 (m, 2H, H2/H4'), 3.75 – 3.67 (m, 11H, TEG (10H)/H6a'), 3.65 (dd, *J* = 10.1, 9.3 Hz, 1H, H3'), 3.60 – 3.56 (m, 2H, H3/H6b'), 3.55 – 3.53 (m, 1H, epoxide), 3.52 – 3.46 (m, 4H, H4/CH_2N_3/H5'), 3.38 (dd, *J* = 9.3, 7.9 Hz, 1H, H2'), 3.21 (d, *J* = 3.9 Hz, 1H, epoxide), 2.33 – 2.27 (m, 1H, H5). ^{13}C NMR (126 MHz, D_2O) δ = 173.2, 103.0 (C1'), 77.7 (C3), 74.8, 74.8

(C5'/C3), 73.8 (C2'), 73.2 (C3'), 70.9 (C2), 70.3, 69.6, 69.5, 69.5, 69.5, 69.3 (OCH_2CH_2O/ O**C**H_2C=O), 60.6 (C6'), 59.8 (C6), 56.6 (epoxide), 55.3 (epoxide), 51.1 (C4'), 50.2 (CH_2N_3), 42.8 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{21}H_{37}N_4O_{13}$ 553.2352 found 553.2349.

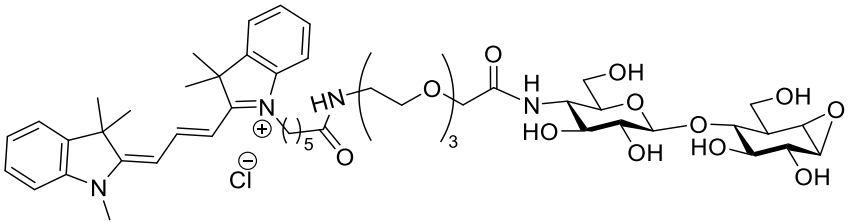
GG Cy5 probe (52)



To **49** (3.1 mg, 5.6 µmol) was added a stock solution of DMSO (0.2 ml) containing THPTA (1.68 µmol), CuI (0.56 µmol) and DIPEA (0.67 µmol). To this solution was added Cy5 alkyne (3.3 mg, 5.9 µmol). The mixture was stirred overnight after which LC-MS analysis indicated full consumption of the starting azide. The product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM NH_4HCO_3 in H_2O). The fractions were concentrated under reduced pressure, diluted with water and lyophilized yielding the compound as a blue solid. (2.8 mg, 2.6 µmol, 46%).

^{1}H NMR (850 MHz, MeOD) δ 8.24 (t, *J* = 12.9 Hz, 2H, alkene), 7.92 (s, 1H, triazole), 7.49 (d, *J* = 7.4 Hz, 2H, phenyl), 7.42 (td, *J* = 7.7, 3.6 Hz, 2H, phenyl), 7.32 – 7.28 (m, 2H, phenyl), 7.28 – 7.25 (m, 2H, phenyl), 6.63 (t, *J* = 12.4 Hz, 1H, alkene), 6.28 (d, *J* = 13.7 Hz, 2H, alkene), 4.58 – 4.56 (m, 2H, OCH_2C**H$_2$**N), 4.42 (s, 2H, TriazoleC**H$_2$**NH), 4.36 (d, *J* = 7.9 Hz, 1H, H1'), 4.10 (m, 3H, H6b/CH_2N=C), 4.01 (s, 2H, O**C**H_2C=O), 3.90 (t, *J* = 5.1 Hz, 2H, OC**H$_2$**CH_2N), 3.83 (dd, *J* = 10.9, 7.1 Hz, 1H, H6a), 3.79 – 3.70 (m, 2H, H4'/H2), 3.69 – 3.59 (m, 15H, TEG10H /H6a'/H3'/CH_3N), 3.56 – 3.49 (m, 2H, H6b'/H5), 3.43 – 3.34 (m, 3H, epoxide/H3/H4), 3.30 – 3.27 (m, 1H, H2'), 3.04 (d, *J* = 3.7 Hz, 1H, epoxide), 2.25 (t, *J* = 7.4 Hz, 2H, HNC=OC**H$_2$**), 2.20 – 2.16 (m, 1H, H5), 1.82 (q, *J* = 7.7 Hz, 2H, HNC=$OCH_2CH_2CH_2$C**H$_2$**), 1.75 – 1.67 (m, 14H, HNC=OCH_2C**H$_2$**/CH_3 4x), 1.48 (q, *J* = 7.9 Hz, 2H, HNC=OCH_2CH_2C**H$_2$**). ^{13}C NMR (214 MHz, MeOD) δ 175.7, 175.4, 174.6, 173.7, 155.5, 155.5, 146.1, 144.3, 143.6, 142.6, 142.5, 129.8, 129.7, 126.6, 126.3, 126.2, 125.0, 123.4, 123.3, 112.1, 111.8, 104.7 (C1'), 104.4 (Cy), 104.3 (Cy), 80.5 (C4), 77.0 (C5'), 76.9 (C3), 75.8 (C2'), 74.9 (C3'), 72.9 (C2), 71.9 (TEG), 71.4 (TEG 2x), 71.3 (TEG), 71.2 (O**C**H_2C=O), 70.3 (O**C**H_2CH_2N), 62.8 (C6'), 62.1 (C6), 56.9 (epoxide), 56.5 (epoxide), 52.9 (C4'), 51.4 (OCH_2**C**H_2N), 44.9 (C5), 44.8 (**C**H_2N=C), 36.5 (HNC=OC**H$_2$**), 35.6 (CH_2NH), 31.5 (CH_3N), 28.2 (HNC=$OCH_2CH_2CH_2$**C**H_2), 28.0 (2x **C**H_3Cq), 27.8 (2x **C**H_3Cq), 27.4 (HNC=OCH_2CH_2**C**H_2), 26.4 (HNC=OCH_2**C**H_2CH_2). HRMS (ESI) m/z: $[M]^+$ calculated for $C_{56}H_{78}N_7O_{14}$ 1072.5601, found 1072.5618.

GG Cy3 probe (50)

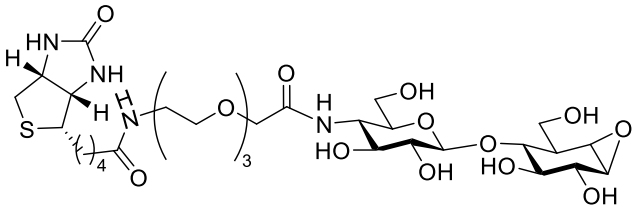


Amine **48** (4.7 mg, 14 µmol) was reacted with stock solution TEG Cy3 **S10** (0.34 ml) according to general procedure B. Providing the product as a red solid contaminated with a small amount of an unknown byproduct (9.7 mg, 9.7 µmol, 69%).

^{1}H NMR (600 MHz, D_2O) δ = 8.48 (t, *J*=13.4, 1H), 7.59 – 7.53 (m, 2H), 7.50 – 7.43 (m, 2H), 7.37 – 7.30 (m, 4H), 6.36 – 6.26 (m, 2H), 4.46 (d, *J*=8.0, 1H), 4.13 – 4.05 (m, 5H), 3.95 – 3.88 (m, 1H), 3.87 – 3.80 (m, 2H), 3.73 – 3.65 (m, 6H), 3.66 – 3.54 (m, 10H), 3.54 – 3.47 (m, 4H), 3.42 – 3.37 (m, 1H), 3.31 – 3.26 (m, 2H), 3.23 (d, *J*=3.8, 1H), 2.33 – 2.27 (m, 1H), 2.28 – 2.22 (m, 2H), 1.90 – 1.82 (m, 2H), 1.73 – 1.70 (m, 12H), 1.69 – 1.62 (m, 2H), 1.41 – 1.32 (m, 2H). ^{13}C NMR (151 MHz, D_2O) δ 177.2, 176.0, 175.5, 173.6, 151.3, 143.3, 142.7, 141.6, 141.5, 129.3, 126.0, 123.0, 122.9, 112.0, 111.7, 103.6, 102.8, 102.7, 78.5,

75.4, 74.4, 73.7, 71.5, 70.8, 70.2, 70.1, 70.0, 69.4, 61.3, 60.5, 57.2, 55.9, 51.7, 49.8, 49.7, 44.3, 43.3, 31.5, 27.9, 27.7, 27.2, 26.0, 25.6. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{51}H_{73}N_4O_{14}$ 965.5118 found 965.5116.

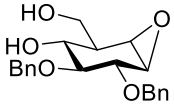
GG biotin probe (51)



Amine **48** (4.7 mg, 14 μmol) was reacted with stock solution TEG biotin **S8** (0.34 ml) according to general procedure B. Providing the product as a white solid. (4.68 mg, 6.2 μmol, 44%).
^{1}H NMR (850 MHz, MeOD) δ = 4.50 (dd, *J*=7.9, 4.0, 1H, biotin), 4.36 (d, *J*=7.9, 1H, H1'), 4.31 (dd, *J*=7.9, 4.5, 1H, biotin), 4.12 (dd, *J*=10.9, 4.1, 1H, 6A), 4.05 (d, *J*=2.4, 2H, O**CH$_2$**C=O), 3.85 (dd, *J*=10.9, 7.1, 1H, 6B), 3.77 – 3.71 (m, 3H), 3.71 – 3.67 (m, 5H), 3.67 – 3.61 (m, 4H), 3.57 – 3.54 (m, 3H), 3.52 (ddd, *J*=10.4, 6.1, 2.1, 1H), 3.42 (dt, *J*=3.7, 1.1, 1H, epox), 3.42 – 3.36 (m, 4H), 3.30 – 3.28 (m, 1H, H2'), 3.22 (ddd, *J*=9.0, 5.9, 4.5, 1H, biotin), 3.05 (d, *J*=3.7, 1H, epoxide), 2.93 (dd, *J*=12.8, 5.0, 1H, biotin), 2.71 (d, *J*=12.7, 1H, biotin), 2.23 (t, *J*=7.4, 1.4, 2H, biotin), 2.21 – 2.17 (m, 1H, H5), 1.77 – 1.71 (m, 1H, biotin), 1.71 – 1.57 (m, 3H, biotin), 1.48 – 1.42 (m, 2H, biotin). ^{13}C NMR (214 MHz, MeOD) δ 176.2, 173.8, 104.7 (C1'), 80.5, 77.0, 76.9, 75.8 (C2'), 74.9, 72.9, 71.8, 71.4, 71.4, 71.2, 71.1, 70.6, 63.4 (biotin), 62.8 (C6'), 62.2 (C6), 61.6 (biotin), 57.0 (biotin), 56.8 (epoxide), 56.5 (epoxide), 52.9, 45.0 (C5), 41.0, 40.3, 36.8, 29.8, 29.5, 26.8. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{31}H_{53}N_4O_{15}S$ 753.3223 found 753.3219.

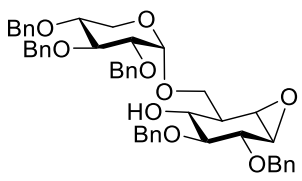
GX probes

2,3-di-*O*-benzyl-cyclophellitol (18)



Diol **53** (102 mg, 0.30 mmol) was dissolved in THF (3 ml, 0.1 M). DMAP (30 mg, 0.24 mmol) and Boc_2O (262 mg, 1.2 mmol) were added and the mixture was stirred overnight. The mixture was diluted with Et_2O and washed with NH4Cl (aq. sat.) $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure.
The residue was dissolved in AcOH (1.3 ml, 0.2 M) and NIS (118 mg, 0.523 mmol) was added and the mixture was stirred overnight. The mixture was diluted with Et_2O and washed with $NaHCO_3$ (aq. sat.), $Na_2S_2O_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The iodide was dissolved in MeOH (3 ml, 0.05 M), K_2CO_3 (96 mg, 0.696 mmol) was added and the mixture was stirred overnight. The mixture was filtered, the solvent evaporated and the residue was dissolved in EtOAc. The solution was washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated. The product was obtained after chromatography (Et_2O/pentane, 9/1, v/v) as a white solid (80 mg, 0.23 mmol, 75%) Spectra matched previously recorded data.[33,36]

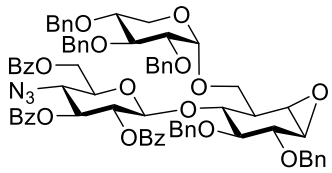
6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-2,3-di-*O*-benzyl-cyclophellitol (21)



Diol **18** (47 mg, 0.132 mmol) was co-evaporated with toluene (3x). DCM (1 ml), DIPEA (30 μl, 0.173 mmol), $OPPh_3$ (160 mg, 0.575 mmol) and 3Å molecular sieves were added and the mixture was stirred for 30 minutes at room temperature.
Acetate donor **56** (80 mg, 0.173 mmol) was co-evaporated with toluene (3x). The dry residue was dissolved in DCM (0.5 ml) followed by the addition of TMSI (25 μl, 0.173 mmol) at room temperature. The mixture was stirred for 30 minutes and turned deep red. The mixture was co-evaporated with toluene (2x) and dissolved in DCM (1 ml) and

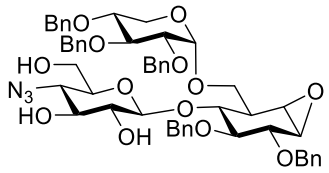
0.8 ml was added to the reaction flask containing **18**. Upon addition the color faded immediately and the reaction was stirred for 23 hours.
The reaction was quenched by the addition of $NaHCO_3$ (aq. sat.). The solution was separated from the solids and diluted with DCM. The layers were separated and the organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The product was isolated after column chromatography (pentane/EtOAc, 85/15, v/v) as a white solid (47 mg, 0.061 mmol, 46%).
^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.42 – 7.18 (m, 25H), 4.95 – 4.60 (m, 11H, CH_2Bn (5x)/H1'), 3.92 – 3.84 (m, 2H, H3'/H6a), 3.81 (d, *J*=8.1, 1H, H2), 3.73 (dd, *J*=9.4, 5.7, 1H, H6b), 3.63 (q, *J*=3.2, 1H,H5a'), 3.59 – 3.51 (m, 2H, H5b'/H4'), 3.47 (dd, *J*=9.6, 3.6, 1H, H2'), 3.43 – 3.38 (m, 2H, epoxide/H3), 3.33 (t, *J*=9.6, 1H, H4), 3.20 (d, *J*=3.7, 1H, epoxide), 2.88 (s, 1H, OH), 2.39 – 2.31 (m, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 139.0, 138.6, 138.4, 138.3, 137.6, 128.7, 128.6, 128.6, 128.6, 128.5, 128.2, 128.2, 128.1, 128.1, 128.0, 128.0, 128.0, 127.7, 97.5 (C1), 83.7 (C3), 81.5 (C3'), 79.8 (C2'), 79.3 (C2), 78.1 (C4'), 75.9 (CH_2Bn), 75.1 (CH_2Bn), 73.7 (CH_2Bn), 73.6 (CH_2Bn), 72.9 (CH_2Bn), 68.1 (C4), 68.0 (C6), 60.2 (C5'), 54.6 (epoxide), 54.2 (epoxide), 41.7 (C5). $^1J_{H,C}$ 4.70 ppm, 97.5 ppm = 168 Hz. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{47}H_{50}O_9Na$ 781.3353, found 781.3362.

6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-4-*O*-(2,3,6-tri-*O*-benzoyl-4-deoxy-4-azido-β-D-glucopyranosyl)-2,3-di-*O*-benzyl-cyclophellitol (19)



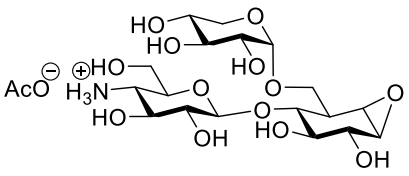
A mixture of **42** (74 mg, 0.121 mmol), Ph_2SO (31 mg, 0.151 mmol) and TTBP (75 mg, 0.303 mmol) was co-evaporated twice with dry toluene and dissolved in dry DCM (1.0 ml). Crushed 3Å molecular sieves were added and the mixture was stirred for 2 hours at room temperature. The mixture was cooled to -60°C and freshly distilled Tf_2O (23 µl, 0.139 mmol) was added. The mixture was allowed to warm to -40°C and was subsequently cooled back to -70°C. **21** (46 mg, 0.061 mmol, co-evaporated trice with dry toluene) was added in DCM (0.5 ml). The mixture was slowly warmed to room temperature overnight. Pyridine (0.05 ml) was added and the mixture was poured over brine. DCM was added and the layers were separated. The organic layer was washed with brine, dried with $MgSO_4$ and filtered. The volatiles were evaporated under reduced pressure and the product was isolated after size exclusion over sephadex LH-20 eluting with DCM/MeOH (1/1, v/v) (79 mg). The product was not completely pure and a yield over two steps is provided after the next step. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{74}H_{71}N_3O_{16}Na$ 1280.4727, found 1280.4735.

6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-4-*O*-(4-deoxy-4-azido-β-D-glucopyranosyl)-2,3-di-*O*-benzyl-cyclophellitol (59)



19 (79 mg) was dissolved in MeOH/DCM (2 ml, 1/1, v/v). NaOMe (10 µl, 5.4M, 0.042 mmol) was added and the mixture was stirred overnight. Solid CO_2 was added the mixture was stirred for a few minutes and the solvent was evaporated under reduced pressure. Column chromatography (DCM/EtOAc, 1/0 -> 4/1, v/v) provided the product (42 mg, 0.045 mmol, 73% over 2 steps).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 – 7.26 (m, 25H), 4.89 (s, 2H, CH_2Bn), 4.83 (d, *J* = 3.5 Hz, 1H, H1 xyl), 4.80 (s, 2H, CH_2Bn), 4.79 – 4.66 (m, 5H, CH_2Bn), 4.61 (d, *J* = 11.6 Hz, 1H, CH_2Bn), 4.27 (d, *J* = 7.7 Hz, 1H, H1 glu), 3.97 (dd, *J* = 9.8, 3.8 Hz, 1H, H6a), 3.94 – 3.84 (m, 2H H3 xyl/H6b), 3.83 (m, 1H, H2), 3.75 – 3.69 (br, 1H, OH), 3.69 – 3.65 (m, 1H, H5a xyl), 3.61 – 3.45 (m, 7H, H4/H3/H5b xyl/H2 xyl/H4 xyl/H6a glu/epoxide), 3.36 – 3.23 (m, 4H, H3 gluc /H2 glu/H6b glu/H4 glu), 3.20 (d, *J* = 3.8 Hz, 1H, epoxide), 3.13 (br, 1H, OH), 2.96 – 2.88 (m, 1H, H5 gluc), 2.47 – 2.41 (m, 1H, H5).^{13}C NMR (126 MHz, $CDCl_3$) δ = 138.9, 138.4, 138.2, 138.2, 137.5, 128.7, 128.6, 128.6, 128.5, 128.5, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.7, 127.4, 102.2 (C1 glu), 97.1 (C1 xyl), 82.3 (C3) , 81.2 (C3 xyl), 79.7 (C2 xyl), 79.5 (C2), 78.1 (C4 xyl), 75.8 (C3 gluc), 75.8 (CH_2Bn), 75.0 (C5 gluc), 74.7 (C4), 74.7 (CH_2Bn), 74.1 (C2 gluc), 73.9 (CH_2Bn), 73.6 (CH_2Bn), 73.2 (CH_2Bn), 66.1 (C6), 61.9 (C6 glu), 61.1 (C4 gluc), 60.5 (C5 xyl), 55.6 (epoxide), 54.1 (epoxide), 41.9 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{53}H_{59}N_3O_{13}Na$ 968.3940, found 968.3959.

6-*O*-(α-D-xylopyranosyl)-4-*O*-(4-deoxy-4-amino-β-D-glucopyranosyl)-cyclophellitol (60)

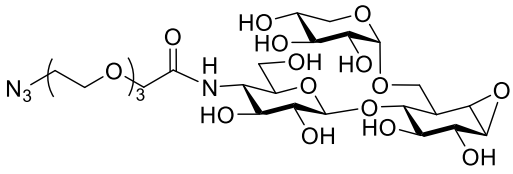


59 (42 mg, 44 µmol) was dissolved in MeCN (1.5 ml). H_2O (150 µl) and polymer bound PPh_3 (84 mg, 3 mmol/g, 250 µmol) were added and the mixture was stirred at 55°C overnight. The reaction was cooled to room temperature, filtered and the solvent was removed under reduced pressure.

Ammonia (5 ml) was condensed at -50°C. Sodium (53 mg, 2.2 mmol) was added. The crude starting material was dissolved in THF (1.5 ml) and *t*-BuOH (0.25 ml, 3.5 mmol) and was added dropwise at -60°C. After 60 minutes the reaction was quenched with AcOH (0.2 ml, 3.52 mmol). The mixture was warmed to room temperature and the ammonia was evaporated. The crude product was purified by size exclusion chromatography over HW-40 eluting with 150 mM NH_4HCO_3 in H_2O yielding the product as a white solid (19 mg, 40 µmol, 91%).

^{1}H NMR (400 MHz, D_2O) δ 4.70 (1H, obscured by HOD peak, H1 xyl), 4.23 (d, *J* = 7.9 Hz, 1H, H1 glu), 3.74 (dd, *J* = 9.5, 3.8 Hz, 1H, H6a), 3.71 – 3.12 (m, 15H), 3.05 (d, *J* = 3.8 Hz, 1H, epoxide), 3.00 (t, *J* =f 10.0 Hz, 1H, H4 glu), 2.36 – 2.26 (m, 1H, H5). ^{13}C NMR (101 MHz, D_2O) δ = 102.7 (C1 glu), 98.3 (C1 xyl), 77.8, 74.2, 73.4, 73.0, 72.3, 71.7, 71.2, 70.6, 69.2, 65.6 (C6), 61.3, 60.2, 56.2 (epoxide), 56.2 (epoxide), 52.1 (C4 glu), 40.7 (C5). $^1J_{H,C}$ 4.23 ppm, 98.3 ppm = 171 Hz, $^1J_{H,C}$ 4.70 ppm, 102.7 ppm = 162 Hz. HRMS (ESI) m/z: $[M+H_3O]^+$ calculated for $C_{18}H_{34}NO_{14}$ 488.1974 found 488.1978.

GX azide probe (61)

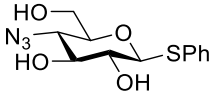


60 (19 mg, 40 µmol) was dissolved in DMF (0.5 ml, 0.1 M) and added to PFP ester **S5** (24 mg, 61 µmol). Et_3N (1 drop) was added and the reaction was stirred overnight. LC-MS indicated full conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM AcOH in H_2O). The fractions were concentrated under reduced pressure, diluted with water and lyophilized to yield the product as a white solid (4.3 mg, 5.6 µmol 14%).

^{1}H NMR (850 MHz, D_2O/MeOD) δ 4.93 (d, *J* = 3.7 Hz, 1H, H1 xyl), 4.41 (d, *J* = 7.9 Hz, 1H, H1 glu), 4.11 (s, 2H, OC**H$_2$**C=ON), 4.02 – 3.99 (m, 1H, H6a), 3.92 (t, *J* = 9.3 Hz, 1H, H6b), 3.86 – 3.82 (m, 2H, H2/H4 glu), 3.77 – 3.71 (m, 10H, TEG), 3.71 – 3.63 (m, 5H, H6a glu/H5a xyl/epoxide), 3.62 – 3.55 (m, 3H), 3.54 – 3.49 (m, 5H, H3/H2 xyl/CH_2N_3), 3.42 (t, *J* = 9.9 Hz, 1H, H4), 3.39 – 3.36 (m, 1H, H2 glu), 3.28 (d, *J* = 3.9 Hz, 1H, epoxide), 2.57 – 2.54 (m, 1H, H5). ^{13}C NMR (214 MHz, D_2O/MeOD) δ 174.0 (amide), 104.1 (C1 glu), 99.6 (C1 xyl), 79.1 (C4), 75.9, 75.6, 74.8 (C2 glu), 74.3, 74.1, 72.5 (C2 xyl), 71.8 (C2), 71.3 (TEG), 70.7 (TEG), 70.6 (TEG), 70.5 (TEG), 70.5, 70.3 (TEG), 66.8, 62.4, 61.7, 57.3 (epoxide), 57.2 (epoxide), 52.2 (C4 glu), 51.2 (CH_2N_3), 42.0 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{26}H_{45}N_4O_{17}$ 685.2774 found 685.2771.

XG probes

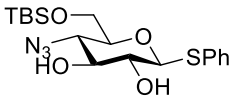
Phenyl 4-deoxy-4-azido-1-thio-β-D-glucopyranoside (S13)



Benzoyl protected building block **42** (1.98 g, 3.25 mmol) was dissolved in MeOH (32 ml) and DCM (7.5 ml). NaOMe (0.12 ml, 5.4 M, 0.65 mmol) was added and the mixture was stirred for 20 hours. The reaction was neutralized with AcOH and dry loaded in silica. Silica with the adsorbed material was loaded on a silica column and eluted (pentane/EtOAc, 7/3 → 6/4, v/v) to provide the product as a white solid (790 mg, 2.63 mmol, 81%).

^{1}H NMR (400 MHz, MeOD) δ 7.56 – 7.52 (m, 2H), 7.33 – 7.22 (m, 3H), 4.59 (d, *J* = 9.8 Hz, 1H, H1), 3.80 (dd, *J* = 12.3, 2.1 Hz, 1H, H6a), 3.69 (dd, *J* = 12.3, 4.5 Hz, 1H, H6b), 3.53 (dd, *J* = 9.6, 8.6 Hz, 1H, H3), 3.41 (t, *J* = 9.8 Hz, 1H, H4), 3.29 – 3.20 (m, 2H, H2/H5). ^{13}C NMR (101 MHz, MeOD) δ 135.0, 132.8, 129.9, 128.4, 89.4 (C1), 80.1 (C5), 78.9 (C3), 73.8 (C2), 63.1 (C4), 62.6 (C6).

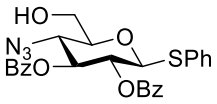
Phenyl 6-*O*-tert-butyldimethylsilyl-4-deoxy-4-azido-1-thio-β-D-glucopyranoside (S14)



Triol **S13** (780 mg, 2.62 mmol) was dissolved in DMF (13 ml, 0.2 M). Imidazole (267 mg, 3.93 mmol) and TBSCl (475 mg, 3.15 mmol) were added and the mixture was stirred overnight. The reaction was diluted with water and extracted with EtOAc (3x) the combine organic layers were washed with water (3x) and brine, dried over $MgSO_4$ and concentrated under reduced pressure yielding a viscous oil (1.17 g) that was used and analyzed without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 – 7.57 (m, 2H, SPh), 7.35 – 7.30 (m, 3H, SPh), 4.50 (d, *J* = 9.7 Hz, 1H, H1), 3.96 (dd, *J* = 11.6, 1.7 Hz, 1H, H6a), 3.88 (dd, *J* = 11.6, 3.9 Hz, 1H, H6b), 3.68 – 3.53 (m, 2H, H3/H4), 3.40 (dd, *J* = 9.7, 8.5 Hz, 1H, H2), 3.29 – 3.24 (m, 1H, H5), 0.98 (s, 9H, *t*-Bu), 0.16 (s, 3H, CH_3Si), 0.15 (s, 3H, CH_3Si). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 132.9, 131.6, 129.0, 128.2, 87.4 (C1), 79.4 (C5), 76.7 (C3), 72.0 (C2), 62.7 (C6), 61.3 (C4), 25.9 (*t*-Bu), 18.4 (*t*-Bu), -5.2 (Me), -5.4 (Me). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{18}H_{33}N_4O_4SSi$ 429.19863, found 429.19861.

Phenyl 2,3-*O*-di-benzoyl-4-deoxy-4-azido-1-thio-β-D-glucopyranoside (62)

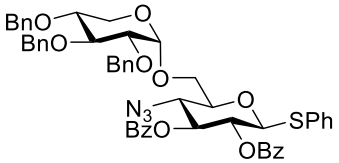


Crude diol **S14** (2.62 mmol) was dissolved in DCM (26 ml, 0.1 M). Pyridine (1.06 ml, 13.1 mmol) and BzCl (0.76 ml, 6.55 mmol) were added and the mixture was stirred overnight. The reaction was quenched with water, poured over $NaHCO_3$ (aq. sat.) and extracted with DCM. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure.

The crude product was dissolved in THF (21 ml, 0.1M), TBAF (5.24 ml, 1 M in THF, 5.24 mmol) was added and the mixture was stirred for 22 hours. The reaction was poured over $NaHCO_3$ (aq. sat.). The mixture was extracted with EtOAc (2x). The combined organic layers were washed with brine (2x), dried over $MgSO_4$ and the volatiles were removed under reduced pressure. The pure product was obtained after chromatography (pentane/Et_2O, 8/2 → 65/35, v/v) as a colorless oil (1.05 g, 2.07 mmol, 79% over 3 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.89 (m, 4H), 7.56 – 7.48 (m, 2H), 7.48 – 7.43 (m, 2H), 7.42 – 7.34 (m, 4H), 7.34 – 7.28 (m, 3H), 5.66 (t, *J* = 9.7 Hz, 1H, H3), 5.36 (t, *J* = 9.7 Hz, 1H, H2), 4.97 (d, *J* = 10.0 Hz, 1H, H1), 4.03 (dd, *J* = 12.4, 2.3 Hz, 1H, H6a), 3.95 (t, *J* = 10.1 Hz, 1H, H4), 3.86 (dd, *J* = 12.4, 4.1 Hz, 1H, H6b), 3.53 (ddd, *J* = 10.3, 4.1, 2.4 Hz, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 165.8, 165.3, 133.6, 133.5, 133.0, 131.9, 130.0, 130.0, 129.2, 129.1, 128.8, 128.6, 128.6, 86.3 (C1), 78.8 (C5), 75.0 (C3), 70.6 (C2), 62.0 (C6), 59.9 (C4). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{26}H_{23}N_3O_6SNa$ 528.11998, found 528.12004.

Phenyl 6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-2,3-*O*-di-benzoyl-4-deoxy-4-azido-1-thio-β-D-glucopyranoside (63)



Acceptor **62** (100 mg, 0.197 mmol) was co-evaporated with toluene (3x). DCM (2 ml), DIPEA (86 µl, 0.493 mmol), $OPPh_3$ (274 mg, 0.985 mmol) and 3Å molecular sieves were added and the mixture was stirred for 1 hour at room temperature.

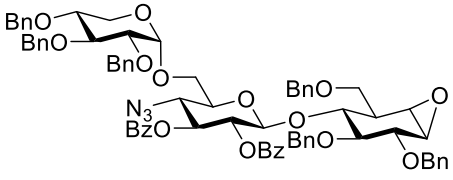
Acetate donor **56**[48] (182 mg, 0.394 mmol) was co-evaporated with toluene (3x). The dry residue was dissolved in DCM (1 ml) followed by the addition of TMSI (59 µl, 0.414 mmol) at room temperature. The mixture was stirred for 30 minutes and turned deep red. The mixture was co-evaporated with toluene (2x), dissolved in DCM (0.5 ml) and added to the reaction flask containing the acceptor. Upon addition the color faded immediately and the reaction was stirred overnight.

The reaction was quenched by the addition of water. The mixture was filtered over celite and diluted with EtOAc. The layers were separated and the organic layer was washed with , $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The product was isolated after column chromatography (pentane/Et_2O, 8/2, v/v) (125 mg, 0.138 mmol, 70%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 – 7.92 (m, 2H), 7.88 – 7.83 (m, 2H), 7.56 – 7.48 (m, 4H), 7.44 – 7.21 (m, 22H), 5.59 (t, *J* = 9.7 Hz, 1H, H3), 5.32 (t, *J* = 9.7 Hz, 1H, H2), 4.96 – 4.87 (m, 3H, H1', CH_2Bn), 4.85

(d, *J* = 10.0 Hz, 1H, H1), 4.75 (m, 2H, CH_2Bn), 4.66 – 4.61 (m, 2H, CH_2Bn), 3.99 – 3.85 (m, 4H, H4/H6ab/H3'), 3.71 – 3.55 (m, 4H, H5ab'/H4'/H5), 3.49 (dd, *J* = 9.6, 3.5 Hz, 1H, H2'). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 165.8, 165.3, 139.1, 138.5, 138.3, 133.9, 133.6, 133.5, 131.7, 130.1, 130.0, 129.3, 129.2, 128.9, 128.7, 128.6, 128.5, 128.5, 128.2, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.7, 97.5 (C1'), 86.5 (C1), 81.2 (C3'), 80.1 (C2'), 78.8, 78.2(C4'/C5), 75.9 (CH_2Bn), 75.0 (C3), 73.6 (CH_2Bn), 73.4 (CH_2Bn), 70.7 (C2), 66.0 (C6), 60.4 (C5' and C4 weak). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{52}H_{49}N_3O_{10}SNa$ 930.3063, found 930.3023.

4-*O*-(6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-2,3-*O*-di-benzoyl-4-deoxy-4-azido-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (20)

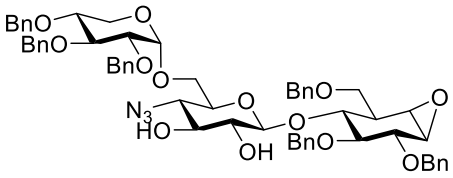


Donor **63** (108 mg, 0.119 mmol), Ph_2SO (33 mg, 0.162 mmol) and TTBP (134 mg, 0.54 mmol) were co-evaporated with toluene (2x). DCM (1.2 ml) and 3 Å molecular sieves were added and the mixture was stirred for 30 minutes at room temperature. The mixture was cooled to -70°C and Tf_2O (0.25 ml, 0.59 M in DCM, 0.148 mmol) was added and the mixture was allowed to warm to -40°C within 30 minutes. The mixture was cooled again to -70°C and acceptor **25** (48 mg, 0.108 mmol) dissolved in DCM (0.5 ml) was added.

The mixture was slowly warm to room temperature overnight, diluted with DCM and poured over brine. The layers were separated and the organic layer was dried over $MgSO_4$. The product was obtained after chromatography (Pentane/EtOAc, 9/1 -> 8/2, v/v) (85 mg, 0.068 mmol, 63%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 – 7.89 (m, 2H), 7.88 – 7.84 (m, 2H), 7.52 – 7.46 (m, 2H), 7.44 – 7.17 (m, 36H), 5.43 (t, *J* = 9.9 Hz, 1H, H3'), 5.30 (dd, *J* = 9.8, 7.9 Hz, 1H, H2'), 4.97 (d, *J* = 12.3 Hz, 1H, CH_2Bn), 4.91 (d, *J* = 3.5 Hz, 1H, H1"), 4.89 – 4.81 (m, 3H, CH_2Bn), 4.80 (d, *J* = 8.0 Hz, 1H, H1'), 4.73 (d, *J* = 11.8 Hz, 1H, CH_2Bn)), 4.65 – 4.57 (m, 4H, CH_2Bn)), 4.55 (d, *J* = 11.4 Hz, 1H, CH_2Bn), 4.32 (d, *J* = 11.8 Hz, 1H, CH_2Bn), 4.15 (d, *J* = 11.8 Hz, 1H, CH_2Bn), 3.94 (t, *J* = 10.1 Hz, 1H, H4'), 3.92 – 3.87 (m, 1H, H3"), 3.86 (d, *J* = 7.4 Hz, 1H, H2), 3.69 (t, *J* = 9.8 Hz, 1H, H4), 3.62 (m, 2H, H6a/H6a'), 3.59 – 3.47 (m, 5H, H5ab"/H6b'/H4"/H3), 3.43 (t, *J* = 8.5 Hz, 1H, H6b), 3.37 – 3.31 (m, 2H, H2"/epoxide), 3.17 (ddd, *J* = 10.2, 3.9, 1.6 Hz, 1H, H5'), 3.09 (d, *J* = 3.7 Hz, 1H, epoxide), 2.18 (dddd, *J* = 9.8, 8.2, 3.5, 1.4 Hz, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 165.8, 165.2, 139.4, 139.0, 138.6, 138.5, 138.2, 137.7, 133.5, 133.4, 130.0, 129.9, 129.3, 129.0, 128.6, 128.6, 128.5, 128.5, 128.5, 128.4, 128.4, 128.4, 128.3, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.6, 127.3, 126.7, 101.6 (C1'), 97.7 (C1"), 83.3 (C3), 81.3 (C3"), 79.9 (C2"), 79.6 (C2), 78.1 (C4"), 75.9 (C4), 75.7 (CH_2Bn), 74.9 (C5'), 74.2 (CH_2Bn), 74.0 (C3'), 73.4 (CH_2Bn), 73.2 (CH_2Bn), 73.1 (C2'), 73.1 (CH_2Bn), 73.0 (CH_2Bn), 68.6 (C6), 65.5 (C6'), 60.4 (C4'/C5" weak), 55.5 (epoxide), 53.3 (epoxide), 42.0 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{74}H_{73}N_3O_{15}Na$ 1266.4934, found 1266.4942.

4-*O*-(6-*O*-(2,3,4-tri-*O*-benzyl-α-D-xylopyranosyl)-4-deoxy-4-azido-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-cyclophellitol (64)

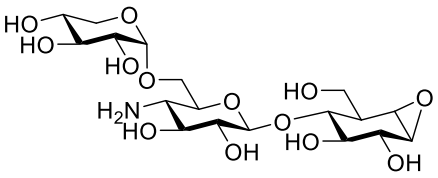


20 (57 mg, 68 µmol) was dissolved in MeOH/DCM (1 ml, 1/1, v/v). NaOMe (10 µl, 5.4M, 42 µmol) was added and the mixture was stirred overnight. NH_4Cl was added and the solvent was evaporated under reduced pressure. Column chromatography (DCM/EtOAc, 1/0 -> 9/1, v/v) provided the product (57 mg, 55 µmol, 81%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.36 – 7.21 (m, 30H), 4.86 (d, *J*=11.7, 1H, CH_2Bn), 4.84 – 4.78 (m, 3H, CH_2Bn/H1"), 4.76 (d, *J*=10.9, 1H, CH_2Bn), 4.70 (d, *J*=11.8, 1H, CH_2Bn), 4.68 – 4.55 (m, 7H, CH_2Bn), 4.41 (d, *J*=7.8, 1H, H1'), 3.90 – 3.77 (m, 5H, H4/H3"/H6ab/), 3.61 – 3.46 (m, 6H), 3.44 – 3.37 (m, 2H), 3.35 (dd, *J*=9.6, 3.5, 1H, H2"), 3.30 – 3.28 (m, 1H, epoxide), 3.15 – 3.09 (m, 2H, H2', epoxide), 3.00 (ddd, *J*=10.1, 4.2, 1.5, 1H), 2.33 – 2.26 (m, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 139.0, 138.8, 138.7, 138.6, 137.5, 128.6, 128.6, 128.5, 128.5, 128.5, 128.5, 128.4, 128.1, 128.1, 128.0, 128.0, 127.8, 127.7, 127.7, 127.6, 127.5, 127.1, 102.9 (H1'), 97.7 (H1"), 83.5, 81.3, 79.8 (H2"), 79.7, 78.1, 75.7, 75.5, 75.0, 74.7,

74.3, 74.2, 73.4, 73.0, 72.7, 69.3, 66.2, 61.2, 60.3, 56.1 (epoxide), 53.1 (epoxide), 41.9 (C5). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{60}H_{69}N_4O_{13}$ 1053.4856, found 1053.4848.

4-*O*-(6-*O*-(α-D-xylopyranosyl)-4-deoxy-4-amino-β-D-glucopyranosyl)-cyclophellitol (65)

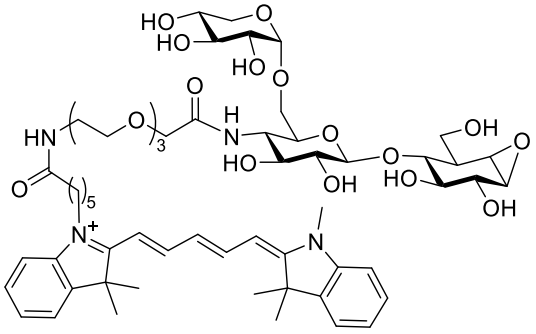


Ammonia (8 ml) was condensed at -50°C. Sodium (77 mg, 3.4 mmol) was added. Starting material **64** (58 mg, 0.056 mmol) was dissolved in THF (2 ml) and *t*-BuOH (0.37 ml, 3.9 mmol) and the mixture was added dropwise at – 65°C. After 40 minutes the reaction was quenched with NH_4Cl (195 mg, 3.6 mmol). The mixture was warmed to room temperature and the ammonia was evaporated.

The crude product was purified by size exclusion chromatography over HW-40 eluting with 1% AcOH in H_2O yielding the product as a white solid (20 mg, 0.043 mmol, 76%).

^{1}H NMR (400 MHz, MeOD) δ 4.84 (d, *J* = 3.7 Hz, 1Hn H1xyl), 4.38 (d, *J* = 7.9 Hz, 1H, H1gluc), 4.11 (dd, *J* = 10.9, 4.1 Hz, 1H, H6a), 3.94 – 3.62 (m, 5H), 3.61 – 3.35 (m, 9H), 3.30 – 3.26 (m, 1H, H2gluc), 3.06 (d, *J* = 3.6 Hz, 1H, epoxide), 2.89 (t, *J* = 9.8 Hz, 1H, H4gluc), 2.23 – 2.16 (m, 1H, H5). ^{13}C NMR (101 MHz, MeOD) δ 105.2 (C1xyl), 100.9 (C1gluc), 81.5, 76.8, 75.6, 75.3, 75.0, 73.7, 73.5, 72.9, 71.4, 69.3, 63.4, 62.0 (C6), 56.7 (epoxide), 56.5 (epoxide), 56.0 (C4gluc), 44.9 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{18}H_{32}NO_{13}$ 470.1868, found 470.1871.

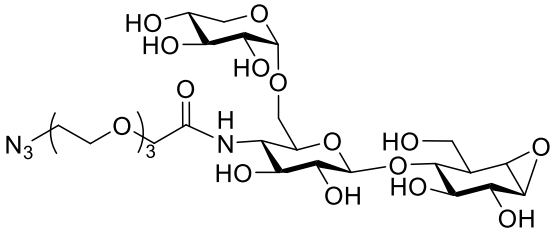
XG Cy5 probe (66)



Amine **65** (4.4 mg, 9.4 μmol) was reacted with stock solution TEG Cy5 **S12** (0.24 ml) according to general procedure B (2.64 mg, 2.2 μmol, 23%).

^{1}H NMR (600 MHz, MeOD) δ = 8.30 – 8.21 (m, 2H), 7.50 (dt, *J*=7.5, 1.5, 2H), 7.44 – 7.39 (m, 2H), 7.32 – 7.23 (m, 4H), 6.64 (t, *J*=12.4, 1H), 6.29 (dd, *J*=13.7, 4.9, 2H), 4.73 (d, *J*=3.6, 1H, H1xyl), 4.32 (d, *J*=8.0, 1H, H1gluc), 4.14 – 4.08 (m, 3H), 4.03 (d, *J*=1.7, 2H), 3.82 (dd, *J*=10.9, 7.2, 1H), 3.79 – 3.58 (m, 16H), 3.57 – 3.46 (m, 5H), 3.45 – 3.39 (m, 2H), 3.39 – 3.32 (m, 7H), 3.05 (d, *J*=3.7, 1H, epoxide), 2.24 (t, *J*=7.4, 2H), 2.22 – 2.17 (m, 1H, H5), 1.86 – 1.80 (m, 2H), 1.76 – 1.66 (m, 14H), 1.51 – 1.44 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 176.0, 175.4, 174.7, 173.5, 144.3, 143.6, 142.7, 142.5, 129.8, 129.7, 126.6, 126.3, 123.4, 123.3, 112.1, 111.8, 105.6, 104.4 (C1gluc), 104.3, 100.0 (C1xyl), 82.7, 77.1, 75.7, 75.1, 74.9, 74.8, 73.9, 72.8, 71.8, 71.7, 71.4, 71.4, 71.2, 71.1, 71.1, 70.5, 68.0, 63.2, 62.4, 56.6 (epoxide), 56.5 (epoxide), 53.3, 50.6, 50.5, 49.8, 49.6, 44.9 (C5), 44.8, 40.3, 36.7, 31.5, 28.2, 28.0, 27.8, 27.4, 26.5. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{58}H_{83}N_4O_{18}$ 1123.5697 found 1123.5690.

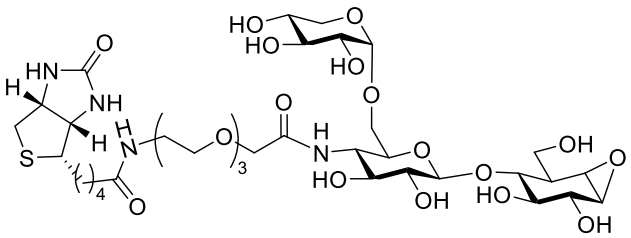
XG azide probe (67)



Amine **65** (4.4 mg, 9.4 μmol) was reacted with stock solution TEG azide **S4** (0.24 ml) according to general procedure B (1.74 mg, 2.54 μmol, 27%).

^{1}H NMR (850 MHz, MeOD) δ 4.74 (d, *J* = 3.6 Hz, 1H, H1xyl), 4.32 (d, *J* = 8.0 Hz, 1H, H1gluc), 4.12 (dd, *J* = 10.9, 4.5 Hz, 1H), 4.03 (d, *J* = 1.9 Hz, 2H), 3.83 (dd, *J* = 10.9, 7.2 Hz, 1H), 3.81 – 3.77 (m, 1H), 3.77 – 3.74 (m, 2H), 3.74 – 3.67 (m, 11H), 3.59 (t, *J* = 9.4 Hz, 1H), 3.57 – 3.52 (m, 2H), 3.49 (t, *J* = 10.7 Hz, 1H), 3.45 – 3.40 (m, 4H), 3.40 – 3.37 (m, 1H), 3.36 – 3.32 (m, 2H), 3.06 (d, *J* = 3.6 Hz, 1H, epoxide), 2.23 – 2.19 (m, 1H, H5). ^{13}C NMR (214 MHz, MeOD) δ 173.5, 105.5 (C1gluc), 100.0 (C1xyl), 82.7, 77.1, 75.6, 75.1, 74.9, 74.8, 73.9, 72.8, 71.9, 71.7, 71.5, 71.4, 71.4, 71.2, 71.0, 68.0, 63.2, 62.4, 56.6 (epoxide), 56.5 (epoxide), 53.3, 51.8, 44.9 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{26}H_{45}N_4O_{17}$ 685.2774 found 685.2768.

XG biotin probe (68)



Amine **65** (4.4 mg, 9.4 μmol) was reacted with stock solution TEG biotin **S8** (0.24 ml) according to general procedure B (2.14 mg, 2,44 μmol, 26%).
^{1}H NMR (850 MHz, MeOD) δ 4.74 (d, *J* = 3.6 Hz, 1H, H1xyl), 4.50 (ddd, *J* = 7.9, 5.0, 0.9 Hz, 1H), 4.34 – 4.29 (m, 2H, H1gluc), 4.12 (dd, *J* = 10.9, 4.5 Hz, 1H, H6a), 4.05 (d, *J* = 2.7 Hz, 2H), 3.84 (dd, *J* = 10.9, 7.1 Hz, 1H, H6b), 3.81 – 3.78 (m, 1H), 3.77 – 3.71 (m, 4H), 3.71 – 3.67 (m, 6H), 3.67 – 3.64 (m, 2H), 3.61 (t, *J* = 9.4 Hz, 1H), 3.58 – 3.52 (m, 4H), 3.50 (t, *J* = 10.7 Hz, 1H), 3.46 – 3.41 (m, 2H, epoxide), 3.41 – 3.37 (m, 3H), 3.36 – 3.34 (m, 2H), 3.34 – 3.32 (m, 2H), 3.24 – 3.20 (m, 1H), 3.06 (d, *J* = 3.6 Hz, 1H, epoxide), 2.93 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.71 (d, *J* = 12.7 Hz, 1H), 2.27 – 2.19 (m, 3H, H5), 1.78 – 1.57 (m, 4H), 1.48 – 1.42 (m, 2H). ^{13}C NMR (214 MHz, MeOD) δ 176.2, 173.5, 105.5 (C1gluc), 100.0 (C1xyl), 82.7, 77.1, 75.7, 75.1, 74.9, 74.8, 73.9, 72.8, 71.8, 71.7, 71.4, 71.4, 71.2, 71.1, 70.6, 68.0, 63.4, 63.2, 62.4 (C6), 61.6, 57.0, 56.7, 56.5 (epoxide), 53.3 (epoxide), 44.9 (C5), 41.0, 40.3, 36.8, 29.8, 29.5, 26.9. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{31}H_{53}N_4O_{15}S$ 753.3223 found 753.3219.

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3 Mechanism-based heparanase inhibitors and activity-based probes

3.1 Introduction

Human heparanase (HPSE), an endo-glycosidase acting on heparan sulfate polysaccharides, is implicated in various diseases and has therefore become an intensively studied potential drug target. In this chapter the chemical synthesis of covalent inhibitors and activity-based probes targeting HPSE is described.

Heparan sulfate biosynthesis and function

The main substrate of HPSE is heparan sulfate (HS, Figure 3.1), a heterogeneously decorated glycosaminoglycan synthesized by virtually all cell types in the body.[1] Its structure and biosynthesis is closely related to heparin which is only produced in mast cells. The polysaccharides are synthesized on core proteins in the Golgi apparatus by glycosyl transfer of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) residues. The growing chain

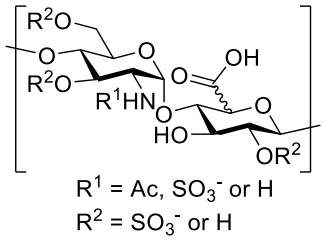


Figure 3.1| General structure of heparan sulfate.

is enzymatically modified, starting with *N*-deacetylation followed by *N*-sulfation by bifunctional *N*-deacetylase/*N*-sulfotransferases (NDST) giving rise to *N*-sulfated (NS) domains. Parts of the chain remain *N*-acetylated (NA domains) and parts become mixed (NA/NS domains). The most common modifications are 2*O*-, 3*O*- and 6*O*-sulfation of GlcNAc/GlcNS, 2*O*-sulfation of GlcA and 5*C*-epimerization of GlcA to L-iduronic acid (IdoA).[2] The complex interplay between the substrate specificities, activities and expression levels of the involved enzymes and the availability of uridine diphosphate (UDP) carbohydrate building blocks and a sulfate donor leads to variable but nonrandom modification of the constructed HS proteoglycan (PG).[3] Heparin consists for almost 90% of NS domains while for HS this is only 60%.[3] Since subsequent modifications mainly occur in NS domains heparin contains mostly IdoA and is densely sulfated while HS contains mostly GlcA and is only moderately sulfated.

The synthesized HSPGs are transported to the cell surface or released into the extracellular matrix (ECM) where they influence the activity and availability of many proteins and small molecules, either by specific sequence recognition or charge interaction.[4] HSPGs also have an important structural function in the ECM where they facilitate intercellular signaling and restrict cell mobility.[5] In their review on the physiological roles of HSPGs, Bishop *et al.* concluded: *'HSPGs interact with so many factors; one would expect few physiological systems to remain unaffected by changes in their composition.'* [6]

Heparanase

The only mammalian glycosidase known to extracellularly modify the composition of HS is heparanase (HPSE). HPSE is a retaining endo-β-D-glucuronidase belonging to GH family 79. The enzyme catalyzes the hydrolysis of the HS polysaccharide into smaller fragments. The protein is expressed as a pre-proenzyme.[7] Cleavage of the signal peptide yields proHPSE which has a 6 kDa 'exo-pocket' loop covering most of the active site cleft. This loop is also found in variable size in other GH79 glucuronidases.[8] The active site residues remain accessible in an exo-

glycosidase like pocket.[9] ProHPSE is secreted and can be endocytosed via multiple receptors on the same or on neighboring cells. After endocytosis the 'exo-pocket' loop is removed by cathepsin L in the lysosome yielding active HPSE as a heterodimer containing an 8 kDa and a 50 kDa subunit. The two catalytic residues are located in a cleft on the large subunit flanked by two HS binding domains (HBDs). The active enzyme is thus present in the lysosome but has also been found in the Golgi, the nucleus and in the extracellular matrix.

Abnormal HPSE activity has been implicated in cancer progression and other pathologies such as inflammation and diabetic nephropathy.[10–12] Pathological effects of HPSE can be a result of its increased enzymatic activity, but non-enzymatic signaling properties are also implicated in some cases.

Heparanase inhibitors

The involvement of HPSE in many physiological processes in human health and disease established HPSE as an interesting therapeutic target and stimulated the generation of different classes of inhibitors of the enzyme.[13–16] The most investigated HPSE inhibitors are densely sulfated oligo- or polysaccharides of which four preparations have made it into clinical trials to date (Figure 3.2). The sulfated oligosaccharides are mixture PI-88 (mupafostat)[17] and the more potent molecule PG545 (pixatimod)[18]. The other two preparations are glycol-split

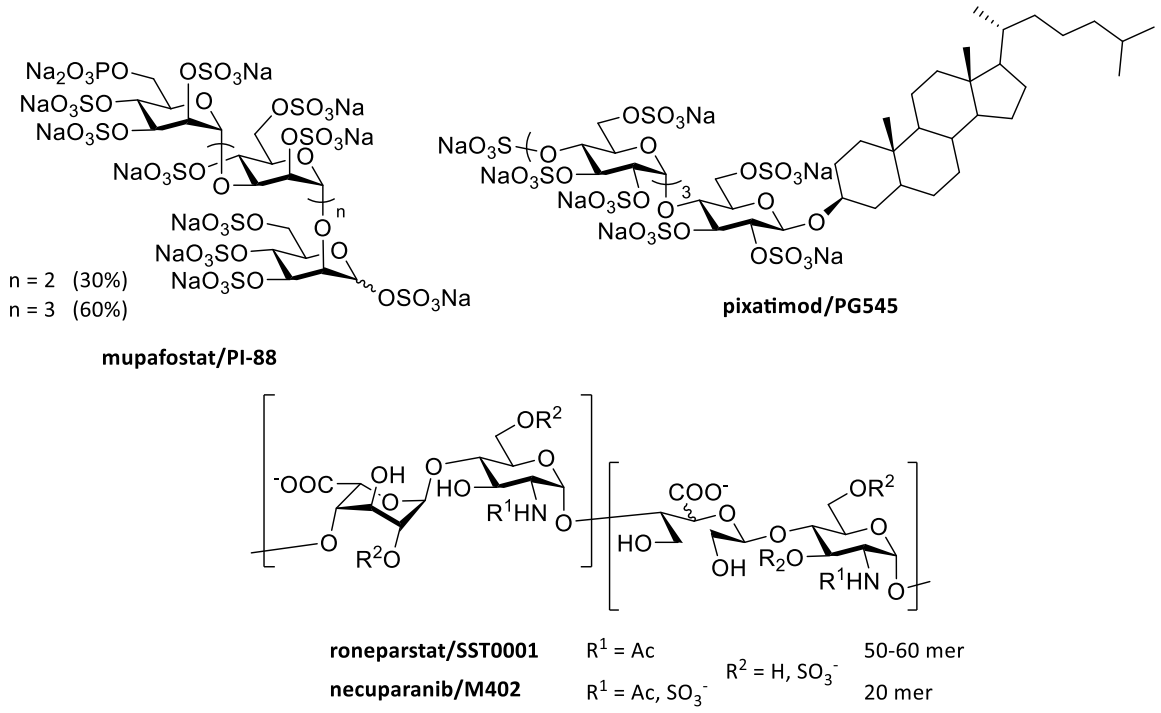


Figure 3.2|HPSE inhibitors examined in clinical trials.

heparin derivatives which are either N-acetylated (roneparstat/SST0001)[19] or N-sulfated (M402/necuparanib)[20]. While these compounds have all shown efficacy in pre-clinical models and an acceptable safety profile in clinical trials the efficacy in anti-cancer clinical trials has so far been moderate. A limitation in optimizing these compounds is their structural similarity to HS and heparin. This similarity makes it difficult to attribute the observed effects to inhibition of HPSE enzymatic activity, blocking of the HPSE HBDs or off-target binding to other proteins with an HBD. The high polarity of the compounds results in fast excretion necessitating high and frequent dosing.

Many small molecules with diverse structures have also been investigated for their potential as HPSE inhibitors, but the lack of a widely used, reliable and rapid inhibition assay limits the development and prohibits the quantitative comparison of the different compound classes.[15,21] Also for these compounds the mode of action, binding in the active site or elsewhere, often remains unclear. The great diversity in molecular structure suggests that the small molecules bind at various sites on the enzyme indicating multiple modes of action, a notion that is supported by molecular docking studies.[22] So far no small molecule has reached the clinic.

Mechanism-based heparanase inhibitors and ABPs

Glucuronic acid configured cyclophellitol aziridine **1** was recently published as an activity-based probe (ABP) targeting retaining β-glucuronidases (Figure 3.3).[9] LC-MS and gel-based activity-based protein profiling (ABPP) in human spleen and platelet lysates and on recombinant proteins revealed labeling of the retaining β-glucuronidases GUSB, proHPSE and HPSE. Retaining exo-β-glucosidases are reported as off-targets.[9,23]

While the labeling kinetics of **1** with exo-glucuronidase GUSB are characterized, the inhibition rate of proHPSE and HPSE is not quantified. As well, the biological significance of the

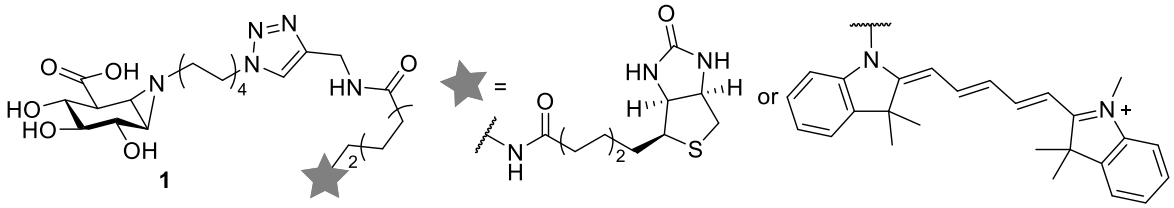
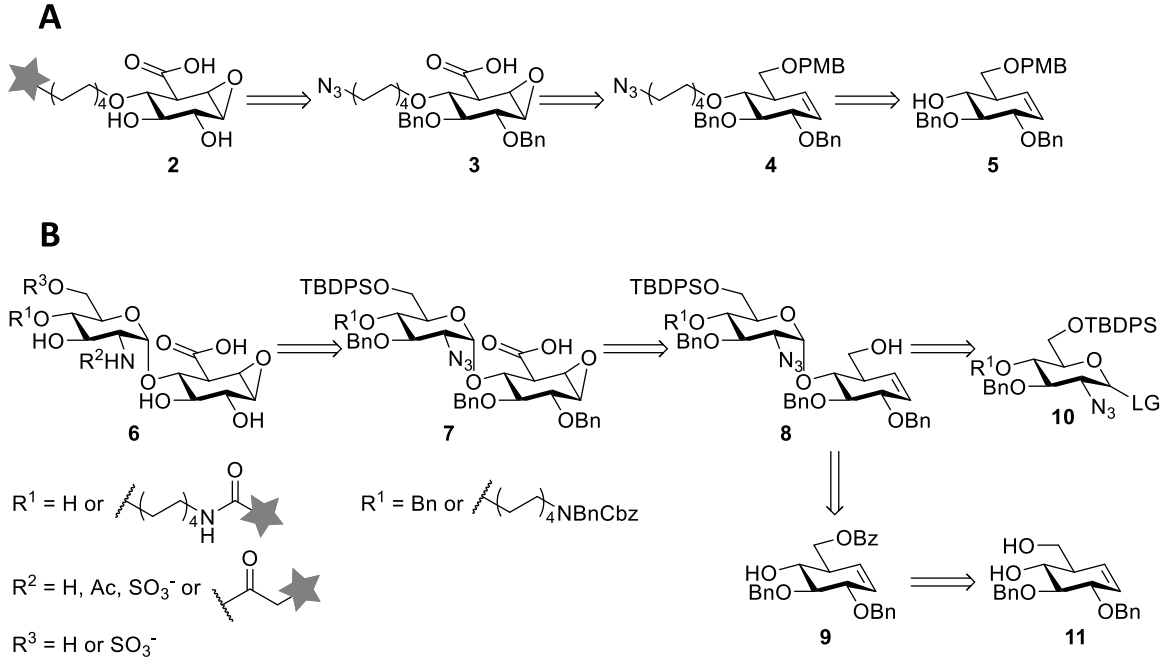


Figure 3.3| Previously reported β-D-glucuronic acid configured ABPs.[9]

reaction of proHPSE with **1** remains unclear because no enzymatic activity of proHPSE on natural substrates has been reported.

In this chapter mechanism-based inhibitors and ABPs to selectively monitor and modulate the hydrolytic activity of HPSE are described. The synthesized molecules are based on the glucuronic acid configured cyclophellitol scaffold. Selective and potent ABPs may ease the detection of low HPSE activities in various tissues and allow to better understand the role of HPSE in pathology. Activity-based protein profiling also allows the screening of HPSE inhibitors and the confirmation of HPSE inhibition *in vivo*.[24] Selective and potent mechanism-based inhibitors may allow the assessment of the therapeutic effect of the inhibition of hydrolytic activity compared to blockage of binding to HBDs. Ultimately selected inhibitors – based both on cyclophellitol-derived covalent inhibitors and compounds discovered through the application of the covalent inhibitor-based ABPs – might serve as lead compounds for the development of anti-cancer therapeutics.[25]

Extending on the results obtained with ABP, it was hypothesized that selectivity over exo-acting enzymes might be obtained by placement of the tag at the non-reducing end leading to design **2** (Scheme 3.1A). This increase in steric bulk at the non-reducing end is hypothesized to prohibit productive interaction with the pocket shaped active sites of GUSB and proHPSE,



Scheme 3.1| Retrosynthetic analysis of the proposed HPSE inhibitors and probes. **A**) non-reducing end capped monosaccharide ABPs. **B**) Disaccharide mimics as inhibitors and ABPs. Stars denote various reporter groups.

while the active site cleft of mature HPSE may be able to accommodate this bulk. ABP **2** could be synthesized from protected epoxide **3** by hydrogenolysis and amide bond formation with appropriate tags. Epoxide **3** would be accessible from cyclohexene **4** by *p*-methoxybenzyl ether (PMB) removal, followed by hydrogen bond directed epoxidation and oxidation. Finally, **4** could be obtained by alkylation of solely ether protected **5**[26].

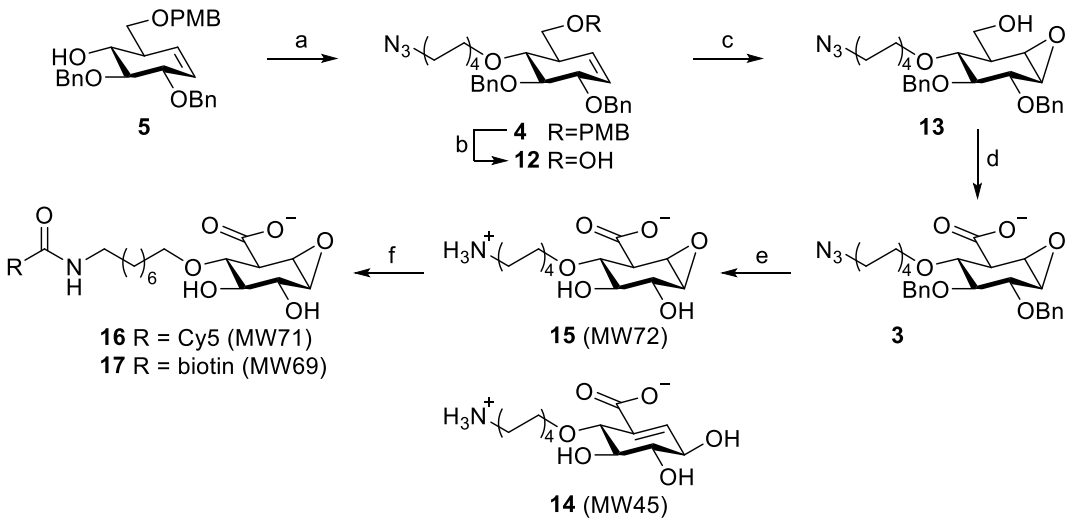
Additionally, a set of HS disaccharide cyclophellitols was designed. Analogous with other endo-glycosidase inhibitors and probes described in this thesis and elsewhere the larger recognition element is expected to increase both selectivity and potency for endo-glycosidases (**6**, Scheme 3.1B).[27,28] Orthogonal protection of specific alcohols and the 2' amine would allow the synthesis of inhibitors with a well-defined sulfation pattern. Orthogonally protected **7** may be a suitable intermediate to access selectively 6'*O*- and 2'*N*-sulfated or acylated derivatives. The 6' alcohol could be temporarily protected as a silyl ether and the 2'amine as an azide. The 2' azide, a non-participating protecting group, would also allow α-selective glycosylation. The tag was envisioned to be introduced at the end of the synthesis so the alkyl amine and the remaining alcohols were masked with benzyl ethers. The epoxide is installed post-glycosylation to allow flexibility in the glycosylation reaction conditions. The epoxide could be obtained from homoallylic alcohol **8** by stereoselective epoxidation followed by oxidation. Stereoselective glycosylation of acceptor **9** with donor **10** would afford pseudo-disaccharide **8**. Cyclohexene **9** can be obtained from previously reported diol **11**[29].

3.2 Results and discussion

In the following sections the synthesis of the monosaccharide probes and disaccharide inhibitors and probes is described. The chapter concludes with a summary of the biological evaluation of the compounds.

Monosaccharide probes

Alkylation of PMB protected cyclohexene **5**[26] with 8-azidooctyl 4-methylbenzenesulfonate[30] afforded **4** (Scheme 3.2). Removal of the temporary PMB protecting group with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) afforded homoallylic alcohol **12**, which was epoxidized in a diastereoselective reaction with meta-chloroperoxybenzoic acid (mCPBA) at 0°C (**13**). Oxidation of the primary alcohol with 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) / bis-acetoxyiodobenzene (BAIB) yielded carboxylic acid **3**. Selective hydrogenolytic benzyl removal of **3** with $Pd(OH)_2/C$ proved difficult but dissolving metal reduction allowed smooth



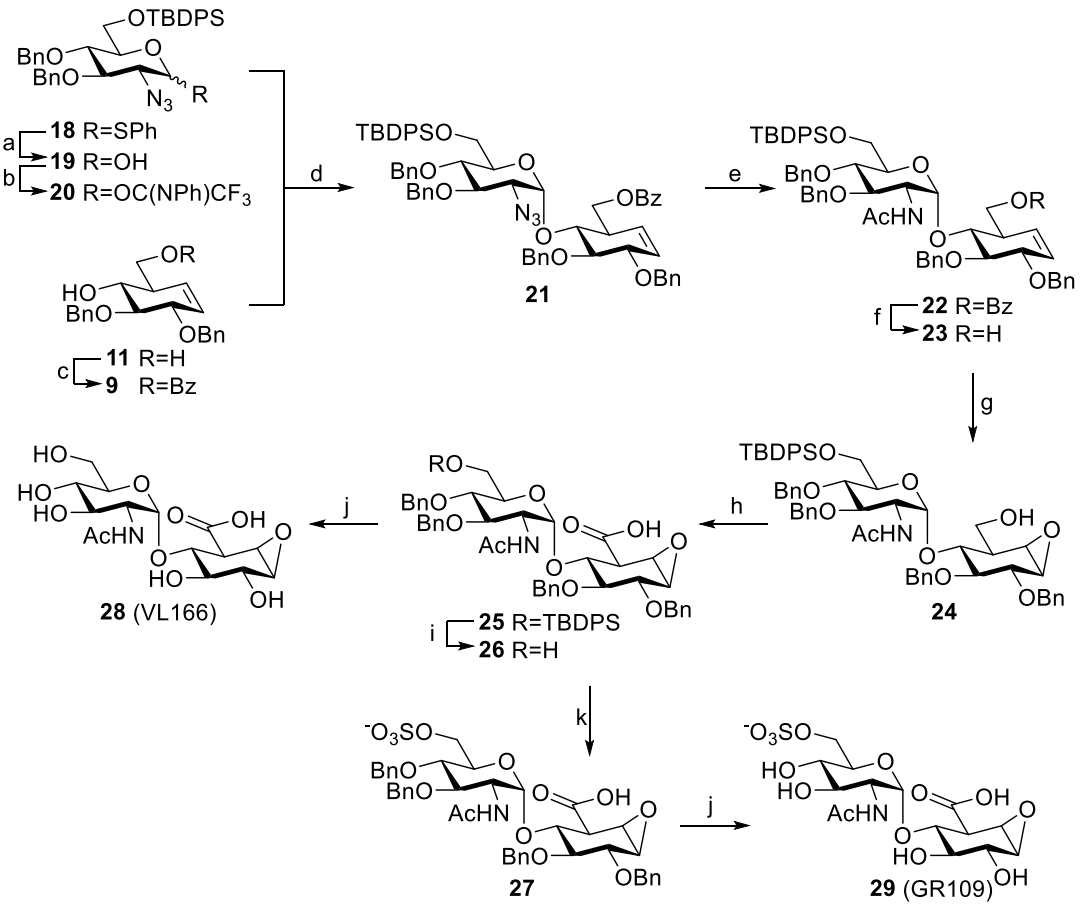
Scheme 3.2| Reagents and conditions: **a**) 8-azidooctyl 4-methylbenzenesulfonate, KHMDS, THF, 71%. **b**) DDQ, DCM, H_2O, 85%. **c**) mCPBA, DCM, 0°C, 87%. **d**) TEMPO, BAIB, *t*-BuOH, DCM, H_2O, 0°C, 90%. **e**) Na (s), NH_3, *t*-BuOH, THF, 28%. **f**) DIPEA, DMF, Cy5-NHS 14% (**16**) or biotin-NHS, 25% (**17**).

removal of the benzyl protecting groups with concomitant reduction of the azide. Quenching the reaction with NH_4Cl, followed by HPLC purification with 50 mM NH_4HCO_3 only afforded elimination product **14.** Quenching with AcOH followed HPLC purification eluting with 50 mM AcOH yielded the desired epoxide **15**. The amine was reacted with the *N*-hydroxysuccinimide activated esters of the Cy5 and biotin tags yielding probes **16** and **17** after HPLC purification.

Early stage azide reduction, sulfated and non-sulfated inhibitors

2-Azido-2-deoxy thioglucosyl donor **18**[31] was hydrolyzed to lactol **19** and converted into *N*-phenyltrifluoroacetimidate **20** (Scheme 3.3). Cyclophellitol alkene acceptor **9** was obtained by selective benzoylation of diol **11**[29,32] using mildly basic conditions. Glycosylation of these two building blocks was optimized to a protocol using a relatively high amount of triflic acid, low temperature and short reaction time. This afforded the product (**21**) in 83% up to 95% yield.

Staudinger reduction followed by acetylation of the liberated amine yielded **22**. The benzoyl ester was removed with NaOMe, setting the stage for stereoselective epoxidation of homoallylic alcohol **23**. Epoxidation with mCPBA afforded the product in a 5:1 diastereomeric mixture favoring the β-configured product (**24**). The epimeric epoxides were difficult to separate by flash column chromatography, therefore a iodocarbonylation sequence was employed to improve the stereoselectivity of the reaction.[33–38] *t*-Butyloxycarbonyl (Boc) protection of the alcohol, followed by activation of the alkene by N-iodosuccinimide (NIS) yields the iodocarbonate with complete stereospecificity. Treatment of this intermediate with



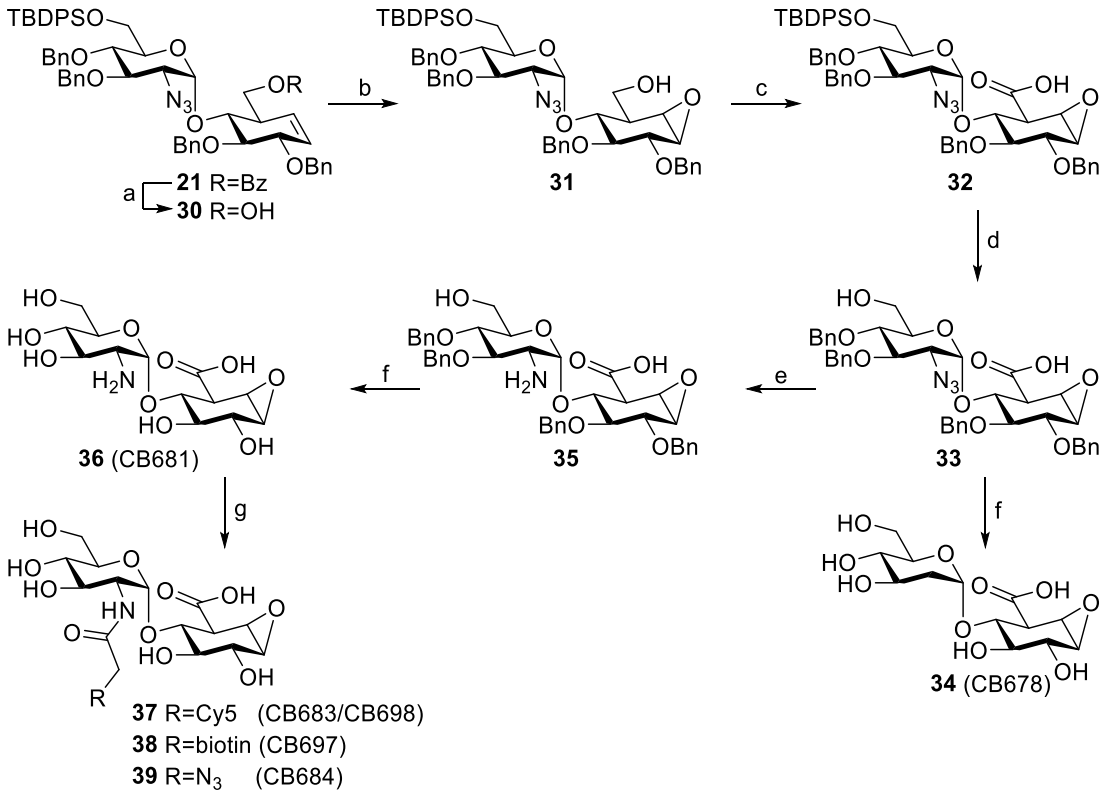
Scheme 3.3| Reagents and conditions: **a**) NIS, acetone/H_2O, 68%. **b**) *N*-phenyltrifluoroacetimidoyl chloride, Cs_2CO_3, DCM, 90%. **c**) BzCl, Et_3N, DCM, 0°C, 82%. **d**) TfOH, DCM, -78°C to -30°C, 83%. **e**) i. PPh_3, H_2O, THF; ii. Ac_2O, pyridine, 89%. **f**) NaOMe, MeOH, DCM, 97%. **g**) i. Boc_2O, DMAP, THF, 76%; ii. NIS, AcOH, DCM, 91%; iii. NaOMe, MeOH, DCM, quant. **h**) TEMPO, BAIB, DCM, *t*-BuOH, H_2O, 80%. **i**) $3HF{\cdot}Et_3N$, Et_3N, THF, 87%. **j**) Na(s), NH_3, *t*-BuOH, THF, 91% for **28**, 87% for **29**. **k**) $SO_3{\cdot}Et_3N$, DMF.

base in methanol generates the epoxide with concomitant deprotection of the primary alcohol providing **24**. The primary alcohol was oxidized with TEMPO/BAIB providing carboxylic acid **25**. The silyl ether was removed with HF triethylamine complex yielding primary alcohol **26**.

Sulfation of the primary alcohol afforded sulfate **27** for which no purification was attempted due to the amphiphilic nature. Dissolving metal hydrogenolysis of **26** and **27** provided the 6'O sulfated and non-sulfated inhibitors **28** and **29** after purification by size exclusion chromatography.

Late stage azide reduction, derivatization on the C2' position

A variation to the above described route was developed to generate diversity on the 2' position (Scheme 3.4). By delaying the azide reduction to the final stage of the synthesis, ABPs with tags on the 2'amine and inhibitors without the 2'acetyl were synthesized. Debenzoylation of **21** with NaOMe provided homoallylic alcohol **30**. Epoxide formation following the same



Scheme 3.4| Reagents and conditions: **a**) NaOMe, MeOH, DCM, quant.; **b**) i. Boc_2O, DMAP, THF, 82%; ii. NIS, AcOH, DCM, 71%; iii. NaOMe, MeOH, DCM, 94%. **c**) TEMPO, BAIB, DCM, *t*-BuOH, H_2O, quant. **d**) $3HF{\cdot}Et_3N$, THF, 86%. **e**) Zn(s), NH_4Cl, MeOH, toluene. **f**) Na(s), NH_3, *t*-BuOH, THF, quant. for **34**, 51% over 2 steps for **36**. **g**) DIC, pentafluorophenol, DIPEA, DMF, Cy5-TEG-COOH or N_3COOH or biotin-TEG-COOH 7% **37**; 10% **38** and 12% **39**.

procedure as executed in the previous section afforded epoxide **31**. TEMPO/BAIB oxidation to carboxylic acid **32** followed by removal of the silyl ether uneventfully afforded **33**. An attempt at dissolving metal hydrogenolysis of the benzyl ethers with concomitant reduction of the azide unexpectedly afforded the 2'deoxy product **34** in quantitative yield. Although the reductive removal of an azide group has been reported as a side reaction[39], no examples of its use as a synthetic utility were found in the literature.

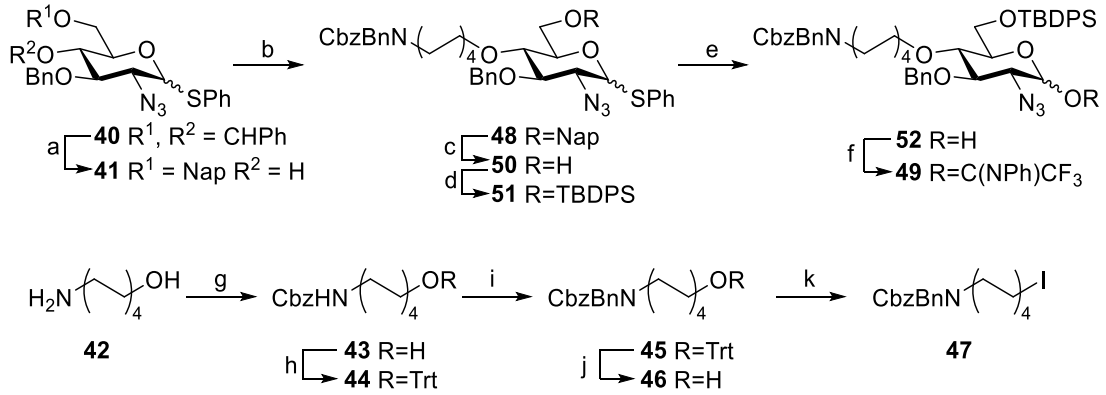
Azide reduction using Zn/NH_4Cl granted access to amine **35**.[40] Filtration over silica was used to remove most of the zinc salts and due to the poor solubility of the product no further purification was attempted. When amine **35** was subjected to dissolving metal hydrogenolysis followed by purification by size exclusion chromatography the expected product **36** was obtained. Attempts to reduce the azide in **33** by Staudinger reduction were incompatible with the epoxide and NMR analysis revealed a product containing an alkene. This product was presumably generated by attack of PPh_3 on the epoxide followed by elimination of PPh_3O resembling Wittig type alkene formation.[41] Selective acylation of **36** with Cy5 or biotin

equipped triethylene glycol (TEG) spacers (Chapter 2) afforded putative ABPs **37** and **38** after HPLC purification. Acylation with 2-azidoacetic acid provided two step ABP **39** after size exclusion chromatography.

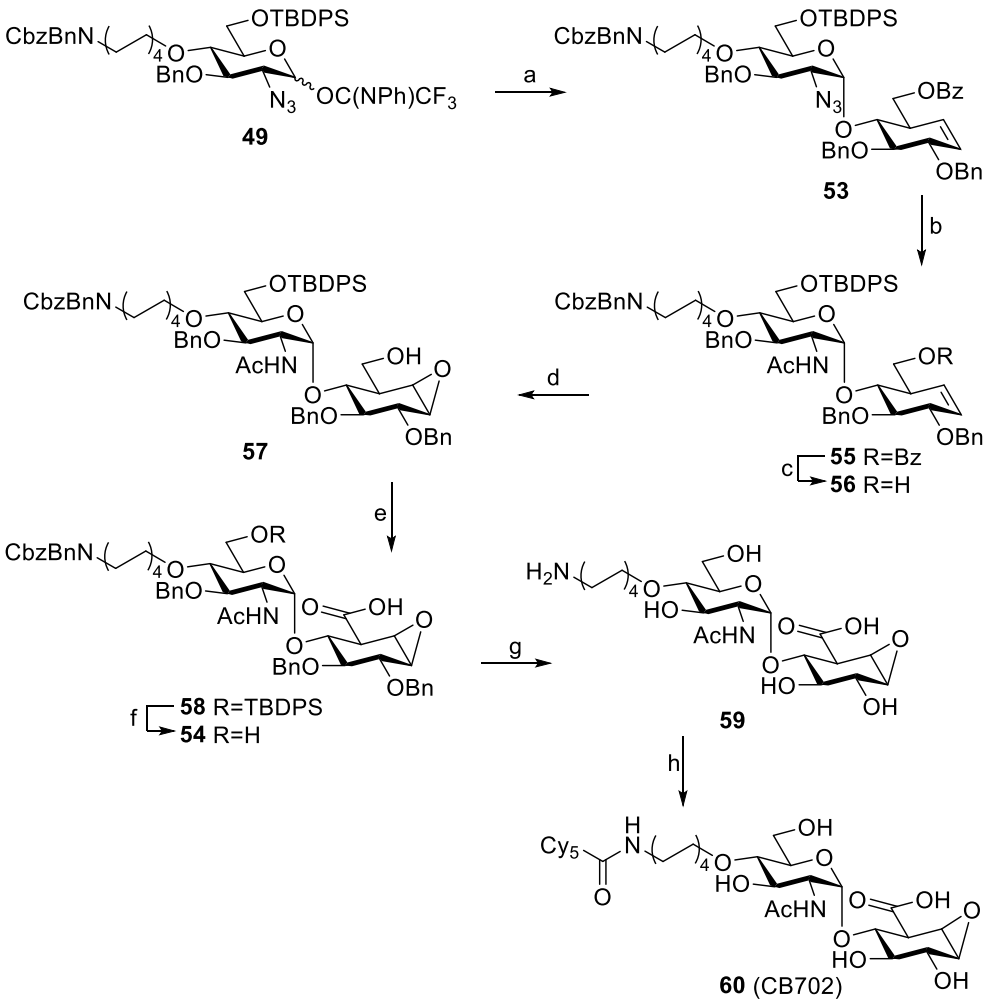
ABPs with a 4' tag

To gain access to disaccharide probes modified at the 4'postion with a reporter entity, a 4'-alkylated donor was synthesized (Scheme 3.5). To this end, the benzylidene acetal on **40**[42] was removed and the resulting diol was regioselectively protected as the naphthyl (Nap) ether using borinate catalysis.[43] The Nap ether was chosen over the *tert*-butyldiphenylsilyl (TBDPS) ether as a base stable protecting group to facilitate smooth alkylation of the secondary alcohol in **41**. The properly protected alkyl iodide was synthesized starting with the benzyloxycarbonyl (Cbz) protection of octanol amine (**42**) yielding **43**. The remaining alcohol was tritylated (**44**) to allow selective benzylation of the carbamate (**45**). The trityl was removed (**46**) and iodination of the resulting alcohol provided iodide **47**. The iodide was reacted with secondary alcohol **41** providing fully protected **48**. Attempts to use the non-benzylated carbamate linker in the alkylation reaction were unproductive due to intramolecular cyclization of the linker.

Application of anomeric *N*-phenyltrifluoroacetimidate derivatives of **48** as a donor in glycosylation reactions with **9** showed no stereoselectivity. Attempts to gain α-selectivity by the addition of α-directing additives such as DMF and 4-formyl morpholine[44] led to diminished yields. The good results obtained using TBDPS protected donor **20** as described in the previous section prompted the synthesis of **49**. The Nap ether in **48** was removed using DDQ (**50**)



Scheme 3.5| Reagents and conditions: **a**) i. CSA, MeOH, DCE, 50°C; ii. NapBr, 2-aminoethyl diphenyl borinate, MeCN, 60°C, 93%. **b**) **47**, NaH, DMF, 88%. **c**) DDQ, DCM, MeOH, 72%. **d**) TBDPSCl, imidazole, DMF. **e**) NIS, acetone/H_2O, DCM, 79%. **f**) *N*-phenyl-trifluoroacetimidoylchloride, Cs_2CO_3, DCM, 94%. **g**) CbzCl, $NaHCO_3$, acetone/H_2O, 88%. **h**) TrtCl, Et_3N, DMF. **i**) BnBr, NaH, TBAI, DMF, 76% over 2 steps. **j**) 3% TFA/H_2O, DCM/MeOH, 84%; **k**) PPh_3, I_2, imidazole, DCM, 97%.



Scheme 3.6| Reagents and conditions: **a**) **9**, TfOH, DCM, -78°C -> -30°C 97%. **b**) i. PPh_3, H_2O, pyridine, THF; ii. Ac_2O, pyr, DCM 98%. **c**) NaOMe, MeOH, DCM, 97%. **d**) i. Boc_2O, DMAP, THF, 82%; ii. NIS, AcOH, DCM, 68%; iii. NaOMe, MeOH, DCM, 88%. **e**) TEMPO, BAIB, *t*-BuOH, DCM, H_2O, 67%. **f**) $Et_3N{\cdot}3HF$, THF, quant. **g**) H_2, Pd/C, H_2O, dioxane, 41%; **h**) Cy5COOH, pentafluorophenyl trifluoroacetate, DIPEA, DMF, 13%.

followed by introduction of the silyl ether (**51**). Hydrolysis of the anomeric thiophenol using NIS afforded lactol **52** which was converted to *N*-phenyltrifluoroacetimidate donor **49**.

Reaction of donor **49** with acceptor **9** provided pseudodisaccharide **53** in good yield and selectivity (Scheme 3.6). The disaccharide was elaborated into partially protected **54** using the '*Early stage azide reduction'* reaction sequence reported for the inhibitors: Staudinger reduction followed by acetylation afforded **55**. Removal of the primary benzoyl (**56**) followed by stereospecific epoxidation via a iodocarbonate intermediate afforded epoxide **57**. TEMPO/BAIB oxidation (**58**) and silyl removal delivered alcohol **54**.

Deprotection of **54** by dissolving metal hydrogenolysis did not lead to the desired product. While these conditions have been successfully applied for the deprotection of diverse cyclophellitol and cyclophellitol aziridine derivatives the contrasting result obtained in this

case is attributed to the *N*-benzyl protecting group. NMR analysis revealed the partial reduction of the *N*-benzyl group to cyclohexadiene or further reduced derivatives as indicated by the presence of non-aromatic double bonds.

Pd/C catalyzed hydrogenolysis of the CBz and benzyl groups in **54** did provide **59**. The reaction was complicated by partial reduction and nucleophilic opening of the epoxide under the reaction conditions. As well, catalyst poisoning by the emerging amine, variable activity of different batches of Pd/C catalyst, the difficulty to monitor this reaction with a multitude of different intermediates and the difficulty of dissolving starting material, intermediates and product in the same solvent made this reaction hard to optimize and reproduce. The reaction was monitored by LC-MS and the obtained mixture was purified by size exclusion (HW-40). The mixture eluted as a broad peak which was collected in three fractions. The pure fraction, based on NMR analysis, was selected for the final reaction.

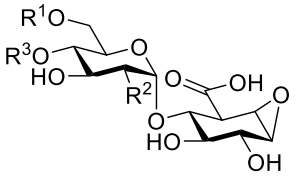
Finally, Cy5COOH was activated as its pentafluorophenyl ester using pentafluorophenyl trifluoroacetate which was directly reacted with **59** to yield ABP **60** after HPLC purification.

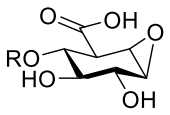
3.3 Biological evaluation

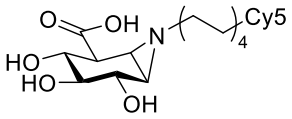
The properties of the synthesized inhibitors and probes (Figure 3.4) were evaluated on recombinant enzymes, in cell lysates and in mice. The experiments were conducted by Liang Wu, Zachary Armstrong and coworkers in the Davies lab at the University of York in the United Kingdom and the Vlodovsky lab at the Technion Israel Institute of Technology in Haifa, Israel.

Evaluation of HPSE inhibitors

The synthesized inhibitors were screened against a panel of recombinant β-glucuronidases by gel-based competitive ABPP using ABP **1** (Figure 3.5). Heparanase was inhibited by **29** at the lowest inhibitor concentration in line with the preference of HPSE for sulfated substrates. The non-sulfated derivative **28** showed approximately ten times lower potency. Substitutions on the 2' position have a profound influence on the potency. Deacetylation of the amine (**36**) is detrimental for the potency in comparison to the GlucNAc derivative (**28**). Also the larger steric bulk of an azidoacetyl substituent (**39**) is not tolerated by HPSE. The potency of inhibitor **34** without a 2' substituent is similar to the naturally occurring NHAc substitution. HPSE is not inhibited by unsubstituted β-glucuronic acid configured cyclophellitol **61**[9] at concentrations of up to 10 μM.







	R^1	R^2	R^3
28	H	NHAc	H
29	SO_3^-	NHAc	H
34	H	H	H
36	H	NH_2	H
37	H	Cy5	H
38	H	Biotin	H
39	H	N_3	H
60	H	NHAc	Cy5

	R
16	Cy5
61	H

1

Figure 3.4| Inhibitors and ABPs used in this section.

ProHPSE has not been reported to have hydrolytic activity but is labeled by ABP **1**. No inhibition by mono- or disaccharide inhibitors (**61**, **28** or **29)** under the tested conditions (up to 10 µM) was observed. Retaining e*xo*-acting β-glucuronidase from *E. coli*[45] belonging to GH family 2 (EcGH2) is not inhibited by the disaccharide inhibitors (**28** and **29**) but is inhibited by the monosaccharide **61** although with moderate potency. The GH79 β-glucuronidase from *Acidobacterium capsulatum*[46] (AcGH79) is inhibited by mono- and disaccharide configured inhibitors (**28**, **29** and **61**) with similar potency, suggesting the enzyme may possess endo-activity in addition to the previously reported exo-activity. The GH79 heparanase from *Burkholderia pseudomallei*[47] (BpHep) is inhibited by the disaccharide inhibitors (**28** and **29**) and not by the mono saccharide **61**. Sulfation does not have a profound effect on the potency for this enzyme. X-ray crystallography of the inhibitor enzyme complexes of **61** with EcGH2 and AcGH79 and **28** and **29** with HPSE and BpHep all show selective alkylation of the catalytic nucleophile of the enzyme (data not shown).

The reactivity of inhibitor **28** towards glutathione, an abundant thiol containing antioxidant in humans, was determined with colorimetric quantitation using Ellman's reagent as a readout (Figure 3.6).[48] The assay with DMSO and iodoacetamide as baseline and positive control showed stability of **28** for at least two hours.

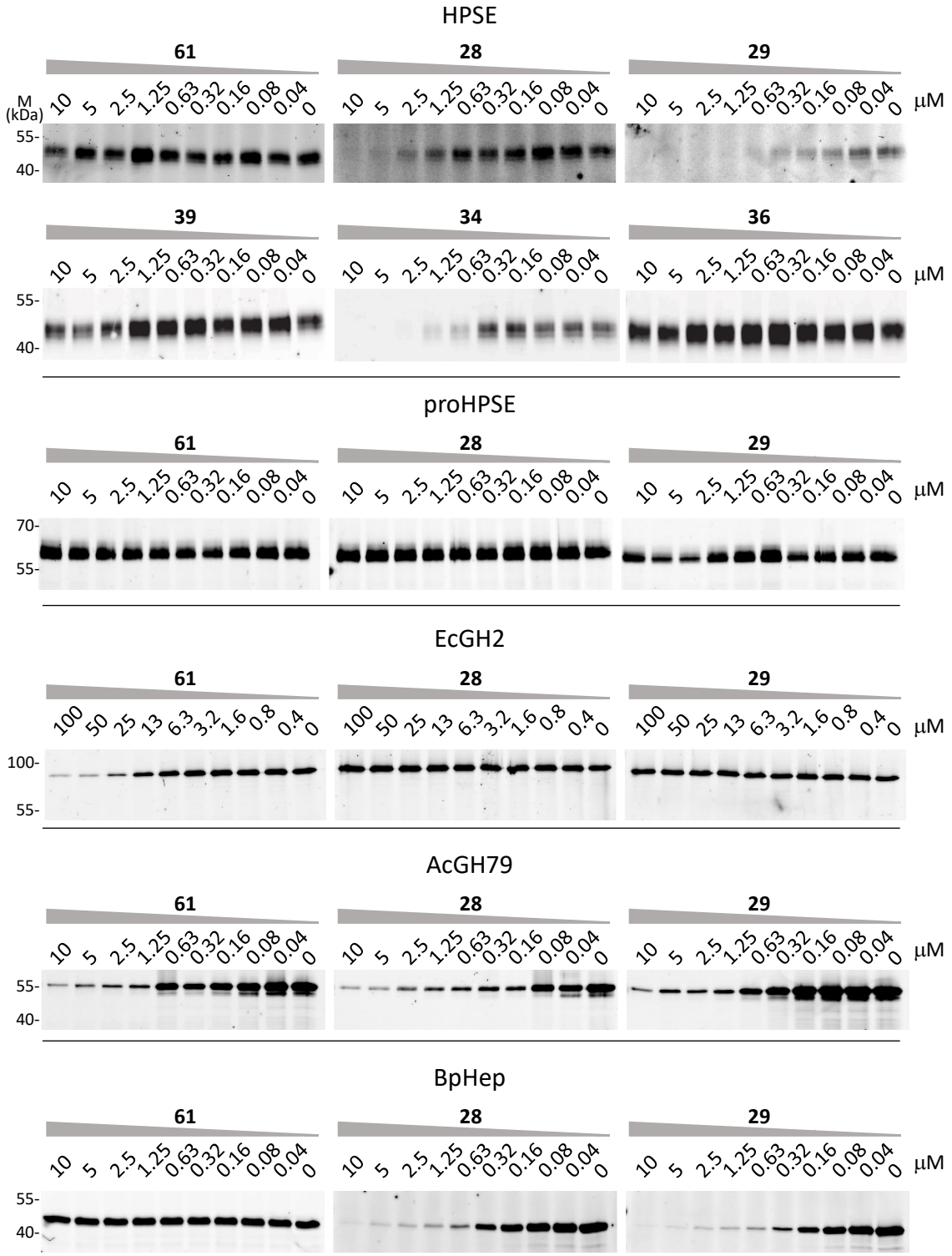


Figure 3.5| Assessment of inhibitor potency by ABP (**1**)-based screening of cyclophellitol-based inhibitors in a panel of recombinant exo- (EcGH2, AcGH79) and endo-(HPSE, BpHep) β-glucuronidases.

Inhibitors **28, 29,** and **61** were selected to be tested in several increasingly sophisticated HPSE inhibition assays. Selectivity and potency of the three inhibitors was estimated in platelet lysate by gel-based ABPP (Figure 3.7A). Monosaccharide **61** inhibited exo-acting GUSB. The

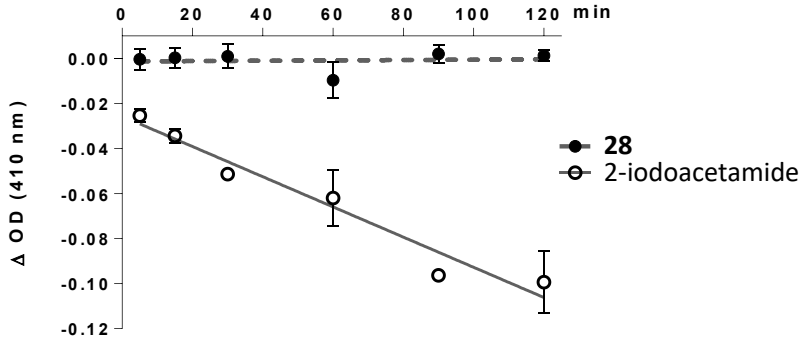


Figure 3.6| Glutathione reactivity assay. All measurements are three replicates corrected for the DMSO control at that time point. Error bars represent one standard deviation.

disaccharide mimics **28** and **29** inhibited HPSE without inhibition of GUSB in the tested concentration of up to 10 µM. The IC_{50} value for **28** (0.5 µM) is roughly ten times higher than that for **29** (0.06 µM) in accord with the results obtained with recombinant HPSE. HPSE IC_{50} values of **28** and **29** were also determined in the fondaparinux digestion assay (Figure 3.7B).[49] Hydrolysis of the synthetic anticoagulant fondaparinux by HPSE is determined by colorimetric quantitation of the hydrolysis product. In contrast to the gel-based assay where **29** was more potent then **28,** the IC_{50} of both compounds was around 0.5 µM in this assay. An undisclosed small molecule reversible inhibitor was used as a positive control.

Compounds **28** and **29** were also equally effective inhibitors of recombinant HPSE mediated hydrolysis of a extracellular matrix (ECM) generated by bovine corneal endothelial cells. [50,51] Hydrolysis of this ECM releases radiolabeled HS fragments that are quantified by size exclusion chromatography. **28** and **29** showed similar potency in this assay. Monosaccharide **61** was not active up to 5 µM (data not shown). Treatment of U87 human glioma (brain cancer) cells with inhibitors **28** or **29** followed by washing and lysis shows only modest inhibition of the naturally expressed HPSE in the above-mentioned ECM degradation assay. Treatment of the U87 cell lysates with the inhibitors does show potent inhibition (data not shown). These results indicate that **28** and **29** do not enter the cell and are thus unable to inhibit intracellular HPSE.

The inhibition of HPSE is expected to result in a diminished ability of the cells to migrate through the ECM. In the case of cancer cells this may reduce their tendency to metastasize. Matrigel invasion assays were performed with **28** and **29** (Figure 3.7C). At 20 µM both compounds show a significant decrease in cell mobility through a basement membrane indicating the inhibition of HPSE by these inhibitors may also have an effect on cell mobility *in vivo*. To verify this **28** was tested in a mouse metastasis model (Figure 3.7D). Balb/c mice were

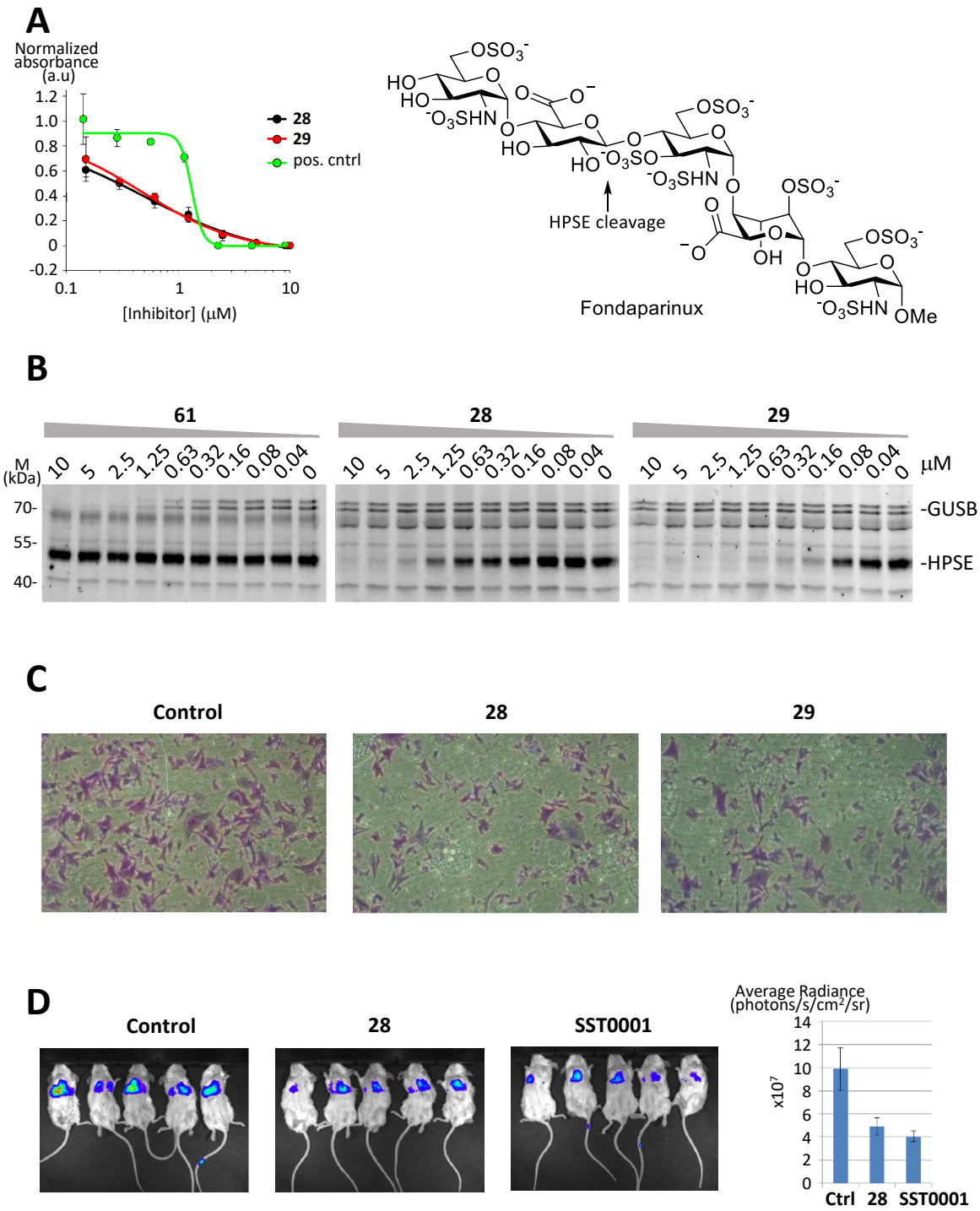


Figure 3.7| A) Inhibition of fondaparinux cleavage by recombinant heparanase. **B**) Selective HPSE and GUSB inhibition in platelet lysate. IC_{50} on HPSE of **28** 534 nM, **29** 56 nM. **C**) Matrigel invasion assay: Representative pictures of the U87 glyoma cells treated with DMSO (neg control) and HPSE inhibitors after passing through a matrigel and crystal violet staining. **D**) Murine metastasis assay result after 14 days.

injected in the tail vein with 4T1 murine mammary carcinoma (breast cancer) cells expressing luciferase. Metastasis to the lungs is quantified by bioluminescent scanning after 14 days. Mice treated with **28** or roneparstat showed a significant reduction in lung metastasis compared to the untreated control.

Evaluation of HPSE ABPs

4'alkylated monosaccharide probe **16** labeled HPSE with reduced potency compared to aziridine probe **1** (Figure 3.8A). ProHPSE was labeled with similar potency so effectively the probe was more selective for proHPSE. Therefore, evaluation of these probes was not pursued further.

2'*N*-tagged disaccharide probe **37** did not show labeling of HPSE. However selective labeling of a 40 kDa protein was observed with this probe in platelets (data not shown). Labeling of a protein with this molecular weight was also observed with **1** and **60** (Figure 3.7B and Figure 3.8C).

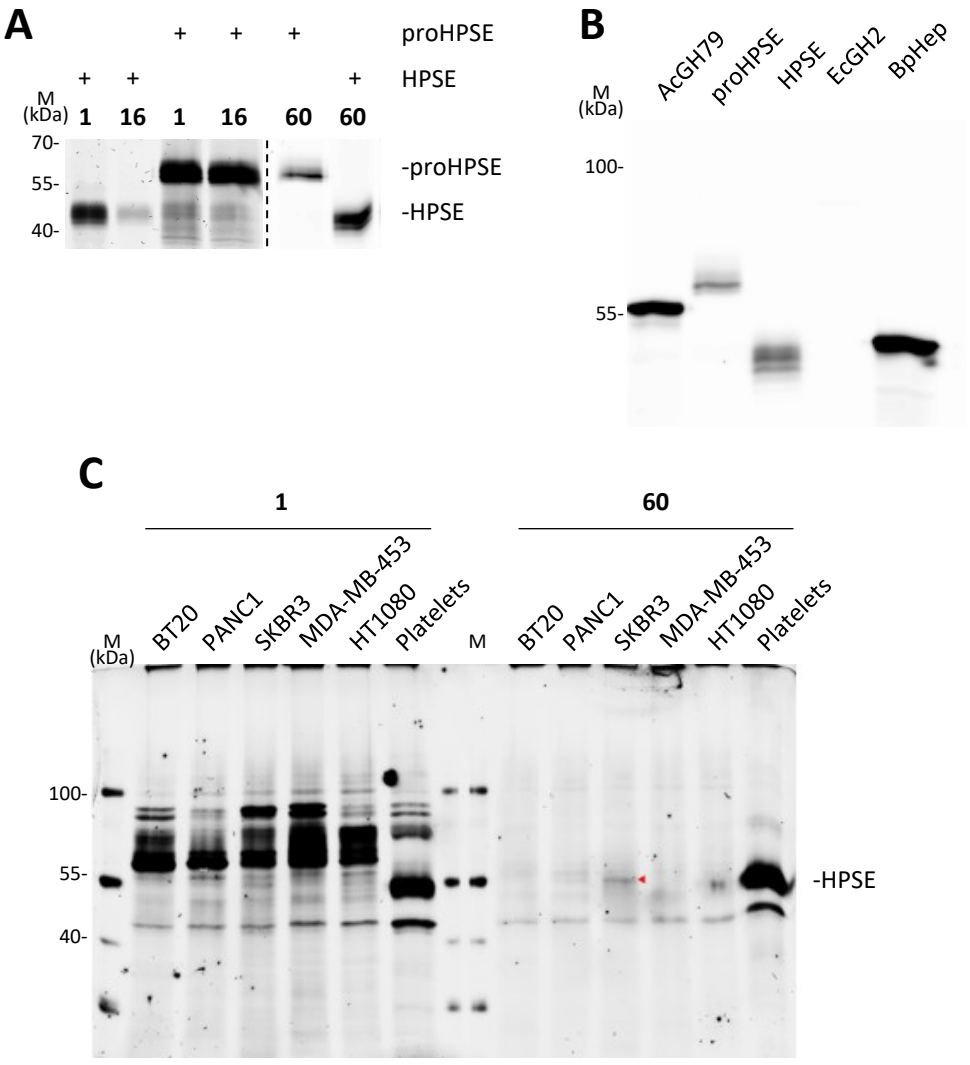


Figure 3.8|A) Labeling of recombinant proHPSE and mature HPSE (100 nM protein) with the indicated probe (100 nM for **1** and **16**, 1 μM for **60**). **B**) Labeling of recombinant human and bacterial β-glucuronidases by **60**. **C**) Comparison of labeling in human lysates with **1** and **60** showing higher selectivity of **60** (both at 1 μM).

On recombinant enzymes, 4'*O*-tagged disaccharide probe **60** showed decreased labeling of proHPSE while maintaining labeling efficiency for mature HPSE indicating increased selectivity for HPSE (Figure 3.8A). ABP **60** efficiently labeled recombinant endo-glucuronidases AcGH79 and BpHep without labeling exo-glucuronidase EcGH2 (Figure 3.8B). Labeling of lysates of human platelets and the cancer cell lines, BT20, SKBR3 and MDA-MB-453 (breast cancers), PANC1 (pancreatic cancer) and HT1080 (fibrosarcoma) with probes **1** and **60** clearly shows the improved HPSE selectivity of **60** (Figure 3.8C). Using both probes HPSE is clearly visible in human platelet lysate as expected. However, in the other lysates the activity of HPSE is too low to distinguish from background and off-target labeling with probe **1**. With probe **60** background and off-target labeling is reduced and HPSE activity can be observed in the SKBR3 cell lysate.

3.4 Conclusion

A set of 4'*O*-alkylated and glycosylated glucuronic acid configured cyclophellitol derivatives is described in this chapter. The synthesis comprises alkylation or glycosylation of a suitably protected cyclophellitol alkene, followed by generation of the glucuronic acid cyclophellitol. The glycosylated derivatives are elaborated into 2'-amino, -NHAc and -deoxy derivatives. Gel-based ABP experiments show sufficient potency for 2'-NHAc and deoxy derivatives but not for the amino derivative. 6'*O*-sulfation is shown to increase inhibitor potency.

Potent and selective HPSE ABPs are generated by 4'*O*-alkylation of disaccharide inhibitors. 4*O*-alkylated cyclophellitol or 2'*N*-acylated disaccharides do not yield potent or selective ABPs for HPSE.

Pseudodisaccharide cyclophellitol derived inhibitors **28** and **29** have shown their ability to inhibit HPSE *in vitro* and *in situ* and **28** was also active *in vivo*. In the future disaccharide inhibitors serve as a starting point for the development of selective mechanism-based HPSE inhibitors as therapeutics. The developed selective ABP may be used to assess HPSE activity in health and disease.

3.5 Acknowledgements

Liang Wu, Zachary Armstrong, Gideon Davies from the University of York, UK and Neta Ilan and Israel Vlodovsky from the Technion Israel Institute of Technology in Haifa, Israel are kindly acknowledged for biological evaluation of the compounds and valuable discussion. Vincent

Lit, Gijs Ruijgrok and Merle Weizenberg are acknowledged for their synthesis work in the context of their MSc internships. Sybrin Schröder is acknowledged for valuable discussion.

3.6 Experimental

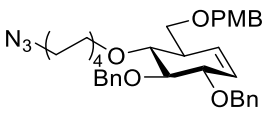
General chemical synthesis procedures are shown in the experimental section of chapter 2.

Glutatione assay

Reduced glutathione (90 µl, 50 µM in 100 mM Tris, pH = 7.5 with 10% acetonitrile) was pipetted in a 96 well plate. **28** (10 µl, 1 mM; final concentration 100 µM in DMSO) or iodoacetamide (positive control, 10 µl, 1 mM; final concentration 100 µM in DMSO) or DMSO (10 µl) was added at several time points (0, 30, 60, 90, 105, 115 minutes). After 5 more minutes Ellman's reagent (100 µl, 0.1 ml 100mM Ellman's reagent in MeOH diluted in 9.9 ml 100mM Tris pH 7.5) was added and absorbance was measured after 15 minutes at 410 nm on a Clariostar plate reader.

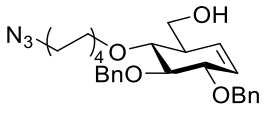
Monosaccharide probes

2,3-di-*O*-benzyl-4-*O*-(8-azidooctyl)-6-*O*-*p*-methoxybenzyl-cyclophellitol alkene (4)



PMB-protected cyclohexene **5**[26] (230 mg, 0.50 mmol) was coevaporated with toluene thrice, after which it was dissolved in THF (1.0 ml). The solution was cooled to 0°C. KHMDS (0.5 M in toluene, 1.5 ml, 0.75 mmol) was added and the mixture was stirred for 1 hour at 0°C. 8-azidooctyl 4-methylbenzenesulfonate[30] (488 mg, 1.50 mmol) was added, the reaction mixture was warmed to rt and stirred for 18 h. The mixture was quenched by the addition of H_2O and diluted with EtOAc. The aqueous phase was extracted with EtOAc (3x) and the combined organic phases were washed with H_2O and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by column chromatography (PE/EtOAc 15/1 -> 4/1, v/v) afforded the product as a colorless oil (219 mg, 0.355 mmol, 71%).
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.40 – 7.21 (m, 12H), 6.89 – 6.84 (m, 2H), 5.71 – 5.62 (m, 2H, Bn), 4.87 (s, 2H, Bn), 4.67 (s, 2H, Bn), 4.49 (d, *J*=11.9, 1H, CH_2PMB), 4.40 (d, *J*=11.9, 1H, CH_2PMB), 4.21 – 4.16 (m, 1H, H2), 3.84 (dt, *J*=8.9, 6.7, 1H, $CH_2C\mathbf{H}_2O$), 3.79 (s, 3H, OCH_3), 3.70 (dd, *J*=10.1, 7.8, 1H, H3), 3.53 (d, *J*=4.0, 2H, H6), 3.44 (t, *J*=9.9, 1H, H4), 3.32 (dt, *J*=8.9, 7.0, 1H, $CH_2C\mathbf{H}_2O$), 3.24 (t, *J*=7.0, 2H, CH_2N_3), 2.50 – 2.38 (m, 1H, H5), 1.66 – 1.13 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.3, 139.1, 138.7, 130.4, 129.5 (alkene), 129.4, 128.5, 128.4, 128.0, 127.9, 127.7, 127.6, 127.0 (alkene), 113.8, 85.4 (C3), 80.9 (C2), 78.7 (C4), 75.3 (Bn), 73.6 ($CH_2\mathbf{C}H_2O$), 72.9 (Bn), 72.2 (CH_2PMB), 68.9 (C6) , 55.3 (OCH_3), 51.5 (CH_2N_3), 44.5 (C5), 30.5, 29.5, 29.2, 28.9, 26.8, 26.2. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{37}H_{47}N_3NaO_5$ 636.3413, found 636.3412.

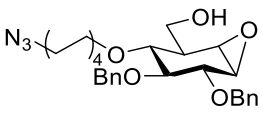
2,3-di-*O*-benzyl-4-*O*-(8-azidooctyl)-cyclophellitol alkene (12)



Cyclohexene **4** (91.0 mg, 0.15 mmol) was dissolved in a mixture of DCM/H_2O (3.0 ml, 19/1, v/v). DDQ (39.0 mg, 0.18 mmol) was added and the reaction mixture was stirred for 18 h at rt. The mixture was diluted with EtOAc and the organic layer was washed with $NaHCO_3$ (sat. aq. (3x)), H_2O and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by column chromatography (PE/EtOAc, 3/1, v/v) afforded the product as colorless oil (62.0 mg, 0.128 mmol, 85%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.24 (m, 10H), 5.74 (dt, *J* = 10.1, 2.5 Hz, 1H, alkene), 5.53 (dt, *J* = 10.2, 1.8 Hz, 1H, alkene), 4.92 – 4.84 (m, 2H, Bn), 4.68 (s, 2H, Bn), 4.21 – 4.13 (m, 1H, H2), 3.97 (dt, *J* = 8.8, 6.9 Hz, 1H, Linker-CH_2O), 3.79 – 3.71 (m, 3H, H6 (2x)/H3), 3.55 (dt, *J* = 8.8, 7.4 Hz, 1H, Linker-CH_2O), 3.47 (t, *J* = 9.7 Hz, 1H, H4), 3.24 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 2.52 – 2.45 (m, 1H, H5), 2.09 – 1.97 (m, 1H, -

OH), 1.73 – 1.49 (m, 4H), 1.41 – 1.16 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 138.5, 128.5, 128.5, 128.0, 127.9, 127.9, 127.8, 127.7, 85.1 (C3), 80.8 (C2), 80.4 (C4), 75.3 (Bn), 73.6 (linker-CH_2O), 72.3 (Bn), 64.2 (C6), 51.6 (CH_2N_3), 45.8 (C5), 30.5, 29.5, 29.2, 28.9, 26.8, 26.1. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{29}H_{39}N_3NaO_4$ 516.2838, found 516.2834.

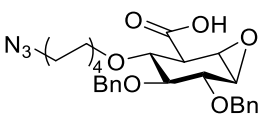
2,3-di-*O*-benzyl-4-*O*-(8-azidooctyl)-cyclophellitol (13)



Homoallylic alcohol **12** (631 mg, 1.28 mmol) was dissolved in DCM (13 ml) and cooled to 0°C. mCPBA (442 mg, 1.97 mmol) was added and the mixture was stirred at 0°C for 18 h. A second portion of mCPBA (574 mg, 2.56 mmol) was added and the mixture was stirred for another 24 h at 0°C. The reaction was diluted with EtOAc and the organic layer was washed with a mixture of $NaHCO_3$ (sat. aq)/$Na_2S_2O_3$ (1/1, v/v, (3x)) and brine. The combined aqueous layers were extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by column chromatography (PE/EtOAc, 5/2, v/v) afforded the product as a colorless oil (571 mg, 1.11 mmol, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.26 (m, 10H), 4.81 (d, *J* = 2.8 Hz, 2H, Bn), 4.74 (dd, *J* = 28.7, 11.4 Hz, 2H, Bn), 4.00 (dd, *J* = 10.8, 5.0 Hz, 1H, H6a), 3.92 – 3.85 (m, 2H, H6b/ linker-CH_2O), 3.80 (dd, *J* = 8.2, 0.5 Hz, 1H, H2), 3.52 – 3.43 (m, 2H, Bn/H3), 3.32 (d, *J* = 3.6 Hz, 1H, epoxide), 3.28 – 3.21 (m, 3H, CH_2N_3/H4), 3.15 (d, *J* = 3.8 Hz, 1H, epoxide), 2.14 (m, 1H, H5), 1.55 (m, *J* = 14.3, 7.0 Hz, 4H), 1.36 – 1.21 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7, 137.7, 128.6, 128.4, 128.1, 128.0, 127.8, 127.7, 84.9 (C3), 79.7 (C2), 76.3 (C4), 75.4 (Bn), 73.8 (linker-CH_2O), 73.3 (Bn), 63.2(C6), 55.9 (epoxide), 53.2 (epoxide), 51.5 (CH_2N_3), 44.0 (C5), 30.4, 29.4, 29.1, 28.9, 26.7, 26.1. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{29}H_{39}N_3NaO_5$ 532.2787, found 532.2782.

2,3-di-*O*-benzyl-4-*O*-(8-azidooctyl)-glucurono-cyclophellitol (3)

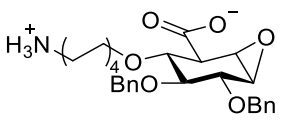


Epoxide **13** (200 mg, 393 µmol) was dissolved in DCM/*t*-BuOH/H_2O (12 ml, 4/4/1, v/v/v). TEMPO (12.3 mg, 78.6 µmol) and BAIB (316 mg, 982 µmol) were added and the reaction was stirred at 0°C for 22 h. The reaction was quenched with $Na_2S_2O_3$ (2 ml, aq. sat.), acidified to pH 3 and extracted with DCM (3x 20 ml). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by column chromatography (PE/EtOAc, 6/1, v/v, 1% AcOH) afforded the product as a colorless oil (185 mg, 0.354 mmol, 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.28 (m, 10H), 4.81 (d, *J* = 1.1 Hz, 2H, Bn), 4.78 (d, *J* = 11.4 Hz, 1H, Bn), 4.71 (d, *J* = 11.3 Hz, 1H, Bn), 3.90 – 3.82 (m, 2H, linker-CH_2O/H2), 3.65 – 3.53 (m, 2H, H4/ linker-CH_2O), 3.52 – 3.44 (m, 2H, epoxide/H3), 3.24 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 3.20 (d, *J* = 3.6 Hz, 1H, epoxide), 2.98 (dd, *J* = 10.0, 1.6 Hz, 1H, H5), 1.61 – 1.44 (m, 4H), 1.36 – 1.18 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.3 (C6), 138.6, 137.6, 128.7, 128.5, 128.2, 128.1, 127.8, 127.8, 84.2 (C3), 79.3 (C2), 75.5 (Bn), 74.9 (C4), 73.8 (linker-CH_2O), 73.5 (Bn), 54.4 (epoxide), 53.8 (epoxide), 51.6 (CH_2N_3), 48.6 (C5), 30.3, 29.3, 29.1, 28.9, 26.7, 25.9. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{29}H_{37}N_3NaO_6$ 546.2580, found 546.2580.

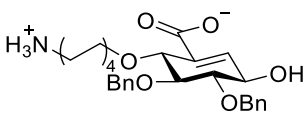
4-*O*-(8-aminooctyl)-glucurono-cyclophellitol (15) and cyclohexene 14

Carboxylic acid **3** (40.0 mg, 76.5 µmol) was dissolved in anhydrous THF, followed by the addition of *t*-BuOH (73 µl, 765 µmol) and a glass-coated stirring bar. The flask was flushed with nitrogen and ammonia (7 ml) was condensed at -60°C. Freshly cut sodium (35.0 mg, 1.53 mmol) was added portion wise at the same temperature under a flow of nitrogen. After stirring for 30 min, the reaction was quenched, warmed to rt and stirred for 1 h. The reaction mixture was concentrated under reduced pressure and coevaporated with H_2O (3x).



Quenching with AcOH (92 µl, 1.61 mmol) and HPLC purification with linear gradient, solutions A: 50 mM AcOH in H_2O and B: CH_3CN yielded **15** (6.7 mg, 21 µmol, 28%).

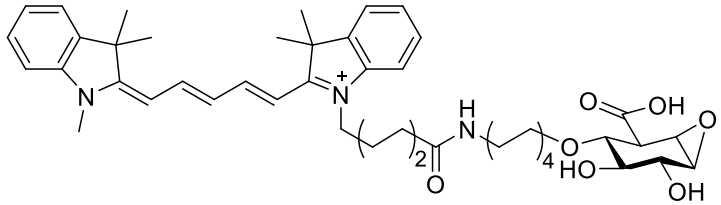
^{1}H NMR (500 MHz, D_2O) δ 3.80 (d, *J* = 8.4 Hz, 1H, H2), 3.76 – 3.70 (m, 1H, linker-CH_2O), 3.61 – 3.55 (m, 1H, linker-CH_2O), 3.49 – 3.44 (m, 2H, H4/epoxide), 3.37 (dd, *J* = 10.4, 8.5 Hz, 1H, H3), 3.15 (d, *J* = 3.7 Hz, 1H, epoxide), 2.97 (t, *J* = 7.6 Hz, 2H, CH_2NH_2), 2.72 (dd, *J* = 9.8, 1.8 Hz, 1H, H5), 1.63 (p, *J* = 7.4 Hz, 2H), 1.49 (d, *J* = 5.5 Hz, 2H), 1.39 – 1.24 (m, 8H). ^{13}C NMR (126 MHz, D_2O) δ 178.0 (C6), 76.9 (C4), 75.8 (C3), 73.0 (CH_2O), 71.2 (C2), 56.5 (epoxide), 55.5 (epoxide), 51.0 (C5), 39.5 (CH_2NH_2), 29.2, 28.0, 27.9, 26.6, 25.3, 25.0. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{15}H_{28}NO_6$ 318.1911, found 318.1913.



Quenching with NH_4HCO_3 (127 mg, 1.61 mmol) and HPLC purification with linear gradient, solutions A: 50 mM NH_4HCO_3 in H_2O and B: CH_3CN yielded **14** (2.20 mg, 6.9 µmol, 9%).

^{1}H NMR (500 MHz, D_2O) δ 5.97 (t, *J* = 1.8 Hz, 1H, alkene), 4.34 – 4.30 (m, 1H, H4), 4.23 – 4.18 (m, 1H, H1), 3.68 – 3.63 (m, 3H, linker-CH_2O/H3), 3.46 – 3.39 (m, 1H, H2), 2.96 (t, *J* = 7.5 Hz, 2H, CH_2NH_2), 1.66 – 1.57 (m, 2H), 1.54 – 1.46 (m, 2H), 1.38 – 1.24 (m, 8H). ^{13}C NMR (126 MHz, D_2O) δ 174.7 (C6), 137.9 (C5), 131.5 (CH alkene), 80.2 (C4), 75.2 (C2), 73.9 (C3), 71.1 (C1), 70.6 (CH_2O), 39.5 (CH_2NH_2), 29.3, 28.1, 27.9, 26.6, 25.3, 25.0. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{15}H_{28}NO_6$ 318.19111 found 318.19109.

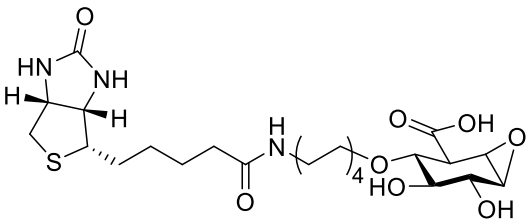
Cy5 probe 16



Amine **15** (10.3 mg, 32.5 µmol) was dissolved in anhydrous DMF (0.5 ml), Cy5-NHS ester (30.0 mg, 48.8 µmol) and DIPEA (10.6 µl, 65.0 µmol) were added. The reaction mixture was stirred for 18 h followed by purification by preparative RP-HPLC (linear gradient, solutions used: A: 50 mM AcOH in H_2O, B: CH_3CN) and lyophilization. The product was obtained as a blue solid (3.50 mg, 4.48 µmol, 14%).

^{1}H NMR (500 MHz, MeOD) δ 8.25 (t, *J* = 13.0 Hz, 2H), 7.49 (d, *J* = 7.5 Hz, 2H), 7.44 – 7.38 (m, 2H), 7.32 – 7.24 (m, 4H), 6.64 (t, *J* = 12.4 Hz, 1H), 6.29 (dd, *J* = 13.7, 6.5 Hz, 2H), 4.11 (t, *J* = 7.4 Hz, 2H), 3.79 – 3.71 (m, 1H), 3.69 (d, *J* = 8.2 Hz, 1H, H2), 3.67 – 3.59 (m, 4H), 3.48 (t, *J* = 9.8 Hz, 1H, H4), 3.39 (s, 1H, epoxide), 3.27 (dd, *J* = 10.1, 8.3 Hz, 1H, H3), 3.12 (t, *J* = 7.0 Hz, 2H), 2.99 (d, *J* = 3.6 Hz, 1H, epoxide), 2.69 (dd, *J* = 8.1, 1.5 Hz, 1H, H5), 2.20 (t, *J* = 7.2 Hz, 2H), 1.87 – 1.79 (m, 2H), 1.73 (s, 11H), 1.71 – 1.66 (m, 2H), 1.55 – 1.37 (m, 9H), 1.29 (s, 13H). ^{13}C NMR (126 MHz, MeOD) δ 155.5, 144.3, 143.6, 142.7, 142.5, 129.8, 129.7, 126.7, 126.2, 123.4, 123.3, 112.1, 111.9, 104.3, 78.2 (C3), 77.9 (C4), 73.9, 73.0 (C2), 56.9 (epoxide), 56.7 (epoxide), 52,1 (C5 from 2D), 50.6, 50.5, 44.8, 40.4, 36.7, 31.5, 31.3, 30.5, 30.3, 28.2, 28.0, 27.9, 27.8, 27.3, 27.0, 26.6. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{47}H_{64}N_3O_7$ 782.4739, found 782.4772.

Biotin probe 17



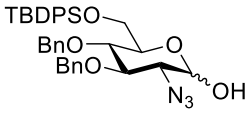
Amine **15** (6.00 mg, 18.9 µmol) was dissolved in anhydrous DMF (0.5 ml). Biotin-NHS ester (6.6 mg, 28 µmol) and DIPEA (6.5 µl, 39 µmol) were added and the reaction mixture was stirred for 18 h. The mixtures was purified by semi-preparative HPLC (linear gradient, 50 mM AcOH in H_2O and CH_3CN) and lyophilized. This afforded the product as a white solid (2.60 mg, 4.79 µmol, 25%).

^{1}H NMR (500 MHz, D_2O) δ 4.62 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.43 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.84 (d, *J* = 8.4 Hz, 1H, H2), 3.79 – 3.74 (m, 1H), 3.62 – 3.56 (m, 1H), 3.56 – 3.53 (m, 1H, epoxide), 3.49 (t, *J* = 10.1 Hz, 1H, H4), 3.44 – 3.39 (m, 1H, H3), 3.36 – 3.31 (m, 1H), 3.23 – 3.13 (m, 3H, epoxide), 3.00 (dd, *J* = 13.1, 5.0 Hz, 1H), 2.87 (d, *J* = 9.8 Hz, 1H, H5), 2.79 (d, *J* = 13.0 Hz, 1H), 2.25 (t, *J* = 7.1 Hz, 2H), 1.78 – 1.55 (m, 4H), 1.55 – 1.46 (m, 4H), 1.46 – 1.35 (m, 2H), 1.35 – 1.23 (m, 8H). ^{13}C NMR (126 MHz, D_2O) δ 176.7 (C6),

76.5 (C4), 75.7 (C3), 73.3, 71.1 (C2), 62.1, 60.3, 56.0 (epoxide), 55.6 (epoxide), 55.5, 49.8 (C5), 39.8, 39.4, 35.6, 29.3, 28.5, 28.4, 28.3, 27.8, 27.7, 26.1, 25.3, 25.2. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{25}H_{42}N_3O_8S$ 544.2693, found 544.2689.

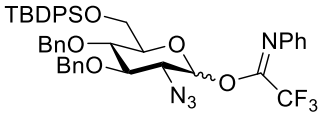
Disaccharide inhibitors

2-Azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranose (19)



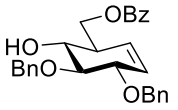
Thioglycoside **18**[31] (3.58 g, 5.00 mmol) was dissolved in acetone/H_2O (50 ml, 9/1, v/v). The mixture was cooled to 0°C and NIS (2.25 g, 10.0 mmol) was added. The reaction was stirred for 4 hours. Upon completion $Na_2S_2O_3$ (aq. sat) was added and the reaction turned colorless. The mixture was concentrated under reduced pressure, the residue was dissolved in EtOAc, washed with $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was obtained after chromatography (Et_2O/pentane, 1/19 -> 1/5, v/v) as a colorless oil (2.13 g, 3.41 mmol, 68%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 – 7.59 (m, 7H), 7.42 – 7.24 (m, 23H), 7.23 – 7.12 (m, 3H), 5.23 (t, *J* = 3.4 Hz, 1H), 4.99 – 4.78 (m, 5H), 4.74 (d, *J* = 10.9 Hz, 1H), 4.66 (d, *J* = 10.8 Hz, 1H), 4.43 (dd, *J* = 7.8, 5.1 Hz, 1H), 4.15 – 3.78 (m, 7H), 3.78 – 3.66 (m, 1H), 3.50 – 3.26 (m, 4H), 1.06 (d, *J* = 1.6 Hz, 14H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.1, 137.9, 137.9, 137.8, 136.0, 135.9, 135.7, 135.7, 133.6, 133.6, 133.1, 133.0, 129.8, 129.8, 129.8, 128.6, 128.6, 128.5, 128.4, 128.3, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.7, 96.1, 92.1, 83.1, 80.2, 78.3, 77.5, 77.2, 76.8, 76.1, 75.8, 75.8, 75.2, 71.9, 67.6, 64.2, 62.8, 62.5, 60.6, 26.9, 19.4, 19.4. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{36}H_{41}N_3O_5Na$ 646.2708, found 646.2702.

2-Azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl *N*-phenyltrifluoroacetimidate(20)



Lactol **19** (2.10 g, 3.37 mmol) was dissolved in DCM (16.8 ml). 2,2,2-trifluoro-*N*-phenyltrifluoroacetimidoyl chloride (0.15 ml, 0.92 mmol) and Cs_2CO_3 (1.32 g, 4.04 mmol) were added and the reaction was stirred overnight at room temperature. The reaction was filtered over celite and concentrated in vacuo. Purification by flash silica column chromatography (Et_2O/pentane, 1/38 -> 1/19, v/v) yielded the product as a colorless oil (2.41 g, 3.03 mmol, 90%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 – 7.58 (m, 4H), 7.51 – 7.18 (m, 19H), 7.16 – 7.00 (m, 1H), 6.81 (d, *J* = 7.8 Hz, 1H), 6.76 – 6.71 (m, 1H), 5.00 – 4.82 (m, 3H), 4.76 (dd, *J* = 10.7, 3.9 Hz, 1H), 4.08 – 3.79 (m, 4H), 3.65 (d, *J* = 10.2 Hz, 1H), 1.07 (d, *J* = 1.0 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.5, 143.3, 137.9, 137.8, 137.7, 137.7, 136.1, 136.0, 135.7, 135.6, 133.6, 133.5, 132.9, 132.8, 130.0, 129.9, 129.9, 129.5, 128.9, 128.8, 128.7, 128.7, 128.7, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 126.5, 124.6, 124.5, 120.6, 119.5, 82.9, 80.4, 77.6, 77.5, 77.2, 77.1, 76.8, 76.5, 76.0, 75.5, 75.5, 74.6, 65.6, 63.2, 62.1, 27.0, 26.9, 19.5. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{44}H_{45}F_3N_4O_5SiNa$ 817.3004 found 817.3005.

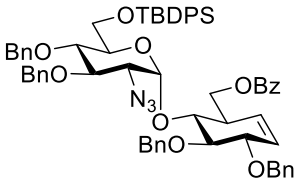
2,3-di-*O*-benzyl-6-*O*-benzoyl-cyclophellitol alkene (9)



Diol **11** (1.36 g, 4.00 mmol) was dissolved in DCM (20 ml). Et_3N (2.79 ml, 20.0 mmol) and benzoyl chloride (0.56 ml, 4.80 mmol) were added at -50°C and the reaction was slowly warmed to room temperature overnight. H_2O and DCM were added and the layers were separated. The organic phase was washed with $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 1/19 -> 1/9, v/v) afforded the product as a colorless oil (1.45 g, 3.27 mmol, 82%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (dd, *J* = 8.3, 1.4 Hz, 2H), 7.60 – 7.49 (m, 1H), 7.49 – 7.27 (m, 12H), 5.80 (dt, *J* = 10.3, 2.2 Hz, 1H, alkene), 5.70 (dt, *J* = 10.3, 2.0 Hz, 1H, alkene), 5.04 (d, *J* = 11.2 Hz, 1H, Bn), 4.80 – 4.61 (m, 3H, Bn), 4.58 (dd, *J* = 10.9, 3.7 Hz, 1H, H6a), 4.44 (dd, *J* = 11.0, 5.5 Hz, 1H, H6b), 4.27 – 4.20 (m, 1H, H2), 3.78 – 3.64 (m, 2H, H4/H3), 2.75 – 2.65 (m, 1H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7 (Bz), 138.9, 138.5, 138.2, 133.2, 129.7, 128.7, 128.6, 128.5, 128.1, 128.1, 128.0, 127.9, 127.8 (alkene),

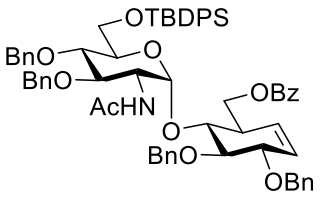
127.4 (alkene), 83.8 (C3), 80.4 (C2), 75.2 (Bn), 71.6 (Bn), 69.9 (C4), 64.4 (C6), 43.4 (C5). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{28}H_{28}NaO_5$ 467.1829, found 467.1832.

2,3-di-*O*-benzyl-4-*O*-(2-Azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6-*O*-benzoyl-cyclophellitol alkene (21)



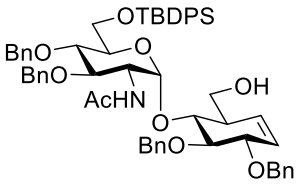
Donor **20** (2.41 g, 3.03 mmol) and acceptor **9** (1.04 g, 2.33 mmol) were co-evaporated with anhydrous toluene (3x). the mixture was dissolved in DCM (15 ml) and activated MS 3 Å were added. The mixture was stirred at room temperature overnight. The mixture was cooled to -78°C. TfOH (0.04 ml, 0.45 mmol) was added and the reaction was warmed to -30°C over 70 minutes and kept at this temperature for 60 minutes. The reaction was quenched with $NaHCO_3$ (aq. sat.), diluted with Et_2O and washed with H_2O, $NaHCO_3$ (aq. sat.), and brine, dried over $MgSO_4$ and filtered. Volatiles were removed under educed pressure and column chromatography (EtOAc/pentane, 1/19->1/9, v/v) afforded the product (2.02 g, 1.92 mmol, 83%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.80 (m, 2H), 7.61 – 7.51 (m, 4H), 7.45 – 7.13 (m, 29H), 5.83 – 5.77 (m, 1H, alkene), 5.71 (d, *J* = 3.9 Hz, 1H, H1'), 5.64 – 5.57 (m, 1H, alkene), 5.08 (d, *J* = 10.7 Hz, 1H, Bn), 4.98 (d, *J* = 10.7 Hz, 1H, Bn), 4.89 – 4.81 (m, 3H, Bn (3x)), 4.73 – 4.62 (m, 3H, Bn (3x)), 4.47 (dd, *J* = 11.1, 3.2 Hz, 1H, H6a), 4.33 – 4.24 (m, 2H, H6b/H2), 4.05 – 3.91 (m, 3H, H3'/H4'/H4), 3.91 – 3.81 (m, 2H, H6a'/H3'), 3.80 – 3.70 (m, 2H, H6b'/H5'), 3.29 (dd, *J* = 10.4, 3.9 Hz, 1H, H2'), 2.78 (br, 1H, H5), 0.98 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2 (OBz), 138.9, 138.3, 138.2, 138.1, 135.9, 135.7, 133.6, 133.2, 133.1, 129.7, 129.7, 129.6, 128.7, 128.6, 128.6, 128.6, 128.4, 128.3, 128.0, 128.0, 127.9, 127.8, 127.8, 127.8, 127.6, 127.6, 98.2 (C1'), 84.4, 81.0 (C2), 80.2 (C4'), 78.1 (C3'), 75.7 (Bn), 75.2 (Bn), 74.8 (Bn), 74.4 (C4), 72.8 (C5'), 71.8 (Bn), 64.5 (C6), 63.6 (C2'), 62.2 (C6'), 43.3 (C5), 27.0 (*t*-Bu)., 19.4 (*t*-Bu). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{64}H_{67}N_3O_9SiNa$ 1072.4539, found 1072.4573.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6-*O*-benzoyl-cyclophellitol alkene (22)



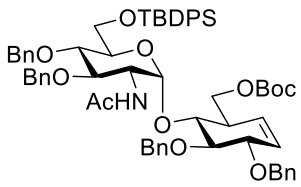
Azide **21** (0.22 g, 0.21 mmol) was dissolved in THF/H_2O (2.6 ml, 17/3, v/v) and PPh_3 (0.14 g, 0.50 mmol) and pyridine (5 µl, 0.06 mmol) were added. The solution was stirred at 50°C for 3 hours. The reaction was concentrated under reduced pressure, dissolved in DCM and dried over $MgSO_4$. The solids were removed by filtration and the filtrate was concentrated under reduced pressure. The residue was coevaporated with toluene and dissolved in DCM (2.0 ml). Acetic anhydride (0.30 ml, 3.1 mmol) and pyridine (0.25 ml, 3.1 mmol) were added and the reaction was stirred overnight. The reaction was cooled to 0°C and quenched with H_2O. The layers were separate and the organic phase was washed with $CuSO_4$ (aq. sat), $NaHCO_3$ (aq. sat) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 9/1 -> 8/2, v/v) afforded the product (0.20 g, 0.19 mmol, 89%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 – 7.92 (m, 2H), 7.72 – 7.63 (m, 4H), 7.42 – 7.13 (m, 29H), 6.62 (d, *J* = 9.7 Hz, 1H, NH), 5.86 – 5.81 (m, 1H, alkene), 5.73 – 5.66 (m, 1H, alkene), 4.98 – 4.77 (m, 5H, H1', Bn (4x)), 4.72 – 4.55 (m, 5H, H6a, Bn (4x)), 4.52 – 4.47 (m, 1H, H6b), 4.38 (td, *J* = 10.0, 3.5 Hz, 1H, H2'), 4.23 – 4.16 (m, 1H, H2), 3.99 – 3.71 (m, 7H), 2.70 – 2.63 (m, 1H, H5), 1.46 (s, 3H, Ac), 1.03 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4 (Ac), 166.2 (Bz), 138.6, 138.3, 137.9, 137.7, 136.0, 135.7, 133.7, 133.2, 133.0, 130.0, 129.8, 129.7, 129.7, 128.8, 128.7, 128.6, 128.6, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.7, 126.8, 100.5 (C1'), 81.8, 81.4, 79.0 (C2), 77.8, 77.4, 75.4 (Bn), 75.3 (Bn), 74.9 (Bn), 73.5, 71.6 (Bn), 64.1 (C6), 62.5 (C6'), 53.5 (C2'), 44.0 (C5), 26.9 (TBDPS), 22.9 (NHAc), 19.4 (TBDPS). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{66}H_{71}NO_{10}SiNa$ 1088.4739, found 1088.4741.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-cyclophellitol alkene (23)



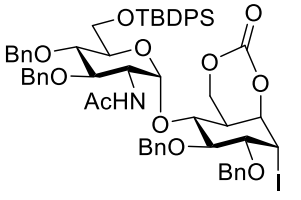
Benzoyl ester **22** (0.52 g, 0.49 mmol) was dissolved in MeOH/DCM (13.5 ml, 4.4/1, v/v). NaOMe (5.4 M in MeOH, 0.03 ml,0.15 mmol) was added and the reaction was stirred overnight. The reaction was quenched with NH_4Cl. Volatiles were removed under reduced pressure. The residue was dissolved in EtOAc and washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 9/1->3/2, v/v) yielded the product (0.46 g, 0.47 mmol, 97%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 16.1, 7.0 Hz, 4H), 7.46 – 7.11 (m, 26H), 6.70 (d, *J* = 9.6 Hz, 1H, NH), 5.90 – 5.82 (m, 1H, alkene), 5.67 – 5.60 (m, 1H, alkene), 4.95 – 4.89 (m, 2H, H1', Bn), 4.83 (d, *J* = 10.6 Hz, 1H, Bn), 4.75 – 4.50 (m, 6H, Bn (6x)), 4.39 – 4.30 (m, 1H, H2'), 4.21 – 4.15 (m, 1H, H2), 3.99 – 3.56 (m, 9H), 2.41 – 2.34 (m, 1H, H5), 1.47 (s, 3H, NAc), 1.05 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4 (NAc), 138.5, 138.1, 137.9, 137.5, 136.0, 135.7, 133.6, 133.1, 129.8, 129.4, 128.7, 128.6, 128.6, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 126.7, 100.2 (C1'), 81.6, 81.2, 78.8 (C2), 78.0, 77.8, 75.2 (Bn), 74.8 (Bn), 73.5, 71.5 (Bn), 62.8 (C6'), 62.5 (C6), 53.3 (C2'), 46.1 (C5), 26.9 (TBDPS), 22.8 (Ac), 19.3 (TBDSP). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{59}H_{67}NO_9SiNa$ 984.4477, found 984.4506.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6-*O*-tert-butyloxycarbonyl-cyclophellitol alkene (S1)



Alcohol **23** (0.72 g, 0.75 mmol) was coevaporated three times with toluene and dissolved in dry THF (5.4 ml, 0.14 M). Boc_2O (0.19 ml, 0.81 mmol) and DMAP (9 mg, 75 µmol) were added and the reaction was stirred at room temperature. After 1h the reaction was cooled to 0 C and quenched with H_2O. The reaction mixture was further diluted with H_2O and extracted with Et_2O. The organic layer was washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated in vacuo. Flash column chromatography (PE/EtOAc, 9/1 -> 2/3, v/v) yielded the product (0.60 g, 0.57 mmol, 76%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 – 7.63 (m, 4H), 7.47 – 7.11 (m, 26H), 6.75 (d, *J* = 9.7 Hz, 1H, NH), 5.82 (dt, *J* = 10.2, 2.5 Hz, 1H, alkene), 5.62 (ddd, *J* = 10.3, 2.6, 1.6 Hz, 1H, alkene), 4.99 – 4.84 (m, 3H, H1', Bn (2x)), 4.80 (d, *J* = 11.1 Hz, 1H, Bn), 4.75 – 4.51 (m, 5H, Bn (5x)), 4.45 – 4.27 (m, 3H, H2', H6ab), 4.23 (dt, *J* = 6.3, 2.3 Hz, 1H, H2), 4.04 – 3.68 (m, 7H), 2.52 (h, *J* = 3.1 Hz, 1H, H5), 1.45 (s, 3H, Ac), 1.42 (s, 9H, Boc), 1.06 (s, 9H, TBDPS). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3 (NAc), 153.4 (Boc), 138.6, 138.3, 137.8, 137.6, 135.9, 135.6, 133.7, 133.0, 129.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.2, 128.2, 128.0, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.7, 127.6, 127.5, 126.8, 100.5 (C1'), 82.1, 81.8, 81.7, 79.3 (C2), 77.7, 75.2 (Bn), 75.1 (Bn), 74.9 (Bn), 73.4, 71.5 (Bn), 66.1 (C6), 62.5 (C6'), 53.5 (C2'), 43.9 (C5), 27.7 (Boc), 26.9 (TBDPS), 22.7 (Ac), 19.3 (TBDPS). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{64}H_{76}NO_{11}Si$ 1062.5182, found 1062.5182.

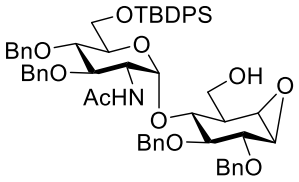
1-iodo-2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6,7-*O*-carbonyl-cyclophellitol alkane (S2)



To a solution of Boc protected **S1** (0.22 g, 0.21 mmol) in DCM/AcOH (1.1 ml, 0.2 M, 2/1, v/v), NIS (0.09 g, 0.41 mmol) was added. After 22h the reaction mixture was diluted with Et_2O and quenched with Et_3N. The organic layer was washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.), $Na_2S_2O_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated in vacuo. Flash column chromatography (PE/EtOAc, 9/1 -> 3/2, v/v) yielded the product (0.21 g, 0.19 mmol, 91%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 – 7.62 (m, 4H), 7.48 – 7.11 (m, 26H), 6.43 (d, *J* = 9.7 Hz, 1H, NH), 5.12 – 4.99 (m, 2H, Bn, H6a), 4.91 – 4.83 (m, 4H, H1', H0, Bn (2x)), 4.72 – 4.63 (m, 4H, H1, Bn (3x)), 4.60 (d, *J* = 10.5 Hz, 1H, Bn), 4.55 (d, *J* = 11.0 Hz, 1H, Bn), 4.41 (td, *J* = 10.0, 3.3 Hz, 1H, H2'), 4.06 (t, *J* = 6.7 Hz,

1H, H6b), 4.01 – 3.82 (m, 5H), 3.82 – 3.72 (m, 2H), 3.13 (dd, *J* = 9.3, 4.0 Hz, 1H, H2), 2.62 (dd, *J* = 10.4, 2.6 Hz, 1H, H5), 1.28 (s, 3H, Ac), 1.06 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 146.8, 138.4, 138.0, 137.0, 136.8, 135.8, 135.5, 133.6, 132.8, 129.9, 129.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 101.4, 82.1, 81.6, 81.4, 77.6, 77.3, 76.7, 76.2, 75.3, 75.0, 74.0, 72.2, 68.3, 62.6, 60.4, 53.3, 35.1, 30.4, 26.9, 22.5, 19.4. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{60}H_{67}INO_{11}Si$ 1132.3523, found 1132.3525.

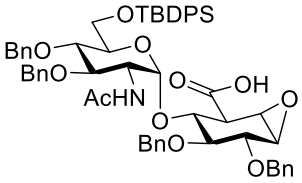
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-cyclophellitol (24)



Iodide **S2** (0.12 g, 0.11 mmol) was dissolved in MeOH/DCM (1.9 ml, 0.06 M, 1.6/1, v/v), NaOMe (4.37 M in MeOH, 0.09 ml, 0.39 mmol) was added and the reaction was stirred for 20 hours. $Et_3N{\cdot}HCl$ was added and the solution was concentrated in vacuo. EtOAc was added and the solution was washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The product was used and analyzed without further purification (0.12 g, 0.12 mmol, quantitative).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 – 7.66 (m, 4H), 7.47 – 7.15 (m, 26H), 6.83 (d, *J* = 9.7 Hz, 1H, NH), 4.95 (d, *J* = 11.0 Hz, 1H, Bn), 4.88 (d, *J* = 3.3 Hz, 1H, H1'), 4.85 – 4.77 (m, 2H), 4.73 – 4.63 (m, 4H), 4.47 (d, *J* = 11.2 Hz, 1H), 4.35 (td, *J* = 9.8, 3.3 Hz, 1H, H2'), 4.12 – 4.05 (m, 1H, H6a), 4.01 – 3.93 (m, 2H, H6b/H6a'), 3.93 – 3.78 (m, 4H, H6b'), 3.72 – 3.52 (m, 3H, H3'/H4), 3.34 – 3.31 (m, 1H, epoxide), 3.15 (d, *J* = 3.7 Hz, 1H, epoxide), 2.03 – 1.97 (m, 1H, H5), 1.44 (s, 3H, NAc), 1.06 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3 (NAc), 138.6, 138.2, 137.5, 137.2, 136.1, 135.7, 133.7, 133.1, 129.8, 128.8, 128.7, 128.6, 128.4, 128.3, 128.2, 128.2, 127.9, 127.9, 127.7, 127.7, 127.6, 100.8 (C1'), 83.1, 81.5, 80.1, 77.6, 75.9, 75.6 (Bn), 75.1 (Bn), 74.9 (Bn), 73.7, 72.8 (Bn), 62.7 (C6'), 61.8 (C6), 56.6 (epoxide), 53.3 (C2'), 52.2 (epoxide), 44.5 (C5), 26.9 (TBDPS), 22.8 (Ac), 19.4 (TBDPS). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{59}H_{67}NO_{10}SiNa$ 1000.4426, found 1000.4452.

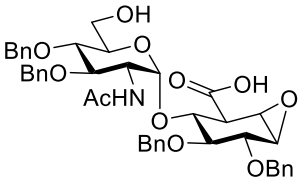
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-glucurono-cyclophellitol (25)



Alcohol **24** (0.13 g, 0.13 mmol) was dissolved in *t*-BuOH/DCM/H_2O (4.9 ml, 5/4/1, v/v). and cooled down to 0°C. TEMPO (4 mg, 0.03 mmol) and BAIB (0.10 g, 0.32 mmol) were added and the reaction was stirred 23 hours. The reaction was diluted with DCM and quenched with $Na_2S_2O_3$ (aq. sat.). The layers were separated and the water layer was acidified with AcOH. The water layer was extracted with DCM (4x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 4/1 -> 1/1, v/v, 1% AcOH) afforded the product (0.10 mg, 0.10 mmol, 80%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 3.4 Hz, 2H), 7.68 (d, *J* = 6.8 Hz, 2H), 7.50 – 7.13 (m, 26H), 6.04 (d, *J* = 9.6 Hz, 1H, NH), 4.97 – 4.74 (m, 6H, Bn(5x)/H1'), 4.69 – 4.53 (m, 3H, Bn (3x)), 4.30 – 4.22 (m, 1H, H2'), 4.08 (d, *J* = 9.7 Hz, 1H), 3.99 (t, *J* = 9.5 Hz, 1H), 3.95 – 3.87 (m, 2H), 3.80 – 3.64 (m, 3H), 3.48 – 3.42 (m, 1H, H2), 3.29 (s, 1H, epoxide), 3.17 (d, *J* = 3.5 Hz, 1H, epoxide), 2.79 – 2.73 (m, 1H, H5), 1.25 (s, 3H, Ac), 1.05 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 170.1 (NHAc/COOH), 138.6, 138.4, 137.6, 137.1, 136.6, 135.8, 133.7, 133.6, 130.0, 129.8, 128.8, 128.8, 128.7, 128.5, 128.4, 128.4, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7, 100.5 (C1'), 81.8, 81.4, 79.1, 77.7, 75.9, 75.6 (Bn), 75.4 (Bn), 75.4 (Bn), 73.2, 72.9 (Bn), 62.2 (C6'), 54.1 (epoxide), 53.7 (epoxide), 53.4 (C2'), 48.7 (C5), 27.0 (TBDPS), 22.5 (Ac), 19.3 (TBDPS). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{59}H_{65}NO_{11}SiNa$ 1014.4219, found 1014.4223.

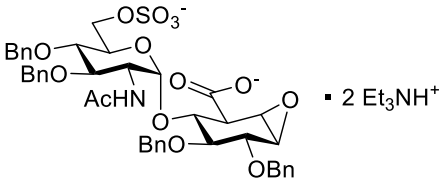
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-D-glucopyranosyl)-glucurono-cyclophellitol (26)



Silyl protected **25** (89 mg, 0.090 mmol) was dissolved in anhydrous THF (1.9 ml). $Et_3N{\cdot}3HF$ (0.046 ml ,0.28 mmol) was added and the reaction was stirred for 20 hours. More $Et_3N{\cdot}3HF$ (0.030 ml, 0.18 mmol) was added and the reaction was stirred 23 hours. The reaction was diluted with DCM and washed with water. The water layer was extracted with DCM (4x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 4/1 -> 9/1, v/v, 1% AcOH) yielded the product (62 mg, 0.082 mmol, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.15 (m, 20H), 6.35 (d, *J* = 9.4 Hz, 1H, NH), 4.96 (d, *J* = 10.7 Hz, 1H, Bn), 4.88 – 4.74 (m, 4H, H1'/Bn (3x)), 4.74 – 4.63 (m, 2H, Bn), 4.63 – 4.49 (m, 2H, Bn), 4.39 – 4.28 (m, 1H, H2'), 4.02 – 3.89 (m, 3H, H4/H2/H6a'), 3.87 – 3.77 (m, 3H, H4'/H5'/H6b'), 3.68 – 3.61 (m, 1H, H3'), 3.49 (t, *J* = 9.3 Hz, 1H, H3), 3.44 – 3.39 (m, 1H, epoxide), 3.22 – 3.16 (m, 1H, epoxide), 2.88 (d, *J* = 8.3 Hz, 1H, H5), 1.19 (s, 3H, Ac). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.2, 172.3 (NAc/COOH), 138.6, 138.3, 137.7, 137.1, 129.1, 128.8, 128.8, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.2, 127.9, 127.6, 127.6, 125.4, 100.5 (C1'), 81.8 (C3'), 81.5 (C3), 79.3, 77.6, 76.7, 75.8 (Bn), 75.2 (Bn), 75.1 (Bn), 73.3, 72.8 (Bn), 61.0 (C6'), 54.4 (epoxide), 53.6 (epoxide/C2'), 49.4 (C5), 22.7 (Ac). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{43}H_{47}NO_{11}Na$ 776.3041 found 776.3058.

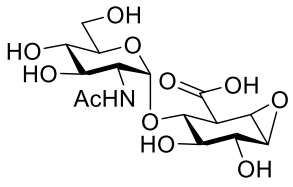
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3,4-di-*O*-benzyl-6-*O*-sulfo-D-glucopyranosyl)-glucurono-cyclophellitol (27)



Alcohol **26** (0.062 g, 0.082 mmol) was dissolved in dry DMF (1.1 ml, 0.08 M). $SO_3{\cdot}Et_3N$ (0.031 g, 0.17 mmol) was added and the mixture was stirred for 4h. Et_3N (0.07 ml, 0.5 mmol) was added, the reaction mixture was diluted with MeOH (2 ml) and concentrated under reduced pressure. The crude mixture was analyzed and used without purification.

^{1}H NMR (400 MHz, MeOD) δ 7.45 – 7.17 (m, 20H), 5.42 (d, *J* = 3.9 Hz, 1H, H1'), 4.90 – 4.59 (m, 8H, Bn), 4.41 (d, *J* = 10.6 Hz, 1H, H6a'), 4.24 (d, *J* = 10.6 Hz, 1H, H6b'), 4.17 (dd, *J* = 10.0, 3.9 Hz, 1H, H2'), 4.08 (t, *J* = 9.7 Hz, 1H, H4), 3.91 – 3.83 (m, 2H, H5'/H4'), 3.78 – 3.63 (m, 3H, H3'/H3/H2), 3.52 – 3.47 (m, 1H, epoxide), 3,33 (under solvent peak, 1H, epoxide) 3.20 (dt, *J* = 8.3, 6.7 Hz, 19H, H5/Et_3N), 1.74 (s, 3H), 1.31 (td, *J* = 7.3, 1.4 Hz, 29H, Et_3N). ^{13}C NMR (101 MHz, MeOD) δ 174.2, 172.9 (NHAc/COOH), 140.1, 139.8, 139.6, 139.0, 129.5, 129.5, 129.4, 129.3, 129.2, 129.0, 128.7, 128.6, 128.5, 128.5, 99.3 (C1'), 84.8 (C3), 82.0, 81.4, 79.0 (C2), 76.2 (Bn), 75.9 (Bn), 75.2 (Bn), 73.3 (Bn), 72.8 (C4), 71.5, 66.8 (C6'), 55.6 (epoxide), 54.2 (C2'), 53.8 (epoxide), 49.4 (C5), 47.9 (Et_3N), 22.9 (Ac), 9.3 (Et_3N). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{43}H_{47}NO_{14}S$ 834.2790, found 834.2785.

4-*O*-(2-*N*-acetyl-2-deoxy-D-glucopyranosyl)-glucurono-cyclophellitol (28)

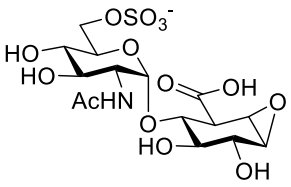


Ammonia (10 ml) was condensed at -70°C. Sodium (17 mg, 0.72 mmol) was added and the solution turned blue. Benzyl protected **26** (14 mg, 0.018 mmol), dissolved in anhydrous THF (2 ml) and *t*-BuOH (0.068 ml, 0.72 mmol) was added. After stirring at -60°C for 15 minutes the blue color faded and more sodium (12 mg, 0.51 mmol) was added. After another 30 minutes the blue color faded again the reaction was quenched with NH_4Cl (66 mg, 1.23 mmol) The ammonia was evaporated, water was added and the compound was desalted by size exclusion over HW-40 (1% AcOH in water).Lyophilization afforded the compound as a white solid (6.42 mg, 0.016 mmol, 91%).

^{1}H NMR (500 MHz, D_2O) δ 5.30 (d, *J* = 3.7 Hz, 1H, H1'), 3.97 – 3.93 (m, 1H, H2'), 3.93 – 3.88 (m, 4H, H4/H6' (2x)), 3.85 – 3.81 (m, 2H), 3.64 – 3.56 (m, 3H, epoxide), 3.32 – 3.29 (m, 1H, epoxide), 2.99 (d, *J* = 9.6 Hz, 1H, H5), 2.13 (s, 3H, Ac). ^{13}C NMR (126 MHz, D_2O) δ 177.6, 174.4 (NAc/COOH), 97.7 (C1'), 76.2,

75.0, 72.0, 71.5, 71.0, 69.7, 60.1 (C6'), 56.2 (epoxide), 55.5 (epoxide), 54.0 (C2'), 50.8 (C5), 22.0 (Ac). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{15}H_{23}NO_{11}Na$ 416.1163, found 416.1164.

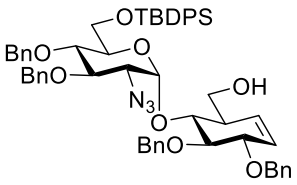
4-*O*-(2-*N*-acetyl-2-deoxy-6-*O*-sulfo-D-glucopyranosyl)-glucurono-cyclophellitol (29)



Ammonia was condensed at -60°C under an inert atmosphere and Na (37 mg, 1.6 mmol) was added. The solution turned dark blue. Benzyl protected **27** (35 µmol, 33 mg) was dissolved in THF/*t*-BuOH (0.6 ml, 2/3, v/v) and added to the solution. After stirring for 45 minutes the reaction was quenched with NH_4Cl (0.10 g, 1.92 mmol). Ammonia was evaporated at room temperature, diluted in water and concentrated under reduced pressure. The compound was desalted by size exclusion over HW-40 (1% AcOH in water). Lyophilization afforded the compound as a white solid (14.9 mg, 0.03 mmol, 87% over 2 steps).
^{1}H NMR (400 MHz, D_2O) δ 5.22 (d, *J* = 3.7 Hz, 1H, H1'), 4.33 (dd, *J* = 11.0, 2.2 Hz, 1H, H6a'), 4.15 (dd, *J* = 11.1, 2.1 Hz, 1H, H6b'), 3.88 – 3.75 (m, 4H, H2'/H5'), 3.70 (dd, *J* = 10.7, 9.1 Hz, 1H), 3.56 (dd, *J* = 10.1, 9.1 Hz, 1H), 3.50 – 3.44 (m, 2H, epoxide), 3.15 (d, *J* = 3.7 Hz, 1H, epoxide), 2.83 (dd, *J* = 9.7, 1.9 Hz, 1H, H5), 1.99 (s, 3H, Ac). ^{13}C NMR (101 MHz, D_2O) δ 180.6, 177.7, 174.3 (NAc/COOH/AcOH), 97.4 (C1'), 76.5, 74.4, 71.6, 70.8, 69.9, 69.0, 66.3 (C6'), 56.1 (epoxide), 55.3 (epoxide), 53.7 (C2'), 50.9 (C5), 22.7 (AcOH), 21.9 (Ac). HRMS (ESI) m/z: $[M+2H]^+$ calculated for $C_{15}H_{24}NO_{14}S$ 474.09120 found 474.09129.

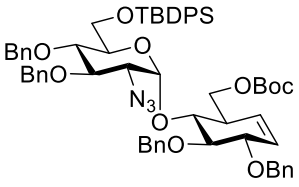
Late stage azide reduction

2,3-di-*O*-benzyl-4-*O*-(2-Azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-cyclophellitol alkene (30)



Ester **21** (0.98 g, 0.93 mmol) was dissolved in MeOH/DCM (1/1, v/v, 18 ml). NaOMe (25 wt%, 0.1 ml, 0.44 mmol) was added and the reaction mixture was stirred overnight. NH_4Cl was added and volatiles were removed under reduced pressure. The crude product was purified using column chromatography (pentane/EtOAc, 1/19 -> 3/17, v/v) yielding the product as a colorless oil (0.91 g, 0.96 mmol, quant.).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 – 7.61 (m, 4H), 7.44 – 7.21 (m, 24H), 7.17 – 7.10 (m, 2H), 5.80 (dt, *J* = 10.2, 2.4 Hz, 1H, alkene), 5.65 (d, *J* = 4.0 Hz, 1H H1'), 5.59 (dt, *J* = 10.2, 2.2 Hz, 1H, alkene), 5.06 (d, *J* = 10.9 Hz, 1H, Bn), 4.95 (d, *J* = 10.9 Hz, 1H, Bn), 4.89 – 4.79 (m, 3H, Bn), 4.71 – 4.57 (m, 3H, Bn), 4.23 (m, 1H, H2), 4.02 – 3.89 (m, 3H, H4/ H3'/H4), 3.88 – 3.80 (m, 3H, H6'ab/H5'), 3.76 – 3.57 (m, 3H, H6ab/H3), 3.29 (dd, *J* = 10.3, 4.0 Hz, 1H, H2'), 2.54 – 2.44 (m, 1H, H5), 1.04 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.0, 138.3, 138.0, 136.0, 135.8, 133.4, 133.1, 129.9, 128.9, 128.6, 128.6, 128.6, 128.5, 128.5, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 98.2 (C1'), 84.2 (C3), 80.9 (C2), 80.3 (C4'), 78.4 (C3'), 75.7 (Bn), 75.3 (Bn), 74.6 (Bn), 74.4 (C4), 72.9 (C5'), 71.7 (Bn), 63.6 (C2'), 63.0 (C6'), 62.6 (C6), 45.5 (C5), 27.0 (TBDPS), 19.4 (TBDPS). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{57}H_{63}N_3O_8SiNa$ 968.4277, found 968.4274.

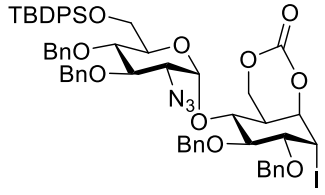
2,3-di-*O*-benzyl-4-*O*-(2-Azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6-*O*-tert-butyloxycarbonyl-cyclophellitol alkene (S3)



Alcohol **30** (0.92 g, 0.93 mmol) was co-evaporated with toluene and subsequently dissolved in THF (9.3 ml, 0.1 M). DMAP (91 mg, 0.74 mmol) and Boc_2O (0.41 g, 1.87 mmol) were added. The reaction was stirred at room temperature for 3 hours. Water was added and the mixture was stirred for 15 minutes. The mixture was extracted with Et_2O (3x), the combined organic layers were washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.) and brine , dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was obtained after column chromatography (Et_2O/pentane, v/v/, 0/1 -> 1/9) as a colorless oil (0.80 g, 0.76 mmol, 82%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 – 7.59 (m, 4H), 7.44 – 7.12 (m, 26H), 5.76 (dt, *J* = 10.2, 2.4 Hz, 1H, alkene), 5.66 (d, *J* = 3.9 Hz, 1H, H1'), 5.57 (dt, *J* = 10.2, 2.3 Hz, 1H, alkene), 5.05 (d, *J* = 10.9 Hz, 1H, Bn), 4.94 (d, *J* = 10.9 Hz, 1H, Bn), 4.91 – 4.83 (m, 3H, Bn), 4.74 (d, *J* = 10.9 Hz, 1H, Bn), 4.70 – 4.60 (m, 2H, Bn), 4.26 – 4.16 (m, 2H, H6a/H2), 4.03 – 3.94 (m, 3H, H3'/H6b/H6a'), 3.93 – 3.81 (m, 4H, H4/H3/H6b'/H4'), 3.75 – 3.69 (m, 1H, H5'), 3.28 (dd, *J* = 10.4, 3.9 Hz, 1H, H2'), 2.69 – 2.61 (m, 1H, H5), 1.25 (s, 9H, Boc), 1.03 (s, 9H, TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.5 (Boc), 139.0, 138.3, 138.1, 136.0, 135.7, 133.8, 133.2, 129.7, 129.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.0, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.5, 98.0 (C1'), 84.3, 82.1 (Boc), 80.7 (C2), 80.3 (C3'), 78.2, 75.7 (Bn), 75.1 (Bn), 74.6 (Bn), 74.1, 72.7 (C5'), 71.8 (Bn), 66.6 (C6), 63.7 (C2'), 62.3 (C6'), 43.0 (C5), 27.7, 27.0 (TBDPS/Boc), 19.4(TBDPS). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{62}H_{71}N_3O_{10}SiNa$ 1068.48009, found 1068.48007.

1-iodo-2,3-di-*O*-benzyl-4-*O*-(2-azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-6,7-*O*-carbonyl-cyclophellitol alkane (S4)

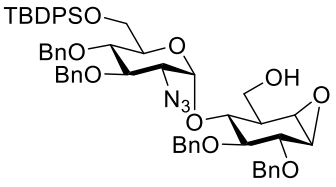


Alkene **S3** (0.80 g, 0.77 mmol) was dissolved in AcOH/DCM (1/2, v/v, 5.1 ml, 0.15 M). NIS (0.35 g , 1.54 mmol) was added and the reaction was stirred for 18 hours in the dark. The mixture was diluted with Et_2O, washed with $Na_2S_2O_3$ (aq.), $NaHCO_3$ (aq. sat. 2x) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was obtained after column chromatography (Et_2O/pentane, 1/4 -> 1/1, v/v) as a foam (0,61 g, 0.55 mmol, 71%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.64 (ddt, *J*=6.6, 2.8, 1.4, 4H), 7.45 – 7.22 (m, 24H), 7.21 – 7.11 (m, 2H), 5.50 (d, *J*=3.9, 1H, H1'), 5.03 (d, *J*=10.7, 1H, Bn), 4.93 – 4.83 (m, 5H, H7/Bn (4x)), 4.72 – 4.63 (m, 2H, Bn (2x)), 4.63 – 4.54 (m, 3H, H1/Bn/H6a), 4.27 (dd, *J*=11.6, 3.3, 1H, H6b), 4.00 – 3.93 (m, 2H, H6a'/H3), 3.94 – 3.82 (m, 4H, H3'/H6b'/H4/H4'), 3.63 (dt, *J*=9.7, 2.3, 1H, H5'), 3.36 (dd, *J*=10.2, 3.9, 1H, H2'), 3.13 (dd, *J*=8.8, 4.0, 1H, H2), 2.79 – 2.73 (m, 1H, H5), 1.06 (s, 9H, TBDPS).

^{13}C NMR (126 MHz, $CDCl_3$) δ 146.7 (carbonate), 138.5, 137.9, 137.8, 137.2, 136.0, 135.8, 133.6, 132.9, 129.9, 129.9, 128.7, 128.5, 128.3, 128.3, 128.2, 128.2, 128.1, 128.1, 127.9, 127.8, 127.7, 127.4, 98.6 (C1'), 83.4 (C3), 81.1 (C7), 80.2 (C3'), 78.0, 77.2 (C2), 75.8 (Bn), 75.4 (Bn), 75.1 (Bn), 74.0, 73.4 (C5'), 72.5 (Bn), 68.6 (C6), 63.6 (C2'), 62.0 (C6'), 34.0 (C5), 29.9 (C1), 27.1 (TBDPS), 19.5 (TBDPS). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{58}H_{66}IN_4O_{10}Si$ 1133.3587, found 1133.3593.

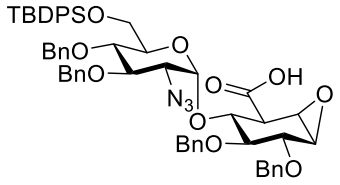
2,3-di-*O*-benzyl-4-*O*-(2-azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-cyclophellitol (31)



Iodide **S4** (0.606 g 0.543 mmol) was dissolved in DCM/MeOH (5.4 ml, 1/1, v/v). NaOMe (4.4M, 0.04 ml, 1.63 mmol) was added and the reaction was left to stir overnight. More NaOMe (4.4M, 0.04 ml, 1.63 mmol) was added and the reaction was stirred for 7 hours. Upon completion the reaction was quenched with NH_4Cl (35 mg, 0.65 mmol). The solvent was removed under reduced pressure and column chromatography (pentane/EtOAc, 85/15 -> 65/35, v/v) afforded the product as an oil (0.492 g, 0.511 mmol, 94%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 – 7.62 (m, 4H), 7.44 – 7.24 (m, 24H), 7.18 – 7.12 (m, 2H), 5.61 (d, *J* = 4.0 Hz, 1H, H1'), 4.99 (d, *J* = 10.9 Hz, 1H, Bn), 4.91 – 4.76 (m, 5H, Bn), 4.69 – 4.61 (m, 2H, Bn), 3.97 – 3.78 (m, 6H, H3'/H6ab/H6ab'), 3.78 – 3.65 (m, 4H), 3.41 – 3.36 (m, 1H, epoxide), 3.24 (dd, *J* = 10.3, 4.0 Hz, 1H, H2'), 3.17 (d, *J* = 3.8 Hz, 1H, epoxide), 2.23 – 2.16 (m, 1H, H5), 1.05 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 138.0, 137.5, 136.0, 135.8, 133.6, 133.2, 129.8, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 128.0, 127.8, 127.7, 127.6, 127.5, 97.9 (C1'), 84.8, 80.5, 80.3 (C3'), 78.3, 75.7 (Bn), 75.2 (Bn), 74.6 (Bn), 72.9 (Bn and CH), 70.4, 63.4(C2'), 62.7, 62.1 (C6/C6'), 56.0 (epoxide), 52.8 (epoxide), 43.2 (C5), 27.0 (TBDPS), 19.4 (TBDPS). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{57}H_{67}N_4O_9Si$ 979.4672, found 979.4669.

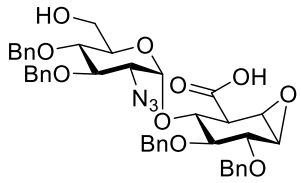
2,3-di-*O*-benzyl-4-*O*-(2-azido-2-deoxy-3,4-di-*O*-benzyl-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl)-glucurono-cyclophellitol (32)



Alcohol **31** (0.492 g, 0.511 mmol) was dissolved in *t*-BuOH/ DCM/H_2O (3.5 ml, 6/4/1, v/v). TEMPO (0.016 g, 0.10 mmol) and BAIB (0.412 g, 1.28 mmol) were added and the reaction was stirred overnight. After TLC analysis (EtOAc/pentane, 1/3 with 0.5% Et_3N, v/v) showed full conversion of the starting material the reaction was diluted with DCM and water. $Na_2S_2O_3$ was added the mixture was shaken and the layers were separated. The water layer was extracted with DCM (4x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was obtained after column chromatography (pentane/EtOAc, 3/17 -> 1/4 v/v, 0.5% AcOH,) as an oil (0.538 g, 0.525 mmol, quant.).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 – 7.63 (m, 4H), 7.43 – 7.25 (m, 24H), 7.21 – 7.16 (m, 2H), 5.38 (d, *J* = 3.7 Hz, 1H, H1'), 4.94 – 4.80 (m, 5H, Bn), 4.76 – 4.62 (m, 3H, Bn), 4.04 (t, *J* = 9.6 Hz, 1H, H4), 4.00 – 3.89 (m, 2H, H3', H6a'), 3.86 – 3.79 (m, 3H, H2/H6b'/H4'), 3.64 (m, 1H, H5'), 3.54 (dd, *J* = 9.6, 8.2 Hz, 1H, H3), 3.29 (dd, *J* = 10.2, 3.8 Hz, 1H, H2'), 3.21 – 3.18 (m, 1H, epoxide), 3.09 (d, *J* = 3.6 Hz, 1H, epoxide), 2.89 (dd, *J* = 9.4, 1.5 Hz, 1H, H5), 1.04 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2 (COOH), 138.7, 138.2, 137.9, 137.4, 136.2, 135.8, 133.6, 133.3, 129.9, 129.8, 128.7, 128.6, 128.6, 128.5, 128.4, 128.2, 128.1, 128.1, 127.9, 127.9, 127.9, 127.8, 127.7, 98.4 (C1'), 83.0, 80.3 (C3'), 79.4, 78.1 (C2/C4'), 75.8 (Bn), 75.2 (Bn), 75.0 (Bn), 73.6 (C4), 73.2 (Bn), 72.5 (C5'), 63.8 (C2'), 62.3 (C6'), 53.9 (epoxide), 53.5 (epoxide), 48.1 (C5), 27.0 (TBDPS), 19.4 (TBDPS). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{57}H_{65}N_4O_{10}Si$ 993.44645 found 993.44650.

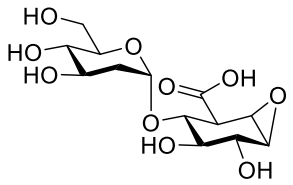
2,3-di-*O*-benzyl-4-*O*-(2-azido-2-deoxy-3,4-di-*O*-benzyl-D-glucopyranosyl)-glucurono-cyclophellitol (33)



Silyl protected **32** (0.525 g, 0.538 mmol) was dissolved in THF (2.7 ml), 3HF·Et_3N (0.44 ml, 2.69 mmol (8.07 mmol HF)) was added and the mixture was stirred for 45 hours. The reaction was poured over water and extracted with DCM (4x). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 35/65 -> 45/55, v/v, 0.5% AcOH) afforded the product as a white solid (0.340 g, 0.461 mmol, 86%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 – 7.23 (m, 20H), 5.41 (d, *J* = 4.0 Hz, 1H, H1'), 4.90 (s, 2H, Bn), 4.87 – 4.79 (m, 3H, Bn), 4.74 (d, *J* = 11.3 Hz, 1H, Bn), 4.63 (dd, *J* = 14.7, 11.2 Hz, 2H, Bn), 4.12 (t, *J* = 9.7 Hz, 1H, H4), 3.93 – 3.85 (m, 2H, H3'/H2), 3.81 (dd, *J* = 11.8, 2.5 Hz, 1H, H6a'), 3.72 (ddd, *J* = 10.1, 4.8, 2.5 Hz, 1H, H5'), 3.64 – 3.58 (m, 2H, H6b'/H3), 3.46 – 3.41 (m, 2H, H4'/epoxide), 3.26 (dd, *J* = 10.3, 3.9 Hz, 1H, H2'), 3.20 (d, *J* = 3.7 Hz, 1H, epoxide), 3.04 (dd, *J* = 9.5, 2.1 Hz, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.8 (C6), 138.8, 137.8, 137.7, 137.4, 128.7, 128.7, 128.6, 128.4, 128.2, 128.2, 128.1, 128.1, 127.5, 127.4, 98.7 (C1'), 83.0 (C3), 80.3 (C3'), 79.6 (C2), 78.4 (C4'), 75.6 (Bn), 75.2 (Bn), 74.9 (Bn), 74.3 (C4), 73.2 (Bn), 72.1 (C5), 63.6 (C2'), 61.9 (C6'), 54.3 (epoxide), 53.7 (epoxide), 48.4 (C5). HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{41}H_{47}N_4O_{10}$ 755.3287 found 755.3284.

4-*O*-(2-deoxy-D-glucopyranosyl)-glucurono-cyclophellitol (34)

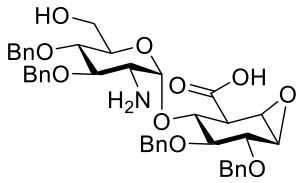


Ammonia (8 ml) was condensed at -70°C. Sodium (31 mg, 1.35 mmol) was added and the solution turned blue. Benzyl protected **33** (50 mg, 0.068 mmol), dissolved in anhydrous THF (2ml). and *t*-BuOH (0.16 ml, 1.69 mmol) was added After stirring at -60°C for 45 minutes the reaction was quenched with NH_4Cl (109 mg, 2.04 mmol) The ammonia was evaporated, water was added and the compound was desalted by size exclusion over HW-40 (1% AcOH in water).Lyophilization afforded the compound as a white solid. (22.7 mg, 0.068 mmol, quant.)

^{1}H NMR (500 MHz, D_2O) δ 5.31 (d, *J* = 3.7 Hz, 1H, H1'), 3.88 (ddd, *J* = 11.9, 9.1, 4.9 Hz, 1H, H3'), 3.84 – 3.76 (m, 4H, H6ab'/H4/H2), 3.67 (dt, *J* = 10.0, 3.1 Hz, 1H, H5'), 3.48 – 3.45 (m, 1H, epoxide), 3.43 (dd, *J*

= 10.2, 8.7 Hz, 1H/H3), 3.35 (dd, *J* = 10.0, 9.2 Hz, 1H, H4'), 3.18 (d, *J* = 3.7 Hz, 1H, epoxide), 2.80 (dd, *J* = 9.8, 1.9 Hz, 1H, H5), 2.19 (ddd, *J* = 13.3, 5.0, 1.3 Hz, 1H, H2a'), 1.64 (ddd, *J* = 13.2, 11.9, 3.9 Hz, 1H, H2'). ^{13}C NMR (126 MHz, D_2O) δ 179.8 (COOH), 97.7 (C1'), 75.7 (C3), 73.7, 71.6 (C5'), 70.4, 70.3 (C4'), 67.6 (C3'), 59.7 (C6'), 55.6 (epoxide), 54.7 (epoxide), 50.4 (C5), 36.4 (C2'). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{13}H_{24}O_{10}$ 354.13947 found 354.13937.

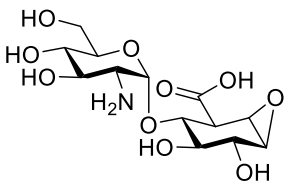
2,3-di-*O*-benzyl-4-*O*-(2-amino-2-deoxy-3,4-di-*O*-benzyl-D-glucopyranosyl)-glucurono-cyclophellitol (35)



Azide **33** (50 mg, 0.068 mmol) was dissolved in MeOH/toluene (2.5 ml, 4/1,v/v). Zn dust (132 mg, 2.02 mmol) and NH_4Cl (144 mg, 2.69 mmol) were added portion wise over the 2 hours. The reaction was stirred for another hour followed by filtration over silica with MeOH/toluene (1/1, v/v). This afforded the poorly soluble product which was analyzed and used without further purification.

^{1}H NMR (400 MHz, MeOD) δ 7.39 – 7.22 (m, 20H), 5.52 (d, *J* = 3.6 Hz, 1H, H1'), 4.79 (d, *J* = 11.3 Hz, 1H, Bn), 4.75 (d, *J* = 11.9 Hz, 1H, Bn), 4.69 (d, *J* = 9.6 Hz, 2H, Bn), 4.54 (d, *J* = 11.3 Hz, 1H, Bn), 4.21 (t, *J* = 9.7 Hz, 1H, H4), 4.00 (d, *J* = 7.1 Hz, 1H, H2), 3.95 – 3.84 (m, 3H, H3'/H6a'/H5'), 3.84 – 3.74 (m, 2H, H3/H6b'), 3.64 (t, *J* = 9.0 Hz, 1H, H4'), 3.50 (dd, *J* = 4.1, 1.6 Hz, 1H, epoxide), 3.28 (d, *J* = 3.7 Hz, 1H,epoxide), 3.23 (dd, *J* = 10.1, 3.6 Hz, 1H, H2'), 3.10 (d, *J* = 9.7 Hz, 1H, H5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{41}H_{46}NO_{10}$ 712.3116 found 712.3112.

4-*O*-(2-amino-2-deoxy-D-glucopyranosyl)-glucurono-cyclophellitol (36)



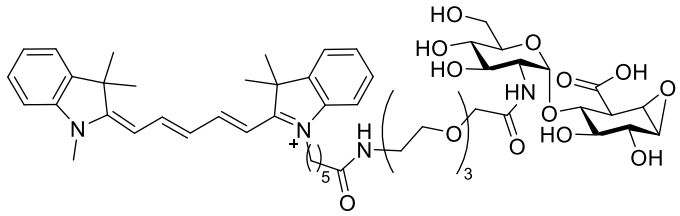
Anhydrous THF (1 ml). and *t*-BuOH (0.32 ml, 3.4 mmol) were added to benzyl protected **35** (crude, 0.068 mmol). The flask was cooled to -70°C and ammonia (5 ml) was condensed directly in the flask. Sodium (63 mg, 2.72 mmol) was added and the solution turned blue. After stirring at -60°C for 45 minutes the reaction was quenched with NH_4Cl (0.18 g, 3.4 mmol) The ammonia was evaporated, water was added and the compound was desalted by size exclusion over HW-40 (1% AcOH in water).Lyophilization afforded the compound as a white solid (12.1 mg, 0.035 mmol, 51% over 2 steps).

^{1}H NMR (500 MHz, D_2O) δ 5.49 (d, *J* = 3.8 Hz, 1H, H1'), 3.93 – 3.85 (m, 3H, H3'/H4/H2), 3.83 (d, *J* = 2.9 Hz, 2H, H6ab'), 3.75 (dt, *J* = 10.1, 2.9 Hz, 1H, H5'), 3.58 – 3.50 (m, 3H, H3/H4'/epoxide), 3.30 (dd, *J* = 10.7, 3.8 Hz, 1H, H2'), 3.21 (d, *J* = 3.7 Hz, 1H, epoxide), 2.90 (dd, *J* = 9.8, 1.8 Hz, 1H, H5). ^{13}C NMR (126 MHz, D_2O) δ 177.7 (C6), 96.1 (C1'), 76.4 (C3), 75.2, 72.1 (C5'), 71.4, 69.7, 69.2 (C4'), 59.7 (C6'), 56.2 (epoxide), 55.4 (epoxide), 54.4 (C2'), 50.9 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{13}H_{22}NO_{10}$ 352.1235, found 352.1238.

General procedure A| Amide coupling reporter tag to warhead PFP method

The appropriate carboxylic acid (50 µmol) was dissolved in DMF (1 ml), 2,3,4,5,6-pentafluorophenol (46 mg, 0.26 mmol), Et_3N (20 µl, 0.26 mmol) and DIC (7.8 µl, 50 µmol) were added and the mixture was stirred for 90 minutes. Part of the stock solution (1.5 eq acid compared to amine) was added to the amine and stirred overnight. LC-MS indicated full conversion and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM AcOH in H_2O). The fractions were concentrated under reduced pressure, coevaporated with water, diluted with water and lyophilized to yield the product.

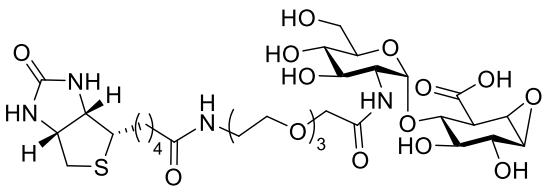
2'-N Cy5 conjugated probe 37



Amine **36** (11.6 mg, 33 µmol) was reacted with Peg Cy5 (Chapter 2) according to general procedure A. This afforded the product as a blue solid (2.35 mg, 2.25 µmol, 7%).

^{1}H NMR (600 MHz, MeOD) δ 8.24 (t, *J* = 13.1 Hz, 2H), 7.49 (dt, *J* = 7.6, 1.5 Hz, 2H), 7.44 – 7.39 (m, 2H), 7.32 – 7.29 (m, 2H), 7.26 (td, *J* = 7.4, 0.9 Hz, 2H), 6.65 (t, *J* = 12.4 Hz, 1H), 6.30 (dd, *J* = 13.7, 3.5 Hz, 2H), 5.23 (d, *J* = 3.8 Hz, 1H), 4.11 (t, *J* = 7.5 Hz, 2H), 4.05 (d, *J* = 15.2 Hz, 1H), 4.00 (d, *J* = 15.2 Hz, 1H), 3.94 – 3.87 (m, 3H), 3.84 – 3.80 (m, 1H), 3.75 – 3.62 (m, 14H), 3.55 (t, *J* = 5.5 Hz, 2H), 3.44 – 3.35 (m, 4H), 3.30 – 3.26 (m, 1H), 2.99 (d, *J* = 3.5 Hz, 1H), 2.76 (dd, *J* = 9.1, 2.2 Hz, 1H), 2.25 (t, *J* = 7.4 Hz, 2H), 1.86 – 1.80 (m, 2H), 1.75 – 1.66 (m, 14H), 1.51 – 1.44 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 178.2, 176.1, 175.3, 174.6, 173.0, 155.5, 144.3, 143.6, 142.7, 142.5, 129.8, 129.7, 126.7, 126.2, 123.4, 123.3, 112.1, 111.8, 104.5, 104.4, 99.4, 77.9, 77.6, 74.1, 73.3, 73.1, 72.6, 71.7, 71.5, 71.4, 71.3, 71.1, 70.6, 63.0, 57.0, 56.6, 55.4, 52.9, 50.5, 50.5, 44.8, 40.3, 36.7, 31.6, 28.2, 28.0, 27.8, 27.4, 26.6. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{53}H_{73}N_4O_{15}$ 1005.5058, found 1005.5067.

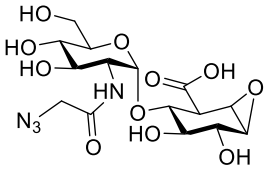
2'-N biotin conjugated probe 38



Amine **36** (14.2 mg, 40.4 µmol) was reacted with Peg biotin (Chapter 2) according to general procedure A. This afforded the product as a white solid (3.00 mg, 3.90 µmol, 10%).

^{1}H NMR (500 MHz, MeOD) δ 5.20 (s, 1H), 4.52 (dd, *J* = 7.9, 4.8 Hz, 1H), 4.33 (dd, *J* = 7.9, 4.4 Hz, 1H), 4.12 – 3.99 (m, 2H), 3.95 – 3.84 (m, 3H), 3.84 – 3.63 (m, 12H), 3.58 (t, *J* = 5.4 Hz, 2H), 3.46 – 3.37 (m, 4H), 3.23 (dt, *J* = 9.8, 5.3 Hz, 1H), 3.04 (d, *J* = 3.4 Hz, 1H), 2.94 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.81 – 2.75 (m, 1H), 2.71 (d, *J* = 12.8 Hz, 1H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.80 – 1.53 (m, 4H), 1.49 – 1.40 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 176.5, 173.1, 99.4, 77.7, 77.7, 74.1, 73.0, 72.9, 72.3, 71.6, 71.3, 71.2, 71.1, 70.6, 63.4, 61.6, 57.1, 57.0, 56.6, 55.3, 49.5, 49.3, 49.2, 49.0, 48.8, 48.7, 48.5, 41.0, 40.3, 36.7, 29.7, 29.4, 26.8, 23.5. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{31}H_{51}N_4O_{16}S$ 767.3009, found 767.3015.

4-*O*-(2-*N*-azidoacetyl-2-deoxy-D-glucopyranosyl)-glucurono-cyclophellitol (39)

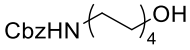


Amine **36** (8.4 µmol) was reacted with N_3AcOH according to general procedure A. The product was purified over HW-40 eluting with AcOH in water (1%, v/v). The fractions were concentrated under reduced pressure, coevaporated with water, diluted with water and lyophilized to yield the product as a white solid (0.45 mg, 1.04 µmol, 12%).

^{1}H NMR (600 MHz, D_2O) δ 5.17 (d, *J* = 3.8 Hz, 1H, H1'), 4.03 (d, *J* = 1.1 Hz, 2H,CH_2N_3), 3.89 (dd, *J* = 10.7, 3.8 Hz, 1H, H2'), 3.79 – 3.71 (m, 5H, H4/H6'ab/H2/H3'), 3.69 (dt, *J* = 10.1, 3.1 Hz, 1H, H5'), 3.50 – 3.45 (m, 3H, H3/H4'/epoxide), 3.19 – 3.13 (m, 1H, epoxide/Et_3N), 2.87 (dd, *J* = 9.5, 1.9 Hz, 1H, H5). ^{13}C NMR (151 MHz, D_2O) δ 178.3, 171.6, 98.5 (C1'), 77.0, 76.0, 72.9 (C5'), 72.2, 71.7, 70.4, 60.9 (C6'), 57.0 (epoxide), 56.4 (epoxide), 54.9 (C2'), 52.7 (CH_2N_3), 51.5 (C5). HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{15}H_{23}N_4O_{11}$ 435.1358, found 435.1358.

Activity-based probes with 4' linker

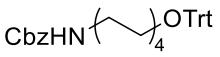
8-Benzyloxycarbonylamino-octan-1-ol (43)



8-Amino-octan-1-ol (**42**) (6.65 g, 45.8 mmol) was dissolved in acetone/water (700 ml, 2/1, v/v). $NaHCO_3$ (11.53 g, 137.3 mmol) was added followed by dropwise

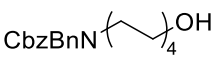
addition of carboxybenzyl chloride (9.78 ml, 68.7 mmol). After TLC showed full conversion the acetone was removed in vacuo. The remaining water layer was extracted with EtOAc (3x). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The product was obtained by column chromatography (EtOAc/pentane, 3/7, v/v) as an oil. (11.26 g, 40.30 mmol, 88%) Spectral data is in accordance with literature.[52]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.28 (m, 5H), 5.09 (s, 2H), 3.63 (t, *J* = 6.6 Hz, 2H), 3.18 (q, *J* = 6.7 Hz, 2H), 1.62 – 1.40 (m, 5H), 1.41 – 1.23 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.5, 136.8, 128.6, 128.3, 128.2, 66.7, 63.1, 41.2, 32.8, 30.1, 29.4, 29.3, 26.7, 25.8. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{16}H_{25}NO_3Na$ 302.1727, found 302.1734.

8-Benzyloxycarbonylamino-1-trityloxy octane(44)



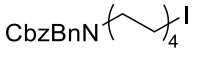
Alcohol **43** (9.8 g, 35 mmol) was dissolved in DMF (58 ml, 0.6 M). Trityl chloride (13.7 g, 175 mmol) and Et_3N (24.5 ml, 175 mmol) were added and the reaction was stirred for 24h. Trityl chloride (4.4 g, 17.5 mmol) and Et_3N (5.0 ml, 36 mmol) were added and the reaction was stirred for 48h. Upon completion the mixture was diluted with H_2O and extracted with Et_2O (3x). The combine organic layers were washed with H_2O (5x) and brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The product was used without further purification.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.41 (m, 6H), 7.37 – 7.25 (m, 11H), 7.25 – 7.19 (m, 3H), 5.09 (s, 2H), 4.71 (s, 1H), 3.17 (q, *J* = 6.7 Hz, 2H), 3.03 (t, *J* = 6.6 Hz, 2H), 1.65 – 1.55 (m, 2H), 1.51 – 1.40 (m, 2H), 1.39 – 1.19 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.5, 144.6, 136.8, 128.8, 128.6, 128.3, 128.2, 127.8, 126.9, 86.4, 66.7, 63.7, 41.2, 30.1, 30.1, 29.5, 29.3, 26.8, 26.3. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{35}H_{39}NO_3Na$ 544.2822, found 544.2830.

8-*N*-benzyl(benzyloxycarbonyl)-1-trityloxy octane(46)



Carbamate **44** (18.3 g, 35 mmol) was dissolved in DMF (96 ml, 0.36 M) and the solution was cooled to 0°C. NaH (60 wt%, 2.9 g, 74 mmol) was added and the reaction was stirred at 0°C for 10 minutes. Benzyl bromide (5.9 ml, 49 mmol) and TBAI (1.3 g, 3.5 mmol) were added and the solution was stirred overnight at room temperature. The reaction mixture was cooled down to 0°C and quenched with H_2O. It was then further diluted with H_2O and extracted with Et_3O (3x). The combined organic layers were washed with H_2O (5x) and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (PE/Et_2O, 98/2 -> 4/1, v/v) yielded the product (16.3 g, 26.7 mmol, 76%, over 2 steps).
Trityl ether **45** (16.3 g, 26 mmol) was dissolved in DCM/MeOH (104 ml, 1/1, 0.25 M). TFA (3% in H_2O, 7.5 ml, 3.0 mmol) was added and the reaction was stirred for 27 hours. More TFA (0.23 ml, 3.0 mmol) was added and the reaction was stirred for another 24 hours. TLC showed complete conversion and the reaction was quenched with $NaHCO_3$, concentrated in vacuo, dissolved in EtOAc and extracted with H_2O (3x) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 9/1 -> 7/3, v/v) afforded the product (8.1 g, 21.9 mmol, 84%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.12 (m, 10H), 5.16 (d, *J* = 13.1 Hz, 2H), 4.48 (d, 2H), 3.57 (t, *J* = 6.7 Hz, 2H), 3.29 – 3.14 (m, 2H), 2.32 (s, 1H), 1.57 – 1.43 (m, 4H), 1.38 – 1.14 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.8, 156.2, 137.9, 136.8, 136.7, 128.5, 128.4, 127.9, 127.8, 127.3, 67.1, 62.7, 50.4, 50.1, 47.2, 46.2, 32.6, 29.2, 29.2, 28.0, 27.6, 26.6, 25.6. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{23}H_{31}NO_3Na$ 392.2196, found 392.2200.

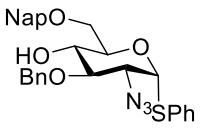
8-*N*-benzyl(benzyloxycarbonyl)-1-iodo-octane(47)



Alcohol **46** (8.1 g, 21.3 mmol) was dissolved in DCM (250 ml, 0.085 M) and cooled to 0°C. PPh_3 (6.7 g, 25.6 mmol), imidazole (1.7 g, 25.6 mmol) and I_2 (6.5 g, 26 mmol) were added to the solution. After stirring for 1 hour at 0 °C the reaction was warmed to rt and stirred for an additional 4 hours. The reaction was quenched with $NaHCO_3$ (aq. sat.) and the water layer was extracted three times with DCM. The combined organic layers were washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.), $Na_2S_2O_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtrated and concentrated

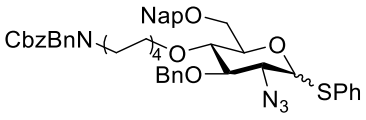
in vacuo. Column chromatography (PE/EtOAc, 24/1 -> 4/1, v/v) yielded the product (10.4 g, 21.6 mmol, 97%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.13 (m, 10H), 5.17 (d, *J* = 11.8 Hz, 2H), 4.49 (d, *J* = 8.0 Hz, 2H), 3.30 – 3.11 (m, 4H), 1.83 – 1.70 (m, 2H), 1.58 – 1.41 (m, 2H), 1.41 – 1.15 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.8, 156.2, 138.0, 128.6, 128.5, 128.0, 127.9, 127.3, 67.2, 50.5, 50.2, 47.2, 46.3, 33.5, 30.4, 29.1, 28.4, 28.1, 27.7, 26.7, 7.4. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{23}H_{30}INO_2Na$ 502.1213, found 502.1216.

Phenyl 2-azido-2-deoxy-3-*O*-benzyl-6-*O*-(2-Naphthylmethyl)-1-thio-α-D-glucopyranoside (41)



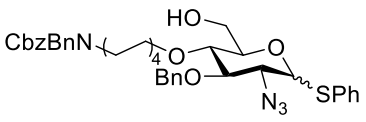
Phenyl 2-azido-2-deoxy-3-*O*-benzyl-4,6-benzylidene-1-thio-α-D-glucopyranoside[42] (**40**, 1.70 g, 3.6 mmol) was suspended in MeOH (30 mL). DCE (5 ml) was added to obtain a clear solution. The solution was heated to 50°C and camphorsulfonic acid (0.42 g, 1.8 mmol) was added. When TLC analysis showed full conversion of the starting material the reaction was cooled down to 0°C and quenched with Et_3N. Solvents were removed in vacuo and the crude residue was dissolved in EtOAc, washed with HCl (aq. 1M), $NaHCO_3$ (aq. sat.), brine and dried over $MgSO_4$.
The solvent was evaporated and the crude diol was dissolved in anhydrous MeCN (15 ml). 2-aminoethyl diphenyl borinate (0.081 g, 0.36 mmol), 2-bromomethyl-naphtalene (1.2 g, 5.4 mmol), KI (0.60 g, 3.6 mmol) and K_2CO_3 (0.55 g, 4.0 mmol) were added. The mixture was heated to 60°C and stirred overnight. The reaction mixture was transferred to a separatory funnel containing EtOAc and H_2O, layers were separated and the water layer was re-extracted three times with EtOAc. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. Column chromatography (pentane/EtOAc, 9/1 -> 3/1, v/v) afforded the product (1.77 g, 3.35 mmol, 93%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.75 (m, 3H), 7.73 – 7.69 (m, 1H), 7.52 – 7.26 (m, 10H), 7.24 – 7.17 (m, 3H), 5.54 (d, *J* = 5.4 Hz, 1H, H1), 4.91 (d, *J* = 11.1 Hz, 1H), 4.80 (d, *J* = 11.1 Hz, 1H), 4.69 (d, *J* = 12.1 Hz, 1H), 4.61 (d, *J* = 12.1 Hz, 1H), 4.34 (dt, *J* = 9.6, 4.2 Hz, 1H, H5), 3.85 (dd, *J* = 10.0, 5.4 Hz, 1H, H2), 3.78 – 3.60 (m, 4H, C6ab/H4/H3), 2.66 (d, *J* = 3.1 Hz, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.9, 135.2, 133.4, 133.2, 133.0, 132.2, 129.1, 128.7, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 126.6, 126.2, 126.0, 125.6, 87.3 (C1), 81.4 (C3), 75.4, 73.7 (Bn/Nap), 72.1 (C4), 71.2 (C5), 69.6 (C6), 63.6 (C2). HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{30}H_{29}N_3O_4SNa$ 550.1771, found 550.1771.

Phenyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-6-*O*-(2-Naphthylmethyl)-1-thio-D-glucopyranoside (48)



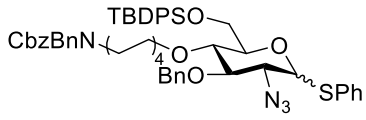
Alcohol **41** (α/β mixture) (2.3 g, 6.2 mmol) and iodide **47** (7.7 g, 16 mmol) were dissolved in dry DMF (13 ml, 0.5 M) and the solution was cooled to 0°C. NaH (60% dispersion in mineral oil, 0.43 g, 10.7 mmol) was added and the reaction was stirred for 18h at rt. The reaction mixture was cooled to 0°C and quenched with water. The water layer was extracted with Et_2O (3x), the combined organic layers were washed with water (5x) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/Et_2O, 49/1 -> 4/1, v/v) yielded the product (4.4 g, 5.4 mmol, 88%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 7.9 Hz, 3H), 7.74 (s, 1H), 7.54 – 7.12 (m, 23H), 5.60 (d, *J* = 5.4 Hz, 1H), 5.17 (d, *J* = 10.2 Hz, 2H), 4.88 (d, *J* = 10.5 Hz, 1H), 4.84 (d, *J* = 10.5 Hz, 1H), 4.78 (d, *J* = 12.1 Hz, 1H), 4.60 (d, *J* = 12.2 Hz, 1H), 4.48 (d, *J* = 9.4 Hz, 2H), 4.33 – 4.27 (m, 1H), 3.91 (dd, *J* = 10.3, 5.4 Hz, 1H), 3.79 (dd, *J* = 10.8, 3.7 Hz, 1H), 3.76 – 3.64 (m, 3H), 3.54 (t, *J* = 9.4 Hz, 1H), 3.39 (q, *J* = 7.0 Hz, 1H), 3.27 – 3.12 (m, 2H), 1.52 – 1.32 (m, 4H), 1.20 – 1.00 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.2, 138.0, 137.8, 135.3, 133.6, 133.2, 133.0, 132.1, 129.1, 128.6, 128.5, 128.5, 128.2, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 126.7, 126.2, 126.0, 126.0, 87.3, 81.8, 78.4, 75.7, 75.7, 73.7, 73.6, 73.6, 73.4, 72.0, 68.3, 67.2, 64.0, 50.5, 50.2, 47.3, 46.3, 30.4, 29.8, 29.5, 29.3, 28.1, 27.7, 26.8, 26.1. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{53}H_{58}N_4O_6SNa$ 901.3969, found 901.3969.

Phenyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-1-thio-D-glucopyranoside (50)



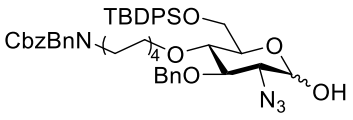
Naphthyl ether **48** (4.8 g, 5.5 mmol) was dissolved in DCM/MeOH (38 ml, 0.15 M, 4/1, v/v) and flushed with N_2 for 15 min. DDQ (3.7 g, 16 mmol) was added and the reaction was stirred for 2.5 hours in the dark. The reaction mixture was concentrated in vacuo and the residue was dissolved in EtOAc. The organic layer was washed with $NaHCO_3$ (aq. sat. 3x) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/Et_2O, 47/3 -> 2/3. v/v) yielded the product (2.6 g, 3.9 mmol, 72%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (dd, *J* = 7.8, 1.7 Hz, 2H), 7.43 – 7.22 (m, 17H), 7.17 (d, *J* = 6.6 Hz, 1H), 5.54 (d, *J* = 5.3 Hz, 1H), 5.17 (d, *J* = 13.2 Hz, 2H), 4.88 (s, 2H), 4.49 (d, *J* = 8.3 Hz, 2H), 4.18 (dt, *J* = 9.9, 3.2 Hz, 1H), 3.84 (dd, *J* = 10.3, 5.3 Hz, 1H), 3.82 – 3.73 (m, 4H), 3.62 – 3.54 (m, 1H), 3.43 (t, *J* = 9.3 Hz, 1H), 3.31 – 3.15 (m, 2H), 1.60 – 1.43 (m, 4H), 1.32 – 1.14 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.8, 132.6, 129.3, 128.6, 128.6, 128.6, 128.2, 128.1, 128.0, 127.9, 87.2, 81.6, 78.5, 75.8, 73.6, 72.7, 67.3, 64.1, 61.6, 30.5, 29.5, 29.3, 26.8, 26.2. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{42}H_{50}N_4O_6SNa$ 761.3343, found 761.3365.

Phenyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-6-*O*-tert-butyldiphenylsilyl-1-thio-α-D-glucopyranoside (51)



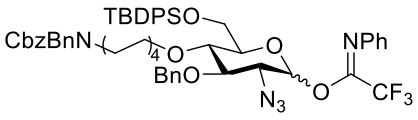
Alcohol **50** (3.4 g, 4.5 mmol) was dissolved in DMF (22.8 ml, 0.2 M). TBDPSCl (2.5 g, 9.1 mmol) and imidazole (1.6 g, 22.8 mmol) were added. After stirring for 17h TLC showed complete conversion and the reaction was quenched with H_2O. The reaction mixture was then diluted further with H_2O and extracted with Et_2O (5x). The combined organic layers were washed with H_2O (5x) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (Pe/Et_2O, 47/3, v/v) yielded the product (4.3 g, 4.4 mmol, 95% contaminated with excess silyl reagent).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.64 (m, 6H), 7.48 – 7.19 (m, 23H), 7.19 – 7.13 (m, 1H), 5.59 (d, *J* = 5.4 Hz, 1H), 5.16 (d, *J* = 11.2 Hz, 2H), 4.88 (s, 2H), 4.48 (d, *J* = 6.8 Hz, 2H), 4.15 (d, *J* = 9.6 Hz, 1H), 3.94 (dd, *J* = 11.5, 3.1 Hz, 1H), 3.91 – 3.78 (m, 3H), 3.74 (t, *J* = 9.6 Hz, 1H), 3.68 – 3.60 (m, 2H), 3.27 – 3.14 (m, 2H), 1.57 – 1.44 (m, 4H), 1.30 – 1.12 (m, 8H), 1.03 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.8, 136.0, 135.6, 134.9, 134.3, 133.7, 133.1, 131.5, 129.8, 129.1, 128.6, 128.5, 128.2, 127.8, 127.7, 127.4, 87.2, 81.9, 78.2, 76.0, 73.6, 73.2, 67.3, 64.2, 62.3, 50.6, 50.2, 47.4, 46.4, 30.6, 29.7, 29.5, 26.9, 26.3, 19.5. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{58}H_{68}N_4O_6SSiNa$ 999.4521, found 999.4539.

2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-6-*O*-tert-butyldiphenylsilyl-D-glucopyranose (52)



Thioglycoside **51** (6.2 g, 6.3 mmol) was dissolved in acetone/H_2O/DCM (49.5 ml, 0.13 M, 9/1/1, v/v). The reaction mixture was flushed with N_2. NIS (3.1 g, 13.5 mmol) was added and the reaction was stirred in the dark for 7 hours. The reaction was quenched with solid $Na_2S_2O_3$. The reaction mixture was concentrated in vacuo and dissolved in EtOAc. The organic layer was washed with $Na_2S_2O_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/Et_2O, 20/1 -> 4/1, v/v) yielded the product. (4.4 g, 5.0 mmol, 79%, α/β = 1/5).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.63 (m, 10H), 7.46 – 7.12 (m, 54H), 5.25 (t, *J* = 3.2 Hz, 1H), 5.17 (d, *J* = 11.4 Hz, 5H), 4.88 – 4.77 (m, 5H), 4.52 – 4.41 (m, 6H), 3.97 – 3.75 (m, 9H), 3.67 – 3.48 (m, 5H), 3.41 – 3.13 (m, 12H), 3.00 (s, 1H), 1.54 – 1.41 (m, 9H), 1.27 – 1.11 (m, 21H), 1.08 – 1.00 (m, 23H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.0, 136.1, 135.7, 129.8, 128.6, 128.3, 128.0, 127.8, 127.7, 96.1, 92.1, 83.1, 80.1, 78.2, 77.6, 76.3, 75.7, 73.4, 72.2, 67.6, 67.3, 64.2, 62.8, 62.6, 30.5, 29.6, 29.4, 27.0, 26.9, 26.2, 19.4. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{52}H_{64}N_4O_7SiNa$ 907.4436, found 907.4436.

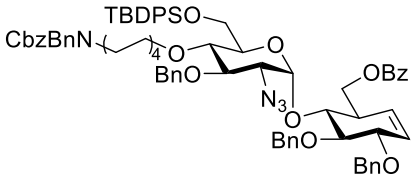
2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-6-*O*-tert-butyldiphenylsilyl-D-glucopyranosyl N-phenyltrifluoroacetimidate (49)



Lactol **52** (3.8 g, 4.3 mmol) was dissolved in DCM (3.5 ml, 0.2 M). 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (1.0 ml, 6.4 mmol) and Cs_2CO_3 (2.1 g, 6.4 mmol) were added. And the mixture was stirred for 6.5 hours. Upon completion the suspension was filtrated over celite and the filtrate was concentrated under reduced pressure. Column chromatography (PE/Et_2O, 20/1 -> 9/10, v/v) yielded the product (4.2 g, 4.0 mmol, 94%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 – 7.64 (m, 4H), 7.46 – 7.02 (m, 26H), 6.80 (d, *J* = 7.7 Hz, 1H), 6.72 (d, *J* = 7.4 Hz, 1H), 5.17 (d, *J* = 10.6 Hz, 2H), 4.94 – 4.79 (m, 2H), 4.49 (d, *J* = 7.4 Hz, 2H), 3.98 – 3.78 (m, 3H), 3.78 – 3.53 (m, 3H), 3.31 – 3.14 (m, 2H), 1.52 (s, 4H), 1.21 (d, *J* = 16.1 Hz, 8H), 1.05 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.0, 137.8, 135.9, 135.6, 133.7, 133.6, 132.9, 129.9, 129.8, 129.4, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.4, 127.4, 127.3, 126.3, 124.5, 124.4, 120.6, 119.4, 82.9, 80.3, 77.6, 76.6, 75.9, 74.7, 73.7, 73.5, 67.2, 65.4, 63.0, 62.0, 50.7, 50.6, 50.2, 47.3, 46.4, 30.5, 30.5, 29.7, 29.6, 29.4, 28.2, 27.8, 26.9, 26.9, 26.3, 19.5.

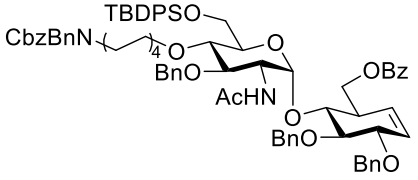
2,3-di-*O*-benzyl-4-*O*-(2-Azido-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-6-*O*-benzoyl-cyclophellitol alkene (53)



Donor **49** (1.3 g, 1.3 mmol) and acceptor **9** (0.39 g, 0.9 mmol) were co-evaporated with toluene (3x) and dissolved in dry DCM (10.6 ml, 0.068 M). 3Å molecular sieves were added and the mixture was stirred for 1 hour. The mixture was cooled to -78°C and TfOH (0.1 M in DCM, 2.8 ml, 0.28 mmol) was added. The reaction mixture was slowly warmed to -30°C within 1.5 hours and stirred for an additional hour at this temperature. The reaction was quenched with Et_3N at -30°C and diluted with DCM. The organic layer was washed with water and the water layer was extracted with DCM (2x). The combined organic layers were washed with brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (Pentane/EtOAc, 20/1 -> 3/1, v/v) yielded the product (1.2 g, 0.88 mmol, 97%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 7.3 Hz, 2H), 7.60 – 7.52 (m, 4H), 7.44 – 7.13 (m, 34H), 5.80 (dt, *J* = 10.2, 2.1 Hz, 1H), 5.68 (d, *J* = 3.9 Hz, 1H), 5.63 – 5.57 (m, 1H), 5.17 (d, *J* = 10.3 Hz, 2H), 5.07 (d, *J* = 10.7 Hz, 1H), 4.98 (d, *J* = 10.7 Hz, 1H), 4.88 (d, *J* = 10.5 Hz, 1H), 4.83 (d, *J* = 10.6 Hz, 1H), 4.71 (d, *J* = 11.5 Hz, 1H), 4.64 (d, *J* = 11.5 Hz, 1H), 4.53 – 4.44 (m, 3H), 4.32 – 4.24 (m, 2H), 4.02 – 3.89 (m, 3H), 3.83 – 3.73 (m, 2H), 3.71 – 3.54 (m, 5H), 3.29 – 3.14 (m, 3H), 2.82 – 2.75 (m, 1H), 1.55 – 1.42 (m, 4H), 1.25 – 1.11 (m, 8H), 0.97 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2, 138.9, 138.2, 138.1, 135.9, 135.6, 133.7, 133.3, 133.0, 129.8, 129.6, 129.6, 129.5, 128.6, 128.6, 128.5, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 98.1, 84.4, 81.0, 80.0, 77.9, 75.6, 74.8, 74.2, 73.3, 72.9, 71.8, 67.2, 64.4, 63.4, 62.1, 50.5, 43.2, 30.5, 29.6, 29.4, 27.0, 26.9, 26.2, 19.4. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{80}H_{90}N_4O_{11}SiNa$ 1333.6268, found 1333.6309.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-6-*O*-benzoyl-cyclophellitol alkene (55)

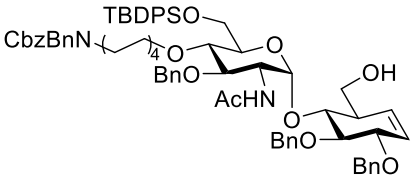


Azide **53** (1.2 g, 0.88 mmol) was dissolved in THF/H_2O (10 ml, 0.085 M, 17/3, v/v). PPh_3 (0.58 g, 2.2 mmol) and pyridine (0.080 ml, 1.0 mmol) were added. The reaction mixture was heated to 50°C and stirred for 3 hours. The reaction mixture was concentrated in vacuo. The residue was dissolved in DCM, dried with $MgSO_4$, and concentrated in vacuo.

The crude product was co-evaporated with toluene and dissolved in DCM (8.8 ml, 0.1 M). Acetic anhydride (1.40 ml, 13.4 mmol) and pyridine (1.2 ml, 13.4 mmol) were added and the reaction mixture was stirred for 16.5 hours. The mixture was cooled to 0°C and quenched with water. The organic layer was washed with $CuSO_4$ (aq. sat.), $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtrated and

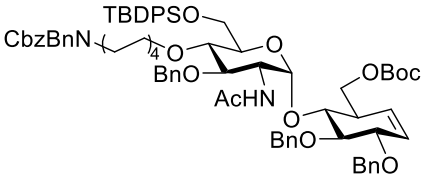
concentrated in vacuo. Column chromatography (PE/EtOAc, 9/1 -> 3/2, v/v) yielded the product (1.1 g, 0.86 mmol, 98%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.91 (m, 2H), 7.74 – 7.64 (m, 4H), 7.48 – 7.42 (m, 1H), 7.39 – 7.19 (m, 33H), 6.64 (d, *J* = 9.6 Hz, 1H), 5.83 (dt, *J* = 10.3, 2.5 Hz, 1H), 5.68 (dt, *J* = 10.3, 2.0 Hz, 1H), 5.18 (d, *J* = 12.6 Hz, 2H), 5.01 – 4.90 (m, 2H), 4.82 (d, *J* = 11.3 Hz, 1H), 4.74 – 4.44 (m, 8H), 4.38 (td, *J* = 9.6, 3.4 Hz, 1H), 4.23 – 4.17 (m, 1H), 3.97 – 3.78 (m, 6H), 3.78 – 3.58 (m, 4H), 3.23 (dt, *J* = 32.6, 7.5 Hz, 2H), 2.68 (t, *J* = 6.2 Hz, 1H), 1.58 – 1.44 (m, 9H), 1.29 – 1.13 (m, 8H), 1.05 (s, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 165.8, 156.6, 155.9, 138.5, 137.8, 137.6, 137.4, 136.7, 135.6, 135.4, 133.5, 132.9, 132.8, 129.7, 129.5, 129.3, 129.2, 128.4, 128.4, 128.3, 128.1, 128.0, 127.8, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 127.1, 126.6, 100.1, 81.4, 81.2, 78.7, 77.5, 76.9, 74.9, 74.6, 73.4, 72.9, 71.2, 66.9, 63.8, 62.3, 53.0, 50.3, 50.0, 47.1, 46.1, 43.7, 30.3, 29.3, 29.1, 27.9, 27.6, 26.7, 26.6, 26.0, 22.6, 19.1. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{82}H_{94}N_2O_{12}SiNa$ 1349.6468, found 1349.6487.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-cyclophellitol alkene (56)



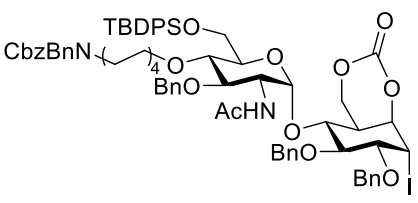
Benzoyl ester **55** (1.1 g, 0.86 mmol) was dissolved in MeOH/DCM (21 ml, 0.04 M, 9.5/1, v/v), NaOMe (4.37 M in MeOH, 0.12 ml, 0.57 mmol) was added and the mixture was stirred for 21.5 hours. NH_4Cl was added and the reaction was concentrated in vacuo. The residue was dissolved in in EtOAc, washed with water and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 4/1 -> 3/2, v/v) yielded the product (1.0 g, 0.83 mmol, 97%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.66 (m, 4H), 7.43 – 7.13 (m, 31H), 6.70 (d, *J* = 9.7 Hz, 1H), 5.88 – 5.81 (m, 1H), 5.66 – 5.60 (m, 1H), 5.17 (d, *J* = 10.8 Hz, 2H), 4.96 – 4.89 (m, 2H), 4.71 (dd, *J* = 11.2, 5.7 Hz, 2H), 4.65 (d, *J* = 11.6 Hz, 1H), 4.57 (d, *J* = 11.6 Hz, 1H), 4.54 – 4.46 (m, 3H), 4.34 – 4.25 (m, 1H), 4.21 – 4.16 (m, 1H), 3.96 – 3.75 (m, 7H), 3.67 – 3.51 (m, 4H), 3.31 – 3.14 (m, 2H), 2.42 – 2.34 (m, 1H), 1.55 – 1.46 (m, 4H), 1.20 (d, *J* = 20.5 Hz, 8H), 1.05 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 138.6, 138.0, 137.9, 137.5, 135.9, 135.8, 135.6, 133.7, 133.1, 129.7, 129.6, 129.5, 128.6, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 127.9, 127.9, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2, 126.6, 100.1, 81.4, 78.9, 77.9, 77.7, 74.9, 74.8, 73.6, 73.1, 71.4, 67.1, 62.7, 62.3, 60.4, 53.1, 50.5, 50.1, 47.2, 46.2, 46.1, 30.4, 29.5, 29.3, 28.1, 27.7, 26.8, 26.8, 26.2, 22.7, 19.3. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{75}H_{90}N_2O_{11}SiNa$ 1245.6206, found 1245.6245.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-6-*O*-tert-butyloxycarbonyl-cyclophellitol alkene (S5)



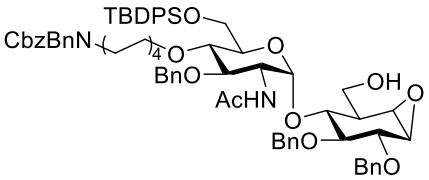
Alcohol **56** (0.53 g, 0.43 mmol) was co-evaporated with toluene (3x) and dissolved in THF (3.2 ml, 0.14 M). Boc_2O (0.38 g, 1.8 mmol) and DMAP (0.16 mM in THF, 0.32 ml, 0.05 mmol) were added. The reaction was stirred for 1 hour and quenched with H_2O. The reaction mixture was diluted with Et_2O and washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 9/1 -> 7/3, v/v) yielded the product (0.47 g, 0.35 mmol, 82%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 – 7.65 (m, 4H), 7.43 – 7.14 (m, 31H), 6.73 (d, *J* = 9.7 Hz, 1H), 5.87 – 5.78 (m, 1H), 5.67 – 5.57 (m, 1H), 5.18 (d, *J* = 7.0 Hz, 2H), 4.94 (d, *J* = 11.1 Hz, 1H), 4.86 (d, *J* = 3.5 Hz, 1H), 4.78 (d, *J* = 11.3 Hz, 1H), 4.73 – 4.44 (m, 7H), 4.42 – 4.17 (m, 4H), 3.96 – 3.56 (m, 10H), 3.31 – 3.06 (m, 2H), 2.52 (s, 1H), 1.58 – 1.37 (m, 16H), 1.28 – 1.12 (m, 8H), 1.05 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 153.4, 138.8, 138.0, 137.9, 137.6, 135.9, 135.7, 135.6, 133.9, 133.1, 129.6, 128.6, 128.6, 128.6, 128.5, 128.3, 128.3, 128.2, 128.0, 128.0, 127.9, 127.7, 127.6, 127.5, 127.3, 126.8, 100.5, 82.1, 81.7, 79.3, 77.7, 77.6, 77.5, 77.2, 76.8, 75.0, 74.9, 73.5, 73.2, 71.5, 67.2, 66.1, 62.5, 53.3, 50.5, 50.2, 47.3, 46.3, 44.0, 30.5, 29.6, 29.4, 27.8, 26.9, 26.8, 26.2, 22.7, 19.4. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{80}H_{99}N_2O_{13}Si$ 1323.6911, found 1323.6910.

1-iodo-2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-6,7-*O*-carbonyl-cyclophellitol alkane (S6)



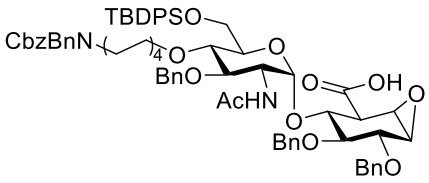
Alkene **S5** (0.47 g, 0.35 mmol) was dissolved in DCM/AcOH (1.8 ml, 0.2 M, 2/1, v/v). NIS (0.16 g, 0.71 mmol) was added and the reaction was stirred for 20 hours. Additional NIS (0.039 g, 0.18 mmol) was added and the reaction was stirred for 2 hours. The solution was diluted with Et_2O and quenched with Et_3N. The organic layer was washed with NH_4Cl (aq. sat.), $NaHCO_3$ (aq. sat.), $Na_2S_2O_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 9/1 -> 3/2, v/v) yielded the product (0.33 g, 0.24 mmol, 68%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 – 7.62 (m, 4H), 7.46 – 7.11 (m, 31H), 6.37 (d, *J* = 9.7 Hz, 1H), 5.17 (d, *J* = 11.3 Hz, 2H), 5.08 (d, *J* = 10.5 Hz, 1H), 5.01 (d, *J* = 12.1 Hz, 1H), 4.87 – 4.77 (m, 3H), 4.71 – 4.44 (m, 7H), 4.34 (td, *J* = 9.9, 3.4 Hz, 1H), 4.04 (dd, *J* = 12.2, 2.7 Hz, 1H), 3.96 – 3.71 (m, 7H), 3.71 – 3.54 (m, 3H), 3.32 – 3.14 (m, 3H), 3.12 (dd, *J* = 9.3, 4.0 Hz, 1H), 2.66 – 2.57 (m, 1H), 1.56 – 1.44 (m, 4H), 1.24 (d, *J* = 4.8 Hz, 11H), 1.04 (d, *J* = 5.9 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 146.8, 138.6, 138.1, 137.1, 136.8, 135.9, 135.6, 133.9, 132.9, 130.0, 129.8, 128.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.0, 127.9, 127.7, 127.6, 101.5, 82.2, 81.7, 81.4, 77.8, 77.4, 76.7, 76.3, 75.0, 74.2, 73.4, 72.3, 68.4, 67.2, 62.7, 53.2, 50.6, 50.2, 47.3, 46.3, 35.2, 30.6, 30.5, 29.7, 29.5, 28.2, 27.8, 27.0, 26.9, 26.4, 22.5, 19.5. HRMS (ESI) m/z: $[M+H]^+$ calculated for $C_{76}H_{90}N_2O_{13}SiI$ 1393.5251, found 1393.5247.

2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-cyclophellitol (57)



Iodocarbonate **S6** (0.28 g, 0.24 mmol) was dissolved in MeOH/DCM (3.4 ml, 0.7 M, 12:5, v/v). NaOMe (4.37 M in MeOH, 0.13 ml, 0.57 mmol) was added and the reaction was stirred for 15.5 hours. The reaction was quenched with $Et_3N{\cdot}HCl$ and concentrated in vacuo. The residue was dissolved in EtOAc, washed with H_2O and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. The product was used without further purification (0.26 g, 0.21 mmol, 88%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.66 (m, 4H), 7.45 – 7.14 (m, 31H), 6.80 (d, *J* = 9.7 Hz, 1H), 5.20 – 5.14 (m, 2H), 4.94 (d, *J* = 11.0 Hz, 1H), 4.86 (d, *J* = 3.5 Hz, 1H), 4.78 (d, *J* = 11.2 Hz, 1H), 4.72 – 4.62 (m, 3H), 4.52 – 4.43 (m, 3H), 4.32 – 4.25 (m, 1H), 4.09 – 4.01 (m, 1H), 3.99 – 3.86 (m, 3H), 3.85 – 3.74 (m, 3H), 3.58 (tdt, *J* = 11.9, 9.1, 5.8 Hz, 5H), 3.35 – 3.31 (m, 1H), 3.30 – 3.13 (m, 3H), 2.03 – 1.96 (m, 1H), 1.56 – 1.44 (m, 5H), 1.43 (s, 3H), 1.30 – 1.14 (m, 8H), 1.05 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 138.6, 138.0, 137.4, 137.2, 136.0, 135.6, 133.7, 133.1, 129.7, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.2, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 127.5, 127.3, 127.2, 100.6, 83.1, 81.3, 80.0, 77.5, 77.4, 77.1, 76.8, 75.8, 75.5, 74.7, 73.8, 73.0, 72.7, 67.2, 62.6, 61.7, 56.5, 53.5, 53.1, 52.2, 50.5, 50.2, 47.3, 46.3, 45.9, 44.5, 30.5, 29.7, 29.6, 29.4, 28.2, 26.9, 26.8, 26.2, 22.7, 19.3. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{75}H_{90}N_2O_{12}SiNa$ 1261.6155, found 1261.6184.

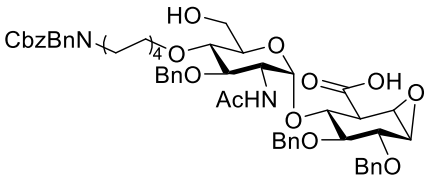
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)6-*O*-tert-butyldiphenylsilyl-α-D-glucopyranosyl)-glucurono-cyclophellitol (58)



Alcohol **54** (0.25 g, 0.21 mmol) was dissolved in *t*-BuOH/DCM/H_2O (7.7 ml, 26 mM, 5/4/1, v/v). TEMPO (7 mg, 0.04 mmol) and BAIB (0.161 g, 0.50 mmol) were added and the solution was stirred for 24 hours. The reaction was diluted with DCM and H_2O and quenched with $Na_2S_2O_3$ (aq. sat.). The water layer was acidified with AcOH and extracted with DCM (4x). The combined organic layers were dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 9/1 -> 1/1, v/v, 1% AcOH) yielded the product (0.17 g, 0.13 mmol, 67%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 – 7.65 (m, 4H), 7.46 – 7.14 (m, 31H), 6.07 (d, *J* = 9.7 Hz, 1H), 5.17 (d, *J* = 13.7 Hz, 2H), 4.93 (dd, *J* = 7.3, 3.7 Hz, 2H), 4.78 (t, *J* = 11.5 Hz, 2H), 4.64 (d, *J* = 11.3 Hz, 1H), 4.57 (dd,

J = 11.1, 5.0 Hz, 2H), 4.49 (d, *J* = 7.4 Hz, 2H), 4.19 (td, *J* = 10.0, 3.6 Hz, 1H), 3.98 – 3.61 (m, 9H), 3.58 (dd, *J* = 10.4, 8.4 Hz, 1H), 3.47 (dd, *J* = 10.1, 8.1 Hz, 1H), 3.32 (t, *J* = 2.9 Hz, 1H), 3.29 – 3.14 (m, 3H), 2.80 (dd, *J* = 8.8, 2.2 Hz, 1H), 1.55 – 1.43 (m, 4H), 1.31 – 1.14 (m, 11H), 1.03 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.6, 138.7, 138.0, 137.6, 137.1, 136.3, 135.8, 133.8, 133.6, 129.8, 129.7, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.4, 127.2, 100.0, 81.6, 81.5, 79.1, 77.5, 75.4, 75.2, 73.2, 73.2, 72.8, 67.2, 62.2, 54.2, 53.6, 53.2, 50.5, 50.2, 48.8, 47.3, 46.3, 30.6, 29.6, 29.4, 28.2, 27.8, 27.1, 27.0, 26.9, 26.8, 26.3, 22.5, 19.3. HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{75}H_{88}N_2O_{13}SiNa$ 1275.5948, found 1275.5986.

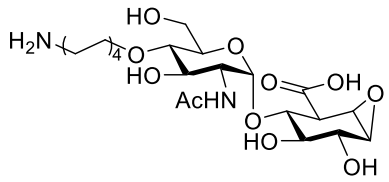
2,3-di-*O*-benzyl-4-*O*-(2-*N*-acetyl-2-deoxy-3-*O*-benzyl-4-*O*-(8-*N*-benzyl(benzyloxycarbonyl)-1-octyl)-α-D-glucopyranosyl)-glucurono-cyclophellitol (54)



Silyl protected **58** (0.60 g, 0.48 mmol) was dissolved in THF (7.2 ml, 0.05 M). $Et_3N{\cdot}3HF$ (0.29 ml, 1.8 mmol) was added and the reaction was stirred for 42 hours. The reaction mixture was diluted with DCM and water and the layers were separated. The water layer was extracted with DCM (4x) and the combined organic layers were dried over $MgSO_4$, filtrated and concentrated in vacuo. Column chromatography (PE/EtOAc, 2/3 -> 0/1, v/v, 1 % AcOH) yielded the product (0.49 g, 0.48 mmol, quant.).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.11 (m, 25H), 6.27 (d, *J* = 9.7 Hz, 1H), 5.17 (d, *J* = 11.6 Hz, 2H), 4.96 (d, *J* = 10.8 Hz, 1H), 4.87 – 4.75 (m, 3H), 4.67 (d, *J* = 11.3 Hz, 1H), 4.56 (t, *J* = 10.8 Hz, 2H), 4.48 (d, *J* = 9.5 Hz, 2H), 4.22 (td, *J* = 10.0, 3.6 Hz, 1H), 3.99 – 3.87 (m, 3H), 3.79 – 3.66 (m, 4H), 3.60 – 3.39 (m, 6H), 3.28 – 3.14 (m, 4H), 2.89 (dd, *J* = 9.0, 2.1 Hz, 1H), 1.50 (d, *J* = 6.7 Hz, 4H), 1.20 (d, *J* = 19.5 Hz, 11H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9, 172.6, 156.9, 156.3, 138.7, 138.0, 137.9, 137.7, 137.1, 137.0, 129.1, 128.7, 128.7, 128.7, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 128.2, 128.0, 127.9, 127.6, 127.5, 127.5, 127.3, 125.4, 100.3, 81.6, 81.5, 79.3, 77.9, 77.5, 77.4, 77.2, 76.8, 76.8, 75.7, 75.0, 73.4, 73.2, 72.8, 67.2, 66.2, 64.3, 61.1, 54.5, 53.6, 53.4, 50.5, 50.2, 49.6, 47.3, 46.3, 43.4, 30.6, 29.8, 29.6, 29.4, 28.2, 27.8, 26.8, 26.2, 22.6. HRMS (ESI) m/z: $[M+H]^+$ calc for $C_{59}H_{71}N_2O_{13}$ 1015.4951, found 1015.4954.

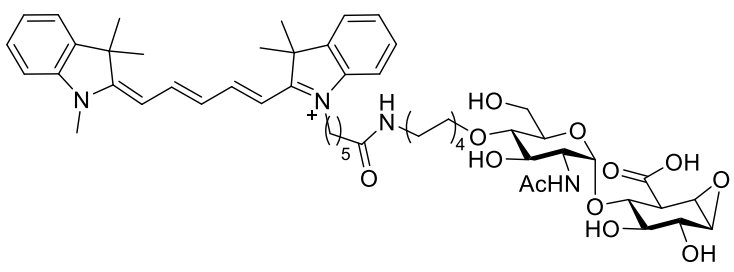
4-*O*-(2-*N*-acetyl-2-deoxy-4-*O*-(8-amino-1-octyl)-α-D-glucopyranosyl)-glucurono-cyclophellitol (59)



Benzyl protected **54** (0.10 g, 0.10 mmol) was dissolved in dioxane/water (10 ml, 0.01 M, 8/5) and flushed with N_2 for 5 min. 10% Pd/C (0.21 g, 0.2 mmol) was added and the suspension was flushed with N_2 for another 5 min. The N_2 balloon was replaced with an H_2 balloon and the solution was flushed with H_2 for 10 min and stirred at rt for 7 hours and 40 minutes. The H_2 balloon was replaced by an N_2 balloon, the reaction mixture was flushed for 5 min, filtrated over celite and concentrated under reduced pressure. Size exclusion chromatography over HW-40 eluting with H_2O 1% AcOH yielded a broad peak that was collected in three fractions. Based on NMR the pure product fraction was selected (0.021 g, 0.041 mmol, 41%).

^{1}H NMR (500 MHz, MeOD) δ 5.11 (d, *J* = 3.8 Hz, 1H), 3.90 – 3.83 (m, 4H), 3.81 – 3.75 (m, 1H), 3.74 – 3.66 (m, 2H), 3.62 (dd, *J* = 11.6, 5.3 Hz, 1H), 3.58 – 3.50 (m, 1H), 3.42 (dd, *J* = 10.0, 8.2 Hz, 1H), 3.39 – 3.36 (m, 1H), 3.10 (dd, *J* = 10.0, 8.8 Hz, 1H), 3.01 (d, *J* = 3.6 Hz, 1H), 2.90 (t, *J* = 7.7 Hz, 2H), 2.74 (dd, *J* = 9.2, 2.1 Hz, 1H), 1.99 (s, 3H), 1.69 – 1.49 (m, 4H), 1.45 – 1.33 (m, 8H). ^{13}C NMR (126 MHz, MeOD) δ 178.3 (weak), 174.0, 99.8, 80.2, 78.2, 77.8, 73.9, 73.7, 73.3, 73.2, 62.7, 57.0, 56.6, 56.1, 52.9, 40.8, 31.1, 29.9, 29.9, 28.7, 27.2, 27.2, 22.8. HRMS (ESI) m/z: $[M+H]^+$ calc for $C_{23}H_{41}N_2O_{11}$ 521.2705, found 521.2703.

4'O Cy5 conjugated probe 60 (CB702)



Cy5 carboxylic acid (11.6 mg, 22.3 µmol) was dissolved in DMF (0.25 ml). DIPEA (12 µl, 70 µmol), and pentafluorophenyl trifluoroacetate (3.8 µl, 22.3 µmol) were added and the mixture was stirred for one hour. LC-MS indicated the presence of starting material so more DIPEA (6 µl, 35 µmol) and pentaflurophenyl trifluoroacetate (3.8 µl, 22.3 µmol) were added. After stirring for 30 minutes water (2 µl) and DMF (0.25 ml) were added and the solution was added to amine **59** (12.2 mg, 23µmol).

The reaction was stirred overnight and the product was purified on semi-preparative HPLC eluting with a linear gradient of solution A (MeCN) in solution B (50mM AcOH in H_2O). The fractions were concentrated under reduced pressure, coevaporated with water, diluted with water and lyophilized to yield the product as a blue solid (2,88 mg, 2,82 µmol, 13%).

^{1}H NMR (850 MHz, CD_3CN) δ 8.02 (t, *J* = 13.1 Hz, 2H), 7.47 – 7.42 (m, 2H), 7.40 – 7.34 (m, 2H), 7.26 – 7.19 (m, 4H), 6.50 (t, *J* = 12.4 Hz, 1H), 6.16 (t, *J* = 13.1 Hz, 2H), 5.01 (d, *J* = 3.9 Hz, 1H), 3.96 (t, *J* = 7.5 Hz, 2H), 3.74 – 3.65 (m, 7H), 3.64 – 3.58 (m, 2H), 3.53 (dd, *J* = 12.0, 5.2 Hz, 1H), 3.49 (s, 5H), 3.38 (dd, *J* = 10.0, 8.4 Hz, 1H), 3.35 (dd, *J* = 3.8, 2.1 Hz, 1H), 3.10 (t, *J* = 9.6 Hz, 1H), 3.06 – 2.99 (m, 3H), 2.69 (dd, *J* = 9.3, 2.1 Hz, 1H), 2.08 (t, *J* = 7.3 Hz, 3H), 1.90 (s, 3H), 1.74 – 1.69 (m, 2H), 1.62 (s, 12H), 1.58 – 1.52 (m, 2H), 1.50 – 1.42 (m, 2H), 1.37 – 1.30 (m, 4H), 1.20 (s, 8H). ^{13}C NMR (214 MHz, CD_3CN) δ 175.5, 174.8, 174.4, 174.2, 154.6, 143.9, 143.2, 142.2, 142.1, 129.6, 126.0, 125.4, 123.2, 123.1, 112.0, 111.8, 103.9, 103.8, 98.3, 98.3, 79.5, 76.8, 76.7, 74.1, 72.5, 72.2, 72.1, 61.6, 57.1, 56.3, 56.3, 55.1, 51.7, 50.0, 50.0, 44.7, 40.0, 36.5, 31.8, 30.5, 29.9, 29.7, 27.7, 27.6, 27.5, 27.3, 26.7, 26.3, 26.1, 23.0. HRMS (ESI) m/z: $[M]^+$ calculated for $C_{55}H_{77}N_4O_{12}$ 985.5523, found 985.5533.

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4 Synthesis of an activity-based probe based on the Psl motif

4.1 Introduction

PslG, a retaining endo-mannosidase expressed by the pathogen *Pseudomonas aeruginosa*, is capable of digesting biofilms, a potential treatment for chronic infections. In this chapter the chemical synthesis and biological evaluation of a putative activity-based probe for PslG is described.

Pseudomonas aeruginosa biofilms

Pseudomonas aeruginosa is an opportunistic Gram negative bacterium that may be present in healthy individuals and that forms a major health concern for hospitalized patients.[1] Following surgery as well as for individuals using a medical device like a catheter or ventilator, *Pseudomonas aeruginosa* can form biofilms on compromised skin area and cause infections.[2]

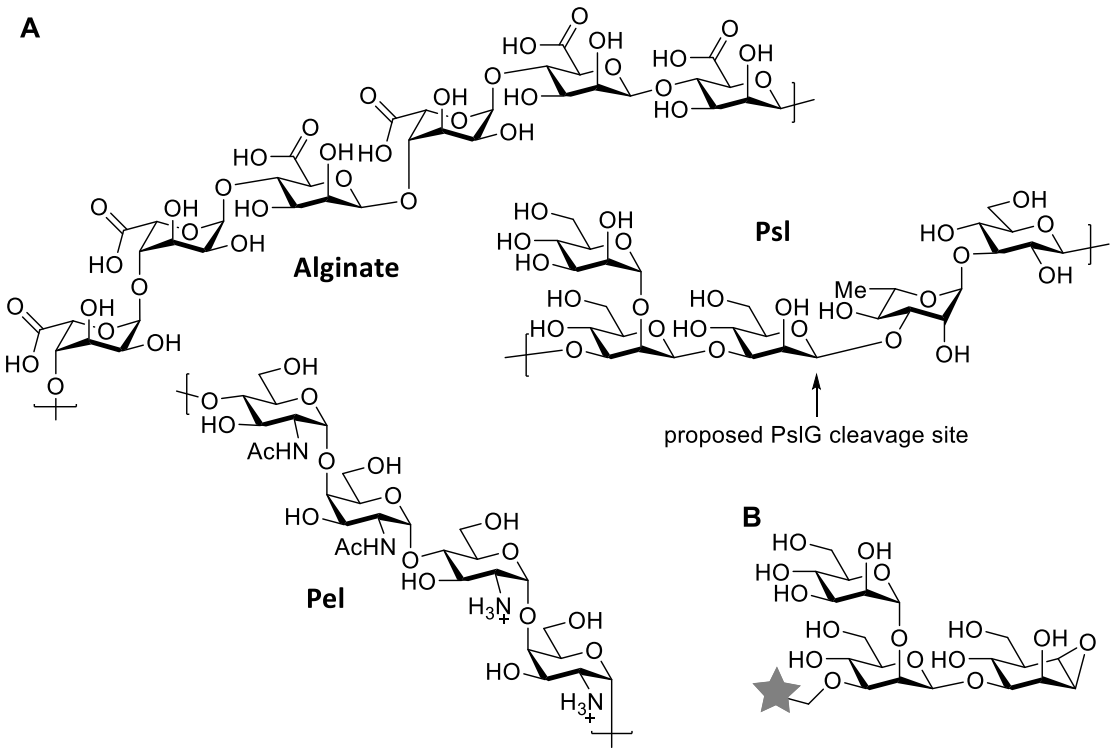


Figure 4.1| A) Simplified structures of Alginate[6] and Pel[8] and the repeating pentamer of the Psl polysaccharides. Based on molecular docking PslG was proposed to hydrolytically cleave Psl at the indicated position. **B**) Envisioned activity-based probe based on the structure of Psl and the proposed catalytic activity of PslG.

Treatment of *Pseudomonas aeruginosa* is difficult when it is embedded in a biofilm because it creates a micro environment shielding the bacterium from antibiotics and protecting it from the host immune system. Even upon eradicating the infective bacteria, the remaining biofilm offers an attractive environment for recolonization by the same or other pathogens leading to chronic infections.[3]

Pseudomonas aeruginosa generates a variety of biofilms containing proteins, DNA and at least three different polysaccharides allowing it to adapt to different environments.[4] Anionic alginate[5,6] is produced by *Pseudomonas aeruginosa* strains with a mucoid phenotype mostly found in the lungs of cystic fibrosis patients and is the most studied polysaccharide excreted by *Pseudomonas aeruginosa*. The structure is composed of β-linked and partially acetylated D-mannuronic and L-guluronic acid residues (Figure 4.1A). Strains isolated from other environments do not incorporate alginate in their biofilm, but incorporate the polysaccharides Pel or Psl instead.[4] Pel consists of partially acetylated D-glucosamine and D-galactosamine and is cationic which allows it to interact with negatively charged DNA in the biofilm.[7,8] Mutant

strains unable to produce Pel do not form pellicles, biofilms formed at the air water interface, a feature after which this particular sugar was named.[9]

Psl, the third polysaccharide produced by *Pseudomonas aeruginosa*, is a neutral polymer of pentameric repeating units consisting of D-mannose, D-glucose and L-rhamnose (Figure 4.1A).[10] The Psl polymer is produced by the combined activities of the enzymes encoded on the *polysaccharide locus*. On the locus are 15 co-transcribed proteins named PslA through to PslO of which 10, PslACDEFHIJKL, are essential for Psl biosynthesis, as determined by mutagenisis. Psl plays an important role in cell surface attachment and biofilm maintenance.[11] Mutant strains unable to synthesize Psl are unable to attach to human cells. Psl or fragments thereof also increase bis-(3',5')-cyclic dimeric GMP (c-di-GMP) levels in *Pseudomonas aeruginosa* leading to increased Psl production.[12] Psl and c-di-GMP both increase tolerance against antibiotics.[13]

PslG

PslG (47 kDa), postulated to be an endo-acting glycosyl hydrolase (GH) belonging to GH family 39, was originally believed to be essential for Psl production but this was later revoked by the same group.[10,14,15] The structure of PslG was reported[14,15] and it was postulated, based on molecular modeling, that PslG is an endo-mannosidase cleaving between the D-mannose and L-rhamnose residue of Psl.[15]

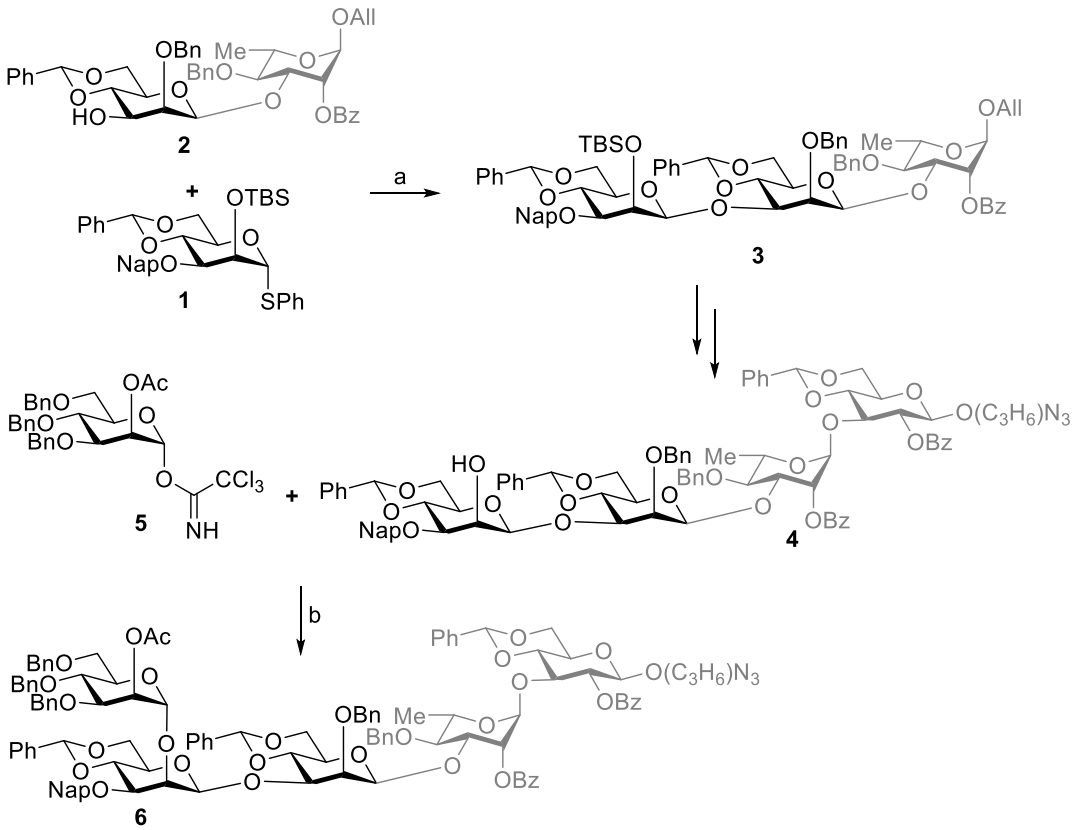
The hydrolase activity of PslG can be exploited to remove existing Psl containing biofilms and prevent biofilm formation by exogenous addition of the enzyme.[16] Exogenous addition of PslG to *Pseudomonas aeruginosa* cultures also leads to higher cell mobility, more random movement of the bacterium and slower microcolony formation.[17] PslG covalently attached to glass slides prevents the attachment of *Pseudomonas aeruginosa* and reduces biofilm formation.[18] These results show that PslG may be used to treat existing biofilms in patients with chronic infections and as a tool to design medical devices that are less susceptible to biofilm attachment. Despite the clinical potential of PslG, molecular understanding of the role endogenous PslG plays in *Pseudomonas aeruginosa* infection and biofilm formation requires more detailed studies.

PslG has been postulated to be responsible for the degradation of improperly processed or transported polymers in the periplasm.[4] However, PslG is not crucial for cell viability.[19] Recent results also suggest that PslG is important for dispersion of the bacterium by partially breaking

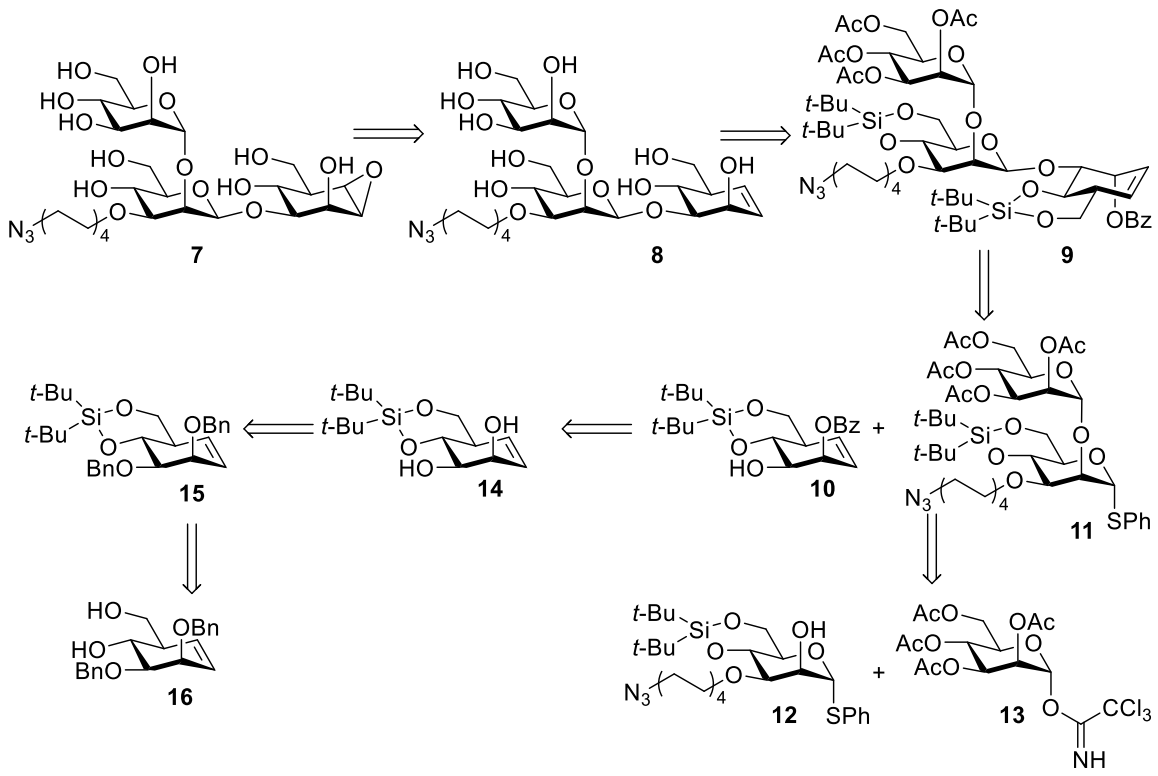
down the Psl polymers in the biofilm in a tightly regulated manner.[20] It could also be hypothesized that PslG is involved in intercellular signaling due to its ability to release complex Psl (fragments) which have been shown to have an inter cellular signaling function.[12]

PslG activity-based probes

A possible way to assess the role of PslG in *Pseudomonas aeruginosa* biology is by monitoring PslG activity *in situ* and *in vivo* by tailored activity-based probes (ABPs). A potent and selective ABP would show the activity of PslG in different cell environments and at different time points during infection and dispersion. It would also facilitate the observation of the direct effects of the inhibition of this activity compared to genetic knockouts. Because of the rare specificity of PslG the activity-based probe may serve as a tool to diagnose *Pseudomonas aeruginosa* infections. The probe may also aid in the improvement of the selectivity and stability of PslG as a therapeutic, by enabling high throughput assays for example by labeling active phage displayed PslG variants. Finally, a PslG ABP could be used for the unbiased screening of various enzyme sources looking for unknown enzymes with similar activities pointing towards undiscovered interactions in microbiology.



Scheme 4.1|Part of the synthesis of Psl fragments by the Boons group. Reagents and conditions: **a**) BSP, DTBMP, Tf_2O, DCM, -60°C, 72%, α/β 1/10. **b**) TMSOTf, DCM, -30°C, 82%.



Scheme 4.2| Retrosynthetic analysis of the trisaccharide activity-based probe.

Cyclophellitol and cyclophellitol aziridine equipped with reporter tags at various positions have proven to be effective scaffolds for exo-glycosidase ABPs.[21–23] More recently it was reported that the elongation of cyclophellitols with the appropriate carbohydrate can yield inhibitors and probes targeting various endo-glycosidases.[24,25] In the context of the work described in this chapter it was hypothesized that suitably configurated and substituted cyclophellitol derivatives equipped with a reporter tag may be effective probes to detect and monitor PslG activities as well. Based on the reported repeating unit and the proposed cleavage site a trisaccharide ABP featuring an electrophilic epoxide warhead as well as a reporter functionality was designed. The reporter tag was positioned at the non-reducing end to prevent possible degradation by exo-mannosidases (Figure 4.1B).

The synthesis strategy was inspired by the only reported chemical synthesis of Psl fragments to date, which was conducted by the Boons lab (Scheme 4.1).[26] For one of the two critical β-mannosylations donor **1** and acceptor **2** are used. This results in trisaccharide **3** in good yield and β-selectivity. **3** was elaborated into tetrasaccharide acceptor **4** which was reacted with trichloroacetimidate donor **5**. This resulted in pentasaccharide **6** containing the complete motif of the designed activity-based probe **7** (Scheme 4.2).

It was envisioned that epoxide **7** might be directly obtained from completely deprotected alkene **8** by hydrogen bond directed epoxidation. By making use of solely silyl ether- and ester-based protective groups it would be possible to conserve the azide during the deprotection sequence. Instead of using a benzylidene acetal as used by Boons to obtain β-selectivity in the mannosylation reaction, a 4,6-*O*-silylene was selected since the Bols group showed that these are also able to induce excellent β-selectivity in mannosylations.[27]

An alkyl spacer bearing an azide was attached on the central mannose to introduce the tag later in the synthesis or allow for two step activity-based protein profiling. Use of the azidosugar without the spacer on the carbohydrate moiety was avoided because of the poor availability of the required D-altrose configured starting material and the reported poor β-selectivity of 3-deoxy-3-azido mannosyl donors.[28] Based on these considerations protected trisaccharide **9** was proposed as intermediate towards **7** (Scheme 4.2).

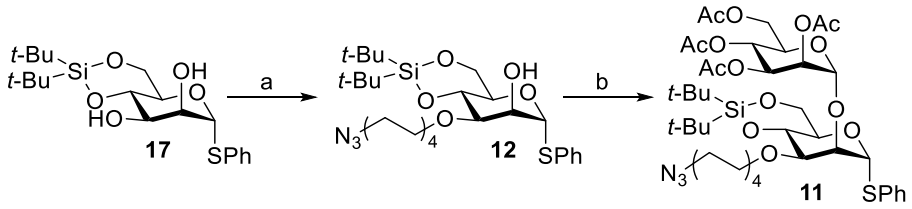
Trisaccharide **9** could be synthesized from cyclohexene acceptor **10** and disaccharide donor **11** to minimize the amount of steps after introduction of the valuable cyclohexene building block. The glycosylation in the Boons synthesis with donor **1** carrying a bulky TBS group on the 2-position is an indication that the glycosylation with the disaccharide could also yield predominantly a β-configured product. The donor could be obtained from acceptor **12** and donor **13** by neighboring group directed 1,2-trans glycosylation.

Acceptor **10** was anticipated to be accessible from diol **14** by selective acylation on the allylic alcohol. The diol could be obtained by debenzylation of fully protected **15,** which in turn could be derived from **16** of which the synthesis has been reported.[29]

4.2 Results and Discussion

Synthesis of a putative PslG activity-based probe

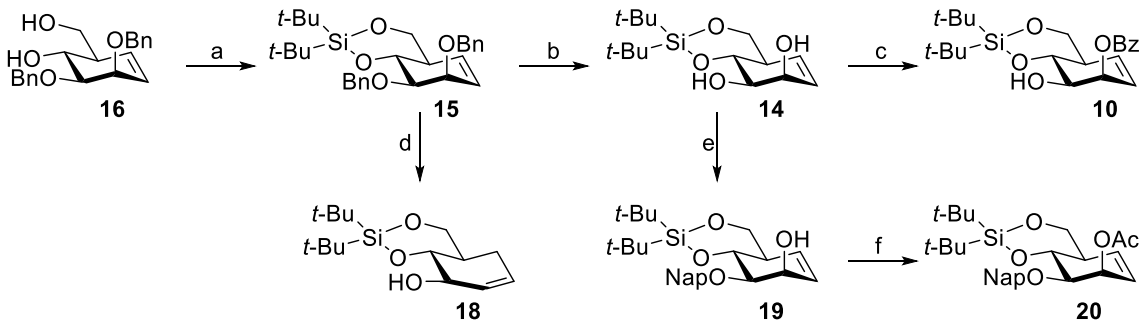
The first aim was to obtain azide tagged disaccharide donor **11** (Scheme 4.3). To this end diol **17**[27] was regioselectively alkylated in a borinate catalyzed reaction with a freshly prepared alkyl triflate under conditions similar to the alkylations described by the Taylor group.[30] Attempts to perform this reaction with the alkyl iodide were sluggish. Glycosylation of **12** with donor **13**[31] afforded disaccharide donor **11** in good yield on a gram scale.



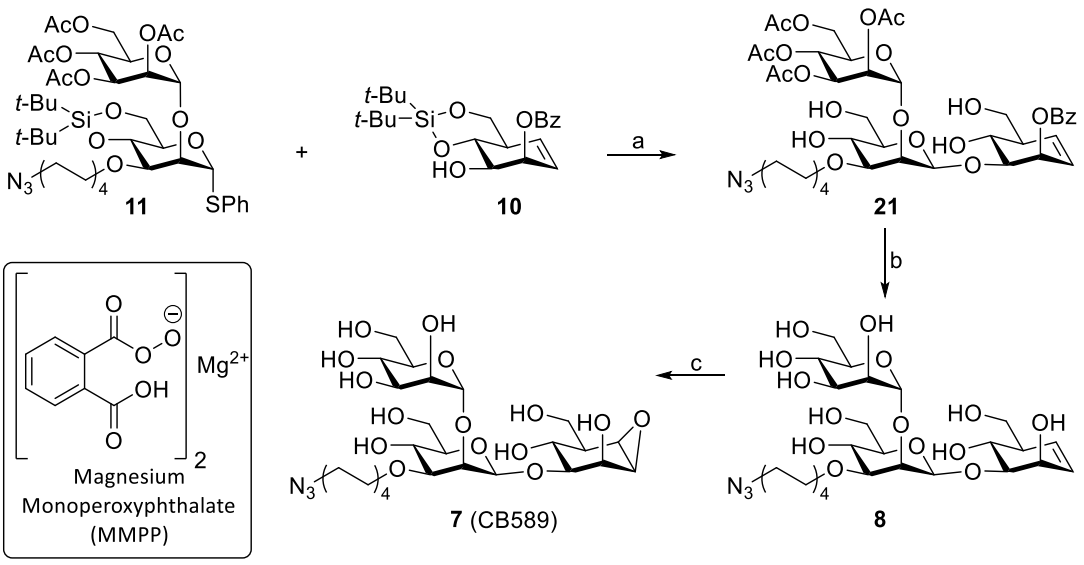
Scheme 4.3| Reagents and conditions: **a**) 8-azidooctyl trifluoromethanesulfonate, 2-aminoethyl diphenylborinate, K_2CO_3, MeCN, 0°C, 80%. **b**) **13**[31], TMSOTf, DCM, -20°C to 5°C, 78%.

The next target was the generation of a suitably protected mannose configured cyclohexene acceptor (Scheme 4.4). To this end diol **16**[29], synthesized based on the chemistry developed by Madsen *et al.*[32] was silylated to obtain **15**. Attempts to remove the benzyl ethers by dissolving metal hydrogenolysis gave low and irreproducible yield. From the conditions studied, reactions with sodium and without a proton source provided the highest yield of the desired product. The mayor observed side product, especially when using lithium and adding a proton source, was alkene **18**, which is consistent with observations made by Birch on his studies on allylic alcohols.[33] The limited solubility of the starting material in ammonia combined with the difficulty in monitoring the progress of these type of reactions by TLC analysis often led to the recovery of a large amount of starting material. Attempts to obtain the product by using lithium naphthalinide[34] or Lewis acidic BCl_3[35] failed as well. Eventually diol **14** was obtained reproducibly in high yield by debenzylation under Lewis acidic conditions using $TiCl_4$.[36]

Selective acylation of the pseudo axial, allylic alcohol in **14** was achieved in moderate yield by borinate catalysis providing **10**.[30] Attempts to orthogonally protect this alcohol with a naphthyl group provided the other regioisomer **19**.



Scheme 4.4|Reagents and conditions: **a**) Di-tert-butylsilyl ditriflate, imidazole, DMF, 73%. **b**) $TiCl_4$, DCM, toluene, 0°C, 82%. **c**) BzCl, 2-aminoethyl diphenylborinate, DIPEA, MeCN, rt, 65%. **d**) Li(s), *t*-BuOH, THF, NH_3. **e**) NapBr, 2-aminoethyl diphenylborinate, KI, K_2CO_3, MeCN. **f**) Ac_2O, pyr, DCM.



Scheme 4.5| Reagents and conditions: **a**) i. Ph_2SO, Tf_2O, TTBP, DCM, cyclohexene, -80°C -> -40°C; ii. $3HF{\cdot}Et_3N$, THF, 35% over 2 steps, 65% based on recovered **10**. **b**) NaOMe, MeOH. **c**) MMPP, NaOH, H_2O, 15% over 2 steps.

The regioselectivity was confirmed by acetylation of the remaining hydroxyl to afford **20** with a chemical shift of the allylic alcohol of 5.65 ppm compared to 3.51 ppm for the starting material. A possible mechanistic explanation for this difference could be that both reactions, the acylation and alkylation, afford mono alkylated and acylated products at the pseudo equatorial 3 OH. In the case of acylation, the acyl group can migrate to the allylic position leading to **10** whereas for alkylation migration is not possible affording **19**. Glycosylation of diol **14** with donor **11** under pre-activation conditions gave mostly the undesired 2*O*-glycosylated product.

Attempts to stereoselectively manipulate the alkene via Boc protection of **14** followed by iodine[37] ,IBr[38], or N-iodosuccinimide[39] induced iodocarbonylation were not productive presumably because of the inflexibility of the locked ring system.

Donor **11** and acceptor **10** were glycosylated under pre-activation conditions (Scheme 4.5).[40] The acceptor was partially recovered but the obtained product was difficult to separate from the hydrolyzed donor side product. This mixture was desilylated and this afforded the pure pseudo trisaccharide **21** after column chromatography. Only the β-configured product was obtained.

Deacylation with NaOMe in MeOH afforded crude **8** which was epoxidized in water with magnesium monoperoxyphthalate and NaOH to force the epoxidation to go via a hydrogen

bond directed mechanism leading to high diastereoselectivity.[41,42] This afforded epoxide **7** after size exclusion purification.

The H2 in the product shows a triplet with a coupling constant of 5.0 Hz in ^{1}H NMR which is exactly the same as the monomeric β-configured epoxide of mannose configured cyclophellitol.[29] The undesired α-configured epoxide shows a double doublet with coupling constant 2.9 and 3.1 Hz in the monomer confirming the formation of the expected β-configured epoxide.

Affinity for- and reactivity with PslG

X-ray crystallography of crystals of recombinant PslG soaked in a solution of epoxide **7** showed noncovalent binding of the alkyl spacer of the probe to a hydrophobic pocket of the enzyme (Figure 4.2). Treatment of recombinant PslG in solution at pH 5 and pH 7 did not lead to covalent attachment of the probe to the enzyme as monitored by ESI-MS of the intact protein.

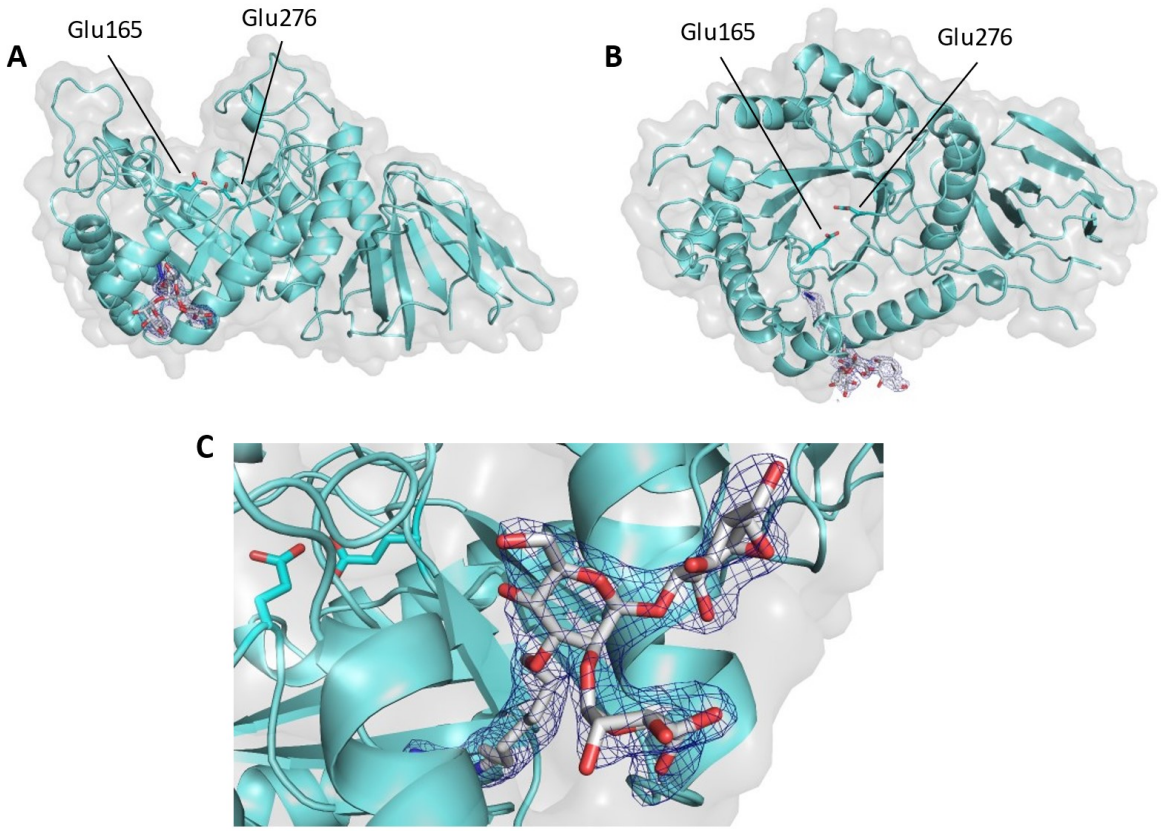


Figure 4.2| Ribbon and surface representation of PslG with **7** noncovalently bound away from the active site. Glu165 (putative acid/base) and Glu276 (putative nucleophile) are shown as stick representation. Electron density of **7** is REFMAC5 maximum-likelihood/σ_A weighted $2F_o-F_c$ map contoured to 0.8 σ (0.19 $e^-/Å^3$. **A**) Side view showing the active side cleft typical of endo-glycosidases. **B**) Top view. **C**) Closeup of **7** bound to PslG.

Attempts to compete binding to the hydrophobic pocket with 0.1 mM or 1 mM *n*-octyl-β-D-glucoside, decyl-β-D-maltoside or dodecyl-β-D-maltoside and the use of relatively high concentrations (5 μM) of probe did not lead to covalent probe binding to recombinant PslG.

4.3 Conclusion

Trisaccharide epoxide **7** was synthesized by a pre-activation protocol from a thioglycoside donor and an epi-cyclophellitol alkene acceptor further expanding the scope of glycosylation chemistry compatible with cyclophellitols and cyclophellitol precursors. The epoxide was generated late stage on a fully deprotected trisaccharide in water with good stereocontrol. The final stereochemistry of the product was confirmed by NMR spectroscopy and X-ray crystallography. The probe did not interact covalently with PslG (GH39).

CAZY[43] (www.cazy.org) GH family 39 contains mainly β-xylosidases (12) and α-L-iduronidases (4). It also contains a multifunctional enzyme (Bgxg1)[44] having β-xylosidase, β-glucosidase and β-galactosidase activities and two arabinosidases releasing disaccharides: α-L-(β-1,2)-arabinobiosidase NF2152 and D-galacto-(α-1,2)-L-arabinosidase NF2523.[45] It also contains one recently reported endo-α-L-rhamnosidase (BN863_22200)[46]. All these enzymes act on carbohydrate substrates with a 1,2-trans glycosidic linkage.

The postulation that PslG is an endo-mannosidase is based on intrinsic tryptophan fluorescence quenching with mannose, but the authors suggest this could also indicate mannose binding away from the active site.[14] In the crystal structure obtained after soaking with 3 M mannose the electron density of the mannose bound in the active side is poor.[14] Based on the results presented in this chapter showing the inability of PslG to interact with **7**, the minimal experimental evidence for the hypothesized cleavage position as well as the activities of the other GH39 family members, the postulated classification of PslG as an endo-mannosidase should perhaps be reconsidered.

4.4 Acknowledgements

Liang Wu, Nicholas McGregor and Gideon Davies from York University, UK are kindly acknowledged for the X-ray and LC-MS experiments and valuable discussion. Thijs Voskuilen and Michaela Ferrari are acknowledged for their synthesis work in the context of their Bachelor and Erasmus internships.

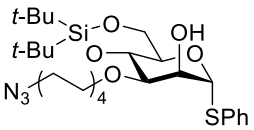
4.5 Experimental

General experimental procedures are shown in the experimental section of chapter 2.

8-azidooctyl trifluoromethanesulfonate

Tf_2O (0.54 ml, 3.22 mmol) was dissolved in DCM (7 ml) and cooled to -20°C. A solution of 8-azido-1-octanol (0.46 g, 2.68 mmol) and pyridine (0.25 ml, 3.22 mmol) in DCM (7 ml) was added and the reaction was stirred for 1 hour at the same temperature. The reaction mixture was diluted with DCM and subsequently washed with cold water and cold brine, dried with $MgSO_4$ and concentrated *in vacuo*. The reagent was used immediately without further purification.

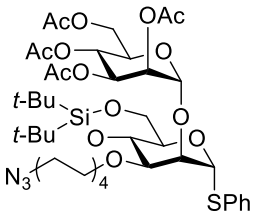
Phenyl 3-(8-azidooctyl)-4,6-*O*-ditertbutylsilyl-1-thio-α-D-mannopyranose (12)



Diol **17**[27] (1.12 g, 2.69 mmol) and K_2CO_3 (0.42 g, 3.0 mmol) were coevaporated with toluene. Freshly prepared 8-azidooctyl trifluoromethanesulfonate (1.22 g, 4.03 mmol) was dissolved in MeCN (7 ml) and added at 0°C. 2-aminoethyl diphenylborinate (0.06 g, 0.27 mmol) was dissolved in MeCN (7 ml) and added to the reaction mixture. DCM (1 ml) was added and the reaction was slowly warmed to rt. After 2 hours the reaction was quenched with $NaHCO_3$ (aq. sat.) and diluted with H_2O. The water layer was extracted with EtOAc (2x) and the combined organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The product was purified by column chromatography. (pentane/EtOAc, 10/1, v/v) (1.21 g, 2.15 mmol, 80%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.42 (m, 2H), 7.34 – 7.23 (m, 3H), 5.55 (d, *J* = 1.3 Hz, 1H, H1), 4.28 – 4.13 (m, 3H, H4/H2/H5), 4.05 – 3.92 (m, 2H, H6ab), 3.89 – 3.81 (m, 1H, CH_2O), 3.76 – 3.69 (m, 1H, CH_2O), 3.50 (dd, *J* = 8.4, 3.4 Hz, 1H, H3), 3.26 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 2.83 (s, 1H, OH), 1.65 – 1.55 (m, 4H, CH_2 (2x)), 1.44 – 1.29 (m, 8H, CH_2 (4x)), 1.06 (s, 9H, (*t*-Bu), 1.04 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 133.9, 131.5, 129.3, 127.6, 87.6 (C1), 79.0 (C3), 75.1, 72.0 (CH_2O), 71.7 , 68.3, 66.5 (C6), 51.6 (CH_2N_3), 30.2 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 27.5 (SiC(**C**H_3)$_3$), 27.2 (SiC(**C**H_3)$_3$), 26.8 (CH_2), 26.1 (CH_2), 22.7 (Si**C**(CH_3)$_3$), 20.1 (Si**C**(CH_3)$_3$). HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{28}H_{47}N_3O_5SSiNa$, 588.2903 found 588.2902.

Phenyl 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-3-*O*-(8-azidooctyl)-4,6-*O*-ditertbutylsilyl-1-thio-α-D-mannopyranose (11)

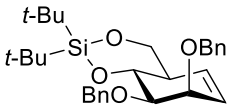


Acceptor **12** (1.21 gram, 2.14 mmol) and donor **13**[31] (1.22 gram, 2.57 mmol) were coevaporated with toluene (3x) and dissolved in DCM (11 ml, 0.2 M). 4Å molecular sieves were added and the mixture was stirred for 30 minutes. The reaction was cooled to -20°C and TMSOTf (0.08 ml, 0.43 mmol) was added. The reaction was allowed to warm to 5°C in 3 hours. The reaction was quenched with Et_3N and diluted with DCM. The organic layer was washed with $NaHCO_3$ (aq sat.). The water layer was extracted with DCM and the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was removed *in vacuo* and the product was isolated by column chromatography (PE/EtOAc, 4/1, v/v) to afford a white sticky solid. (1.38 g, 1.67 mmol, 78%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.42 (m, 2H), 7.37 – 7.25 (m, 3H), 5.46 (d, *J* = 1.4 Hz, 1H, H1), 5.38 (dd, *J* = 3.4, 1.8 Hz, 1H, H2'), 5.33 (dd, *J* = 9.9, 3.4 Hz, 1H, H3'), 5.21 (t, *J* = 9.8 Hz, 1H, H4'), 5.16 (d, *J* = 1.8 Hz, 1H, H1), 4.26 – 4.15 (m, 4H, H5/H4/H2/H6a), 4.07 – 3.97 (m, 4H, H6b/H6ab'/H5'), 3.85 (dt, *J* = 9.3, 6.4 Hz, 1H, C**H**$_2$O), 3.62 (dt, *J* = 9.3, 6.3 Hz, 1H, C**H**$_2$O), 3.53 (dd, *J* = 8.8, 3.1 Hz, 1H, H3), 3.26 (t, *J* = 7.0 Hz, 2H, C**H**$_2$N$_3$), 2.15 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.90 (s, 3H, OAc), 1.64 – 1.49 (m, 4H, spacer CH_2 (2x)), 1.40 – 1.28 (m, 8H, spacer CH_2 (4x)), 1.09 (s, 9H, *t*-Bu), 1.03 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.8 (OAc), 170.0 (OAc), 169.8 (OAc), 169.7 (OAc), 133.9 (SPh), 131.4 (SPh), 129.4 (SPh), 127.8 (SPh), 99.6 (C1'), 87.8 (C1), 79.2 (C3), 78.4, 75.4, 72.3 (CH_2O), 69.4 (C2'), 69.2, 69.0, 68.9 (C3'), 66.5 (C4'), 66.4 (C6), 62.7 (C6), 51.6 (CH_2N_3), 30.2 (spacer), 29.4 (spacer), 29.2 (spacer), 28.9 (spacer), 27.6 (*t*-Bu), 27.2 (*t*-Bu), 26.8 (spacer), 26.1 (spacer), 22.8 (*t*-Bu), 21.0 (OAc), 20.8 (OAc), 20.8

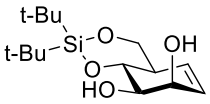
(OAc), 20.6 (OAc), 20.1 (*t*-Bu). HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{42}H_{65}N_3O_{14}SSiNa$ 918.3862, found 918.3854.

2,3-*O*-benzyl-4,6-*O*-ditertbutylsilyl-mannosecyclophellitolalkene (15)



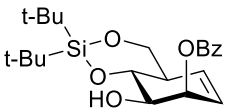
Alkene **16**[29] (1.1 g, 3.23 mmol) was coevaporated with toluene (2x), imidazole (0.9 g, 13.2 mmol) was added and the mixture was dissolved in DMF (32 ml, 0.1 M). The solution was cooled to 0°C, di-tert-butyl-silyltriflate (2.8 ml, 8.7 mmol) was added dropwise and the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with MeOH and the product was extracted with Et_2O (2x), the organic phase was washed with HCl (1 M), $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$ and volatiles were removed under reduced pressure. The product was obtained after column chromatography (PE/EtOAc, 20/1, v/v) as a colorless oil. (1.14 g, 2.36 mmol, 73%)
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.26 (m, 10H), 5.69 (ddd, *J* = 9.8, 5.0, 3.0 Hz, 1H, alkene), 5.30 (dd, *J* = 9.8, 1.8 Hz, 1H, alkene), 5.06 (d, *J* = 12.4 Hz, 1H, CH_2Bn), 4.92 (d, *J* = 12.4 Hz, 1H, CH_2Bn), 4.82 (d, *J* = 12.4 Hz, 1H, CH_2Bn), 4.74 (d, *J* = 12.4 Hz, 1H, CH_2Bn), 4.43 (dd, *J* = 10.3, 9.1 Hz, 1H, H4), 4.08 (dd, *J* = 10.4, 4.6 Hz, 1H, H6a), 4.03 (t, *J* = 4.4 Hz, 1H, H2), 3.87 (dd, *J* = 12.0, 10.3 Hz, 1H, H6b), 3.52 (dd, *J* = 10.2, 4.3 Hz, 1H, H3), 2.53 – 2.44 (m, 1H, H5), 1.09 (s, 9H, *t*-Bu), 1.05 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 139.7, 139.2, 128.4, 128.4, 128.2, 127.7, 127.7, 127.6, 127.5, 127.3, 81.3 (C3), 75.6 (C4), 74.0 (CH_2Bn), 73.4 (CH_2Bn), 73.2 (C2), 68.5 (C6), 45.7 (C5), 27.6 (*t*-Bu), 27.4 (*t*-Bu), 22.9 (*t*-Bu), 20.0 (*t*-Bu). HRMS (ESI) m/z: $[M+NH_4]^+$ calc for $C_{29}H_{44}O_4SiN$ 498.3034, found 498.3033.

4,6-*O*-ditertbutylsilyl-mannocyclophellitolalkene (14)



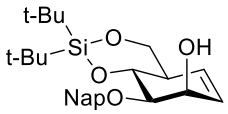
Alkene **15** (0.99 g, 2.06 mmol) was dissolved in DCM (20 ml, 0.1 M) and cooled to 0°C. A solution of $TiCl_4$ (1 M in toluene, 8.24 ml, 8.24 mmol) was added slowly. After 20 minutes, the reaction was quenched by careful addition of $NaHCO_3$ (aq. sat.). The obtained suspension was filtered over celite. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the product was isolated by column chromatography (Pentane/EtOAc, 9/1 to 7.5/2, v/v) as a colorless oil. (0.51 g, 1.7 mmol, 82%)
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.89 (ddd, *J* = 9.8, 4.8, 2.9 Hz, 1H, alkene), 5.42 (dd, *J* = 9.9, 1.8 Hz, 1H, alkene), 4.39 (t, *J* = 4.5 Hz, 1H, H2), 4.13 (dd, *J* = 10.4, 4.8 Hz, 1H, H6a), 4.02 (t, *J* = 9.7 Hz, 1H, H4), 3.83 (dd, *J* = 12.0, 10.4 Hz, 1H, H6b), 3.65 (dd, *J* = 10.1, 4.5 Hz, 1H, H3), 3.11 (s, 1H, OH), 2.87 (s, 1H, OH), 2.56 – 2.45 (m, 1H, H5), 1.05 (s, 9H, *t*-Bu), 1.01 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 128.0 (alkene), 127.7 (alkene), 74.1 (C4), 73.7 (C3), 68.1 (C6), 66.1 (C2), 43.8 (C5), 27.6 (*t*-Bu), 27.2 (*t*-Bu), 22.8 (*t*-Bu), 20.0 (*t*-Bu). HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{15}H_{28}O_4SiNa$, 323.1649 found 323.1647.

2-*O*-benzoyl-4,6-*O*-ditertbutylsilyl-mannocyclophellitolalkene (10)



Diol **14** (0.28 g, 0.94 mmol) was dissolved in MeCN (4.8 ml, 0.2 M). DIPEA (0.82 ml, 4.72 mmol), BzCl (0.33 ml, 2.83 mmol) and 2-aminoethyl diphenylborinate (21 mg, 0.094 mmol) were added and the mixture was stirred for 17 hours at rt. The reaction was diluted with Et_2O and washed with HCl (1 M), $NaHCO_3$ (aq. sat.) and brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under educed pressure. The product was obtained by column chromatography (Pentane/Et_2O, 95/5 to 85/15, v/v) as an orange oil. (250 mg, 0.611 mmol, 65%)
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 – 8.02 (m, 2H), 7.60 – 7.52 (m, 1H), 7.44 (m, 2H), 5.93 (ddd, *J* = 9.7, 5.0, 2.9 Hz, 1H, alkene), 5.83 (td, *J* = 4.8, 1.1 Hz, 1H, H2), 5.55 (dd, *J* = 9.7, 1.9 Hz, 1H, alkene), 4.24 – 4.15 (m, 2H, H6a/H4), 3.94 – 3.84 (m, 2H, H6b/H3), 2.63 – 2.53 (m, 1H, H5), 1.09 (s, 9H, *t*-Bu), 1.03 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 166.2 (PhCOO), 133.1 (Bz), 130.4 (Bz), 129.9 (Bz), 129.7 (alkene), 128.5 (Bz), 125.5 (alkene), 74.7 (C4), 72.5 (C3), 68.4 (C2), 68.1 (C6), 44.2 (C5), 27.6 (*t*-Bu), 27.2 (*t*-Bu), 23.0 (*t*-Bu), 20.0 (*t*-Bu). HRMS (ESI) m/z: $[M+H]^+$ calc for $C_{22}H_{33}O_5Si$ 405.2092, found 405.2089.

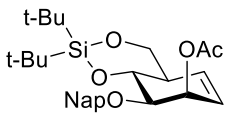
3-*O*-2-methylnaphtalene-4,6-*O*-ditertbutylsilyl-mannocyclophellitolalkene (19)



Diol **14** (0.129 g, 0.430 mmol) was coevaporated with toluene and dissolved in MeCN (2.0 ml). 2-aminoethyl diphenylborinate (0.01 g, 0.04 mmol), K_2CO_3 (0.071 g, 0.52 mmol), KI (0.071 g, 0.43 mmol) and NapBr (0.11 g, 0.53 mmol) were added and the solution was stirred at rt overnight. The reaction was quenched with water and extracted with Et_2O (2x). The combined organic phase was washed with $NaHCO_3$ (aq. sat.) and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was analyzed without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 – 7.76 (m, 4H), 7.60 – 7.52 (m, 1H), 7.51 – 7.42 (m, 2H), 5.81 (ddd, *J* = 9.8, 4.8, 2.9 Hz, 1H, alkene), 5.33 (dd, *J* = 9.8, 1.8 Hz, 1H, alkene), 5.21 (dd, *J* = 11.9, 0.8 Hz, 1H, CH_2Nap), 4.98 (d, *J* = 11.9 Hz, 1H, CH_2Nap), 4.37 – 4.25 (m, 2H, H3/H4), 4.10 (dd, *J* = 10.4, 4.7 Hz, 1H, H6a), 3.85 (dd, *J* = 12.0, 10.4 Hz, 1H, H6b), 3.51 (dd, *J* = 10.1, 4.6 Hz, 1H, H2), 3.05 (s, 1H, OH), 2.57 – 2.47 (m, 1H, H5), 1.10 (s, 9H, *t*-Bu), 1.06 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 136.3, 133.3, 133.1, 128.3, 128.0, 127.9 (alkene), 127.8, 127.6 (alkene), 126.7, 126.2, 126.1, 126.0, 126.0, 79.8 (C2), 75.2 (C3), 74.2 (CH_2Nap), 68.4 (C6), 66.8 (C4), 44.8 (C5), 27.6 (*t*-Bu), 27.3 (*t*-Bu), 22.8 (Cq *t*-Bu), 20.0 (Cq *t*-Bu).

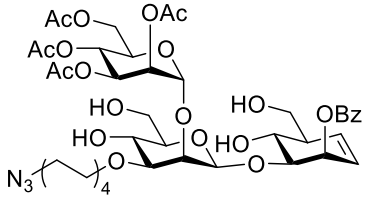
2-*O*-acetyl-3-*O*-(2-methylnaphtalene)-4,6-*O*-ditertbutylsilyl-mannocyclophellitolalkene (20)



Crude alcohol **19** was dissolved in pyridine (1 ml) and Ac_2O (1 ml) and stirred overnight. The was concentrated under reduced pressure, coevaporated with toluene and analyzed without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.78 (m, 4H), 7.57 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.49 – 7.42 (m, 2H), 5.70 (ddd, *J* = 9.5, 5.0, 2.9 Hz, 1H, alkene), 5.65 (t, *J* = 4.8 Hz, 1H, H2), 5.42 (dd, *J* = 9.6, 1.9 Hz, 1H, alkene), 5.03 – 4.93 (m, 2H, CH_2Nap), 4.27 (dd, *J* = 10.3, 9.1 Hz, 1H, H4), 4.11 (dd, *J* = 10.4, 4.6 Hz, 1H, H6a), 3.87 (dd, *J* = 12.0, 10.4 Hz, 1H, H6b), 3.58 (dd, *J* = 10.3, 4.6 Hz, 1H, H3), 2.11 (s, 3H, OAc), 1.11 (s, 9H, *t*-Bu), 1.05 (s, 9H, *t*-Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.8 (OAc), 136.5 (alkene), 133.3, 133.1, 129.5, 129.1, 128.3, 128.2, 128.0, 127.8, 126.4, 126.1, 126.0, 125.8, 125.4 (alkene), 78.1 (C3), 75.2 (C4), 73.5 (CH_2Nap), 68.3 (C6), 67.6 (C2), 45.1 (C5), 27.6 (*t*-Bu), 27.4 (*t*-Bu), 22.9 (Cq *t*-Bu), 21.4 (OAc), 20.0 (Cq *t*-Bu).

2-*O*-benzoyl-3-*O*-(2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-3-*O*-(8-azidooctyl)-β-D-mannopyranosyl)-mannocyclophellitolalkene (21)



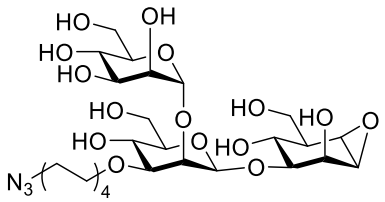
Disaccharide donor **11** (0.20 g, 0.22 mmol), diphenyl sulfoxide (0.045 g, 0.22 mmol) and TTBP (0.15 g, 0.59 mmol) were coevaporated with toluene (2x). The dry starting materials were dissolved in DCM (2 ml), 4Å molecular sieves were added and the mixture was stirred at rt for 30 minutes. The reaction was cooled to -72°C and Tf_2O (0.3 M in DCM, 0.7 ml, 0.21 mmol) was added. The reaction was warmed to -60°C over 30 minutes and was subsequently cooled to -80°C. Acceptor **10** (0.060 g, 0.15 mmol) and cyclohexene (0.08 ml, 0.74 mmol) were dissolved in DCM (1 ml) and added slowly to the reaction mixture. The reaction was allowed to warm to -40°C and was quenched with Et_3N at that temperature. The mixture was diluted with EtOAc. The molecular sieves were removed and the solution was washed with $NaHCO_3$ (aq. sat.) and brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. Column chromatography (Pentane/EtOAc, 90/10 to 84/16, v/v) yielded a mixture of product (**9**) and donor (**11**) (85 mg) and unreacted acceptor **10** (26 mg, 43%).

The product mixture was taken up in THF (5 ml) and $3HF{\cdot}Et_3N$ (0.15 ml, 6,59 mmol) was added. The reaction was stirred overnight. More $3HF{\cdot}Et_3N$ (0.15 ml, 6.59 mmol) was added and the reaction was stirred for 5 hours. The mixture was diluted with THF and $CaCO_3$ (1.0 g, 10 mmol) was added. The suspension was stirred for 30 minutes before it was filtered over celite. The solvent was removed *in vacuo* and the pure product was obtained by column chromatography (DCM/Acetone, 1/0 to 2/8, v/v) as a colorless oil. (47 mg, 0.053 mmol, 35%, 65% based on recovered acceptor)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 – 7.98 (m, 2H, Bz), 7.60 – 7.54 (m, 1H, Bz), 7.45 (m, 2H, Bz), 5.92 – 5.82 (m, 3H, alkene (2x)/H2), 5.34 (dd, *J* = 10.1, 3.4 Hz, 1H, H3”), 5.22 – 5.14 (m, 2H, H4”/H2”), 4.90 (d,

J = 2.0 Hz, 1H, H1”), 4.80 (s, 1H, H1’), 4.25 (dt, *J* = 10.0, 3.3 Hz, 1H, H5”), 4.12 (dd, *J* = 10.0, 3.5 Hz, 1H, H3), 4.09 – 3.99 (m, 2H, H6a”/H4), 3.99 – 3.79 (m, 6H, H2’/H6ab/H6ab’/H4’), 3.60 (dt, *J* = 9.1, 6.5 Hz, 1H, spacer), 3.55 – 3.41 (m, 2H, H6b”/spacer), 3.38 (ddd, *J* = 9.7, 5.0, 3.0 Hz, 1H, H5’), 3.30 (dd, *J* = 9.4, 2.5 Hz, 1H, H3’), 3.26 (t, *J* = 7.0 Hz, 2H, CH_2N3), 2.53 (m, 1H, H5), 2.08 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.96 (s, 3H, OAc), 1.59 (m, 4H, spacer), 1.39 – 1.27 (m, 8H, spacer). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.7, 170.2, 169.8, 166.5, 133.6, 133.4, 129.8, 129.7, 128.8, 123.6 (alkene), 97.7 (C1”), 97.3 (C1’), 82.2 (C3’), 77.3 (C3), 76.6 (H5’), 72.8 (C2’), 70.2 (spacer), 69.5 (C2”), 69.2 (3”), 68.6 (C5”/C4), 66.7 (C4’), 66.1 (C4”), 65.7 (C2), 64.9 (C6’), 62.3 (C6”), 62.1 (C6), 51.6 (CH_2N_3), 46.7 (C5), 29.8, 29.4, 29.1, 28.9, 26.8, 26.0 (spacer 6x), 21.0, 20.9, 20.8 (OAc 4x). $^1J_{H,C}$ (H1”) 4.90 ppm, 97.7 ppm = 171 Hz, $^1J_{H,C}$ (H1’) 4.80 ppm, 97.3 ppm = 154 Hz. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{42}H_{59}N_3O_{19}Na$ 932.3640, found 932.3654.

3-*O*-(2-*O*-(α-D-mannopyranosyl)-3-*O*-(8-azidooctyl)-β-D-mannopyranosyl)-β-mannocyclophellitol (7)



Alkene **21** (17 mg, 19 µmol) was dissolved in MeOH (0.5 ml) a catalytic amount of NaOMe was added and the reaction was monitored by LC-MS. Upon completion the reaction was quenched with AcOH and the solvent was evaporated under reduced pressure. The crude product (**8**) was dissolved in H_2O (1 ml), NaOH (20 mg, 500 µmol) and magnesium monoperoxyphthalate (80%, 21 mg) were added. The mixture was stirred for 5 hours followed by purification on HW40 (150 mM NH_4HCO_3, H_2O). This yielded the product after elution of the salts. (1.83 mg, 2.9 µmol, 15%)

^{1}H NMR (850 MHz, D_2O) δ 5.04 (d, *J* = 1.7 Hz, 1H, H1”), 4.70 (H1’, obscured by HDO), 4.43 (t, *J* = 5.0 Hz, 1H, H2), 4.38 (d, *J* = 2.7 Hz, 1H, H2’), 4.08 (ddd, *J* = 10.2, 5.6, 2.3 Hz, 1H, H5”), 3.95 (dd, *J* = 3.5, 1.7 Hz, 1H, H2”), 3.89 (dd, *J* = 11.1, 4.4 Hz, 1H, H6a), 3.86 – 3.82 (m, 2H, H6a’/H3”), 3.79 (dd, *J* = 12.1, 2.3 Hz, 1H, H6a”), 3.75 (dd, *J* = 11.2, 8.0 Hz, 1H, H6b), 3.72 – 3.64 (m, 4H, H3/H6b’/H6b”/spacer), 3.58 (t, *J* = 8.6 Hz, 1H, H4”), 3.57 – 3.51 (m, 3H, H4’/H4/spacer), 3.47 (dd, *J* = 4.1, 2.1 Hz, 1H, epoxide), 3.45 – 3.42 (m, 2H, epoxide/H3’), 3.32 (ddd, *J* = 9.4, 6.7, 2.3 Hz, 1H, H5’), 3.24 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 2.06 (tdd, *J* = 8.2, 4.4, 2.2 Hz, 1H, H5), 1.58 – 1.49 (m, 4H, spacer), 1.34 – 1.23 (m, 8H, spacer). ^{13}C NMR (214 MHz, D_2O) δ = 100.4 (C1”), 98.3 (C1’), 81.9 (C3’), 79.1 (C3), 76.8 (C5’), 72.4 (C5”), 71.5 (C2’), 70.6 (spacer), 70.3 (C3”), 70.0 (C2”), 66.7 (C4”), 66.0 (C4’), 64.2 (C4), 63.6 (C2), 61.0 (C6’/C6”), 60.7 (C6), 55.6 (epoxide), 53.4 (epoxide), 51.2 (CH_2N_3), 44.0 (C5), 28.8, 28.3, 28.1, 27.9, 25.8, 25.1 (spacer 6x). $^1J_{H,C}$ (H1”) 5.04 ppm, 100.4 ppm = 172 Hz, $^1J_{H,C}$ (H1’) 4.70 ppm, 98.3 ppm = 160 Hz. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{27}H_{47}N_3O_{15}Na$ 676.2905, found 676.2914.

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Summary and future prospects

5

In this dissertation covalent glycosidase inhibitors and activity-based probes (ABPs) for retaining endo-glycosidases are described. The design of the molecules is based on the natural product cyclophellitol. C*hapter 1* highlights previous applications of cyclophellitol derivatives, discusses mechanistic and conformational aspects of the irreversible reaction of cyclophellitol with retaining glycosidases and provides an overview of activity-based protein profiling (ABPP) protocols.

The subsequent chapters describe the chemical synthesis and biological evaluation of inhibitors and probes for xyloglucan active retaining glycosidases, human heparanase and PslG from *Pseudomonas aeruginosa*. This final chapter briefly summarizes the results and suggests directions for future research projects.

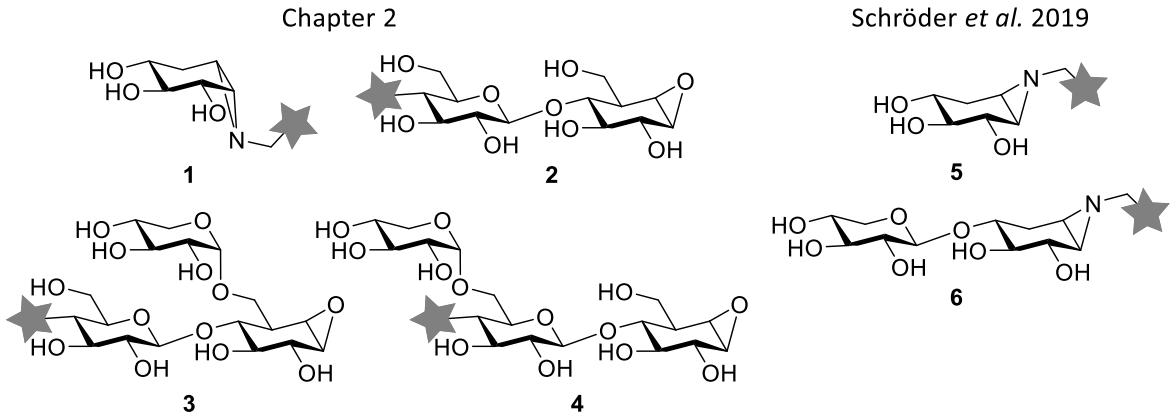


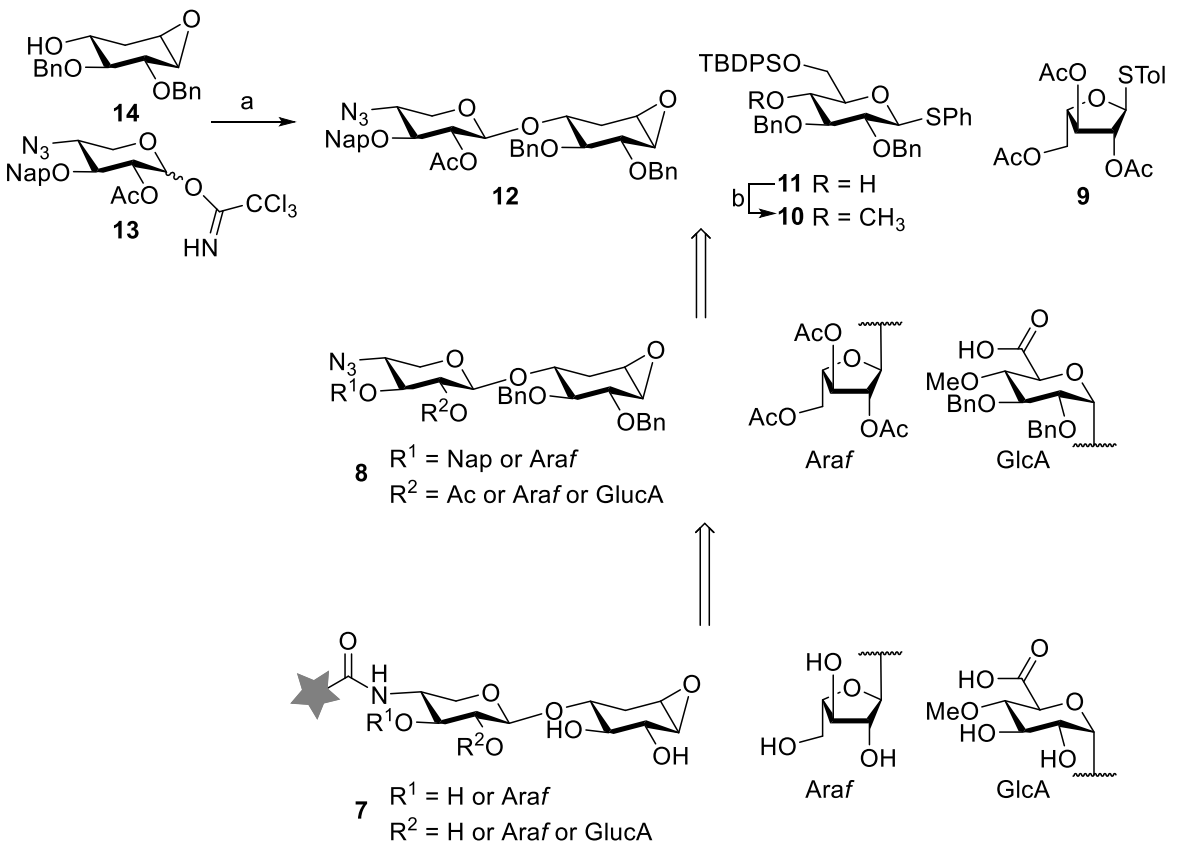
Figure 5.1| Activity-based probes for enzymes acting on xyloglucan as described in chapter 2 and xylan as described in the literature[5].

Glycosidases targeting plant glycans

In *chapter 2* activity-based probes for retaining glycosidases acting on various glycosidic linkages in xyloglucan are presented (**1**-**4**, Figure 5.1). The oligomeric probes, **2, 3** and **4** were synthesized by chemical glycosylation under pre-activation conditions, using cyclophellitol epoxide acceptors.

ABPP in *Aspergillus niger* secretomes showed no cross reactivity between monosaccharide and oligosaccharide probes suggesting selective labeling of endo-glycosidases. Profiling with **2**, **3** and **4** also showed distinct labeling for the three probes. This indicates that glucanases and xyloglucanases with distinct substrate specificities can be distinguished with these probes. To assess the specificity of the probes in more depth, the probes can be used for ABPP of a variety of fungal secretomes and the labeled proteins can be analyzed by SDS-PAGE and by LC-MS proteomics. Together with further characterization of the synthesized probes these future studies may also reveal uncharacterized enzymes with desired characteristics, and provide new insights in fungal physiology.

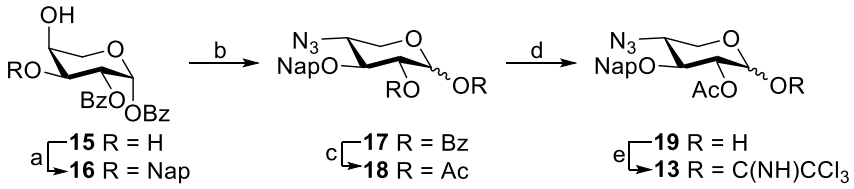
ABPP of xylanases with specificity for xylan with specific branching such as arabino-[1] and glucurono-xylanases[2–4] could be envisaged via a similar approach as an extension of previously developed xylosidase (**5**) and xylanase (**6**) ABPs.[5] The synthesis of probes **7** via protected **8** has been partially conducted (Scheme 5.1) with the aim to test this hypothesis. Orthogonal protection of the non-reducing end xylose allows selective introduction of arabinofuranose and 4-*O*-methyl glucuronic acid on the 2'and 3'position. L-Arabinofuranose donor **9**[6] and 4-*O*-methyl glucose donor **10** may be suitable to introduce the respective side chains. Orthogonal



Scheme 5.1| Reagents and conditions: **a**) TMSOTf, DCM, -30°C, 61%. **b**) MeI, NaH, THF, quant.

silyl ether protection of **10** allows selective oxidation to glucuronic acid post-glycosylation. **10** was synthesized from **11**[7] by reaction with iodomethane. Pseudodisaccharide **12** was obtained by glycosylation of donor **13** with acceptor **14**[5].

Donor **13** was obtained starting from partially benzoylated L-arabinopyranose **15**[8] (Scheme 5.2). A naphthyl (Nap) ether was selectively introduced employing tin ketal catalysis affording **16**. The axial 4-OH was activated as a triflate and substituted with NaN_3 affording D-xylose configured **17**. Selective removal of the anomeric benzoyl group was unsuccessful but removal of both benzoyl esters followed by acetylation afforded **18** on which selective anomeric deprotection was achieved, affording lactol **19.** The lactol was transformed into trichloroacetimidate donor **13**.

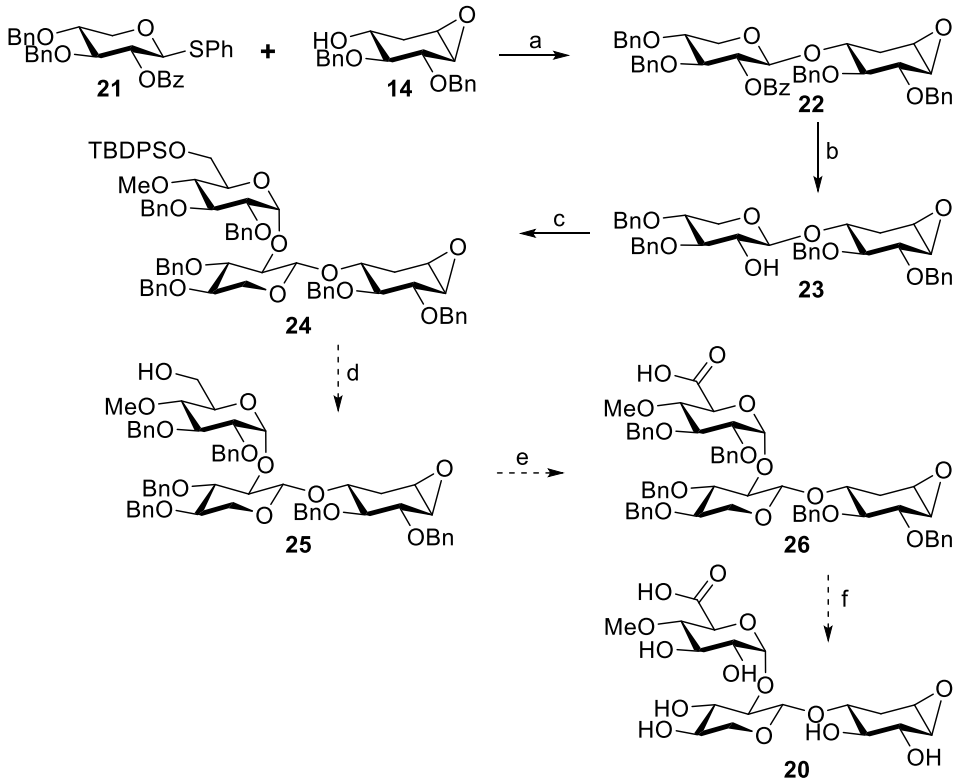


Scheme 5.2| Reagents and conditions: **a**) i. Bu_2SnO, toluene; ii. NapBr, CsF, DMF, 56%. **b**) i. Tf_2O, pyr, DCM -20°C; ii. NaN_3, DMF, 90%. **c**) i. NaOMe, MeOH, DCM; ii. Ac_2O, pyr, 88%. **d**) piperidine, THF, 68%. **e**) CCl_3CN, K_2CO_3, DCM, 90%.

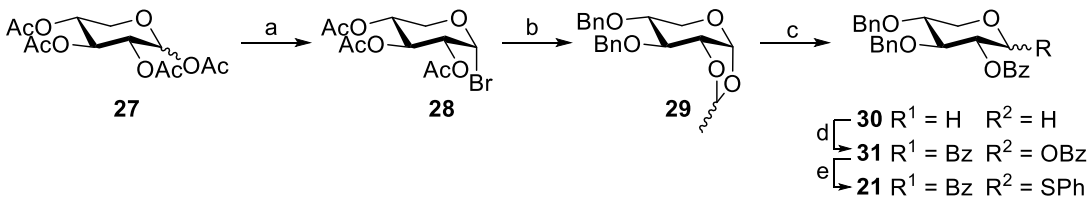
The synthesis of glucuronoxylanase inhibitor **20** was started as well (Scheme 5.3). Glycosylation of donor **21** with acceptor **14** provided pseudodisaccharide **22**. Removal of the benzoyl ester under basic conditions afforded alcohol **23**. Glycosylation of pseudodisaccharide acceptor **23** with donor **10** under pre-activation conditions provided product **24** in 30% yield. N-iodosuccinimide (NIS)/TMSOTf mediated glycosylation in DCM/Et_2O afforded the same product in 16% yield. In both cases only the α-configured product was observed.

To complete the synthesis three more steps could be executed. The silyl protecting group in pseudotrisaccharide **24** may be deprotected with a fluorine source to provide primary alcohol **25**. TEMPO/BAIB oxidation may provide carboxylic acid **26** which could be deprotected to provide inhibitor **20** by either dissolving metal or Pd catalyzed hydrogenation conditions.

Donor **21** was synthesized from peracetylated D-xylose (**27**) (Scheme 5.4). Treatment with HBr provided bromide **28** which was reduced to a diastereomeric mixture of anomeric ethylidenes. Removal of the acetyl esters under basic conditions followed by benzyl ether formation afforded **29**. The acetal masking group was hydrolyzed under acidic conditions and the resulting diol was benzoylated providing **31**. Lewis acid catalyzed introduction of an anomeric thiophenol provided thioglycoside donor **21** as an anomeric mixture.



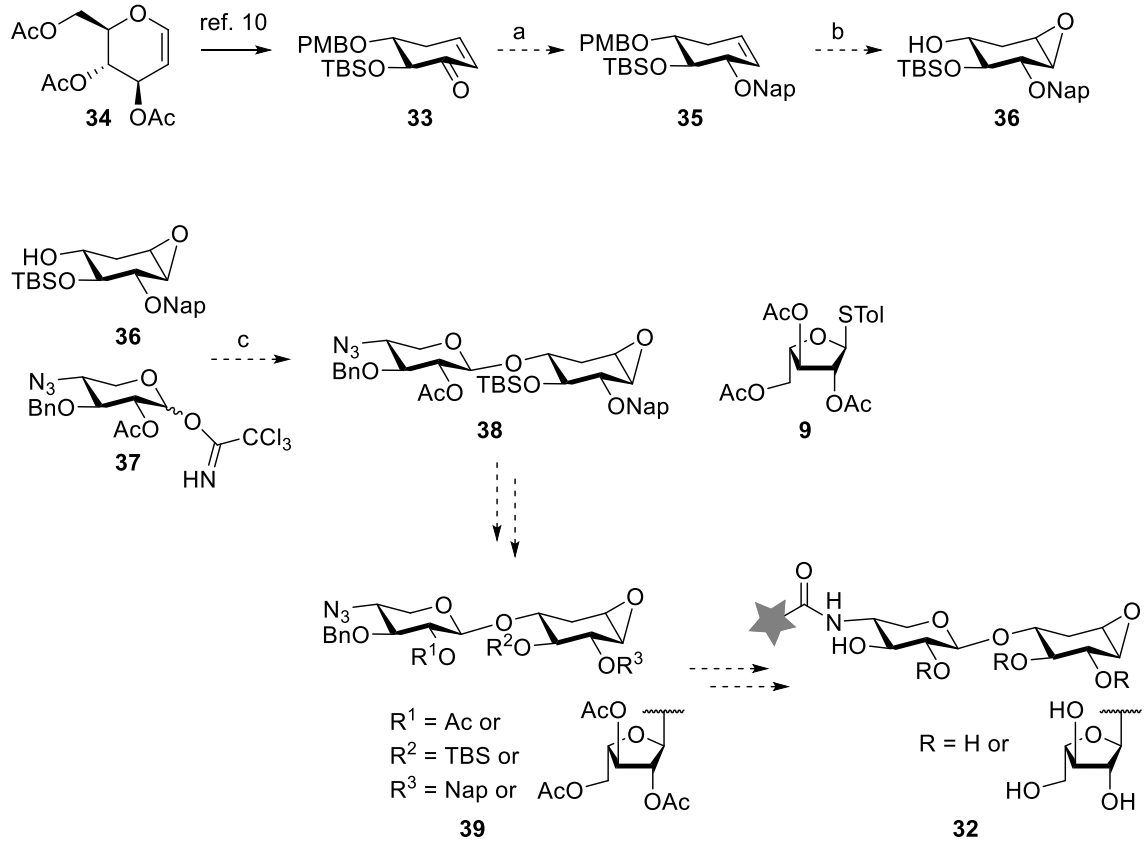
Scheme 5.3| Reagents and conditions: **a**) TMSOTf, NIS, DCM, -70°C to -50°C, 73%. **b**) NaOMe, MeOH, DCM, 78%. **c**) **10**, Tf_2O, Ph_2SO, TTBP, DCM, Et_2O, -70 to -50°C, 30%. Proposed reagents and conditions: **d**) 3HF·Et_3N, THF. **e**) TEMPO, BAIB, DCM, *t*-BuOH, H_2O. **f**) Na(s), *t*-BuOH, NH_3, THF.



Scheme 5.4| Reagents and conditions: **a**) HBr, AcOH, DCM. **b**) i. $NaBH_4$, TBAI, MeCN; ii. NaOMe, MeOH; iii. BnBr, NaH, TBAI, DMF, 44% over 4 steps. **c**) H_2SO_4, H_2O, dioxane; **d**) BzCl, pyr, DCM 67% over 2 steps. **e**) HSPh, $BF_3{\cdot}Et_2O$, DCM, 79%.

A set of activity-based probes with L-arabinofuranose substitution on the xylose configured cyclophellitol (**32**, Scheme 5.5) could also be of interest. The structures could be useful to discover and study arabinoxylanases with specificity for substituted xylosides in the active site such as CTXyl5A[9] from *Clostridium thermocellum*.

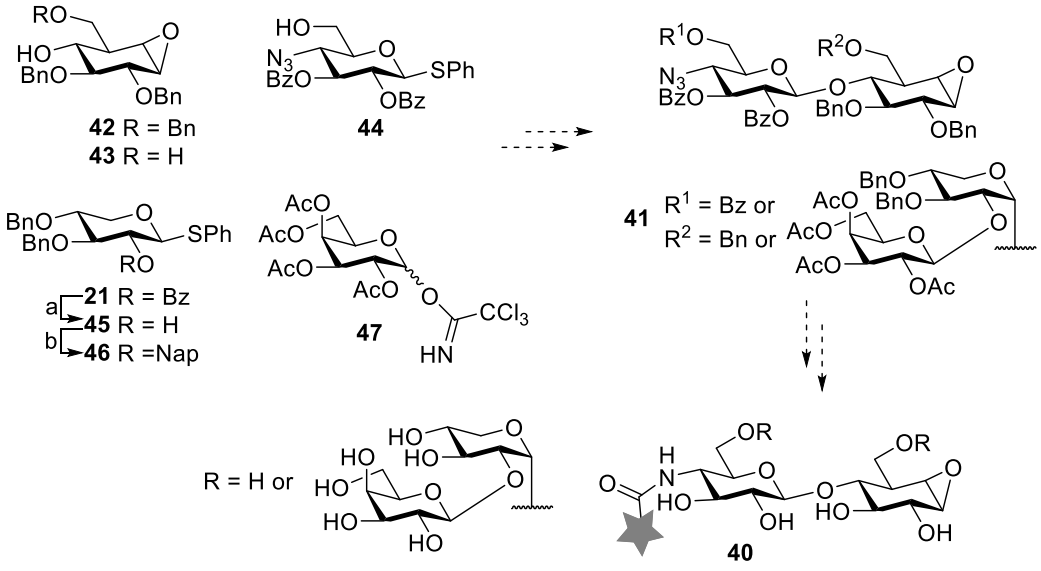
Pivotal to the successful synthesis of these structures is the choice of suitable orthogonal protecting groups on the xylose configured cyclophellitol building block. *p*-Methoxybenzyl (PMB) and silyl ether protected enone **33**[10], of which a synthesis has been reported starting from readily accessible peracetylated glucal (**34**), may be a suitable starting material. Luche reduction[11] could provide the allyl alcohol which can be protected with a naphthyl ether



Scheme 5.5| Proposed reagents and conditions: **a**) i. $NaBH_4$, $CeCl_3$, MeOH; ii. NapBr, NaH, DMF. **b**) i. DDQ, H_2O, DCM; ii. 1,1,1-trifluoroacetone, Oxone, $NaHCO_3$. **c**) TMSOTf, DCM.

providing **35**. Selective removal of the PMB protecting group by either HCl/HFIP[12], DDQ[13] or ceric ammonium nitrate (CAN)[14] followed by epoxide formation with *in situ* generated methyl(trifluoromethyl)dioxirane could provide acceptor **36**. Donor **37**, which could be prepared analogous to **13** (Scheme 5.2), could be reacted with acceptor **36** to provide orthogonally protected pseudodisaccharide **38**. Treatment with a nucleophilic base, fluorine source or DDQ, would allow selective removal of the acetyl, silyl ether or naphthyl ether protecting groups respectively. Glycosylation of the liberated alcohol(s) with arabinofuranose donor **9**[6] could provide a set of protected inhibitors with diverse arabinofuranosylation patterns on the 2O, 3O and 2'O position (**39**). Removal of the remaining protecting groups and reduction of the azide would allow introduction of fluorophores or affinity tags by amide bond formation using the functionalized triethylene glycol spacers and reaction conditions as described in chapter 2.

A final suggestion for future synthesis of ABPs in the biomass utilization field would be the extension of the XG and GX configured probes described in chapter 2 with a β-D-galactose residue because it has been reported that some xyloglucanases have increased reactivity towards more extensively branched substrates (Scheme 5.6).[15] A set of LG, GL and LL configured probes (**40**) may be constructed via **41** using cyclophellitol building blocks **42** and **43,** and 4-deoxy-4-azido-glucoside **44** described in chapter 2 together with orthogonally protected D-xylose configured building blocks **21**, **45** and **46** and D-galactose donor **47**[16].



Scheme 5.6| Reagents and conditions: **a**) NaOMe, MeOH, DCM quant. **b**) NapBr, NaH, DMF, 55%.

Heparanase

Heparanase (HPSE) is the only known human endo-glycosidase that modifies heparan sulfate in the extracellular matrix. Overexpression of HPSE is implicated in a wide range of pathologies inciting the development of HPSE inhibitors and activity assays. A set of mechanism-based covalent and irreversible inhibitors of HPSE and HPSE activity-based probes are presented in *chapter 3*. The HPSE inhibitors are synthesized via common intermediate **48** (Figure 5.2). By selective oxidation and deprotection the set of inhibitors **49**-**53** was synthesized. The relative potency of the inhibitors was assessed by competitive ABBP. 6'O sulfated inhibitor **49** was found to be the most potent inhibitor followed by **50** and **51**. Inhibitors **52** and **53** were found to be significantly less potent.

Inhibitors **54** and **55** may also be synthesized from **48**. With these inhibitors the effect of *N*-sulfation on inhibitor potency could be determined. 6'O and 2'N sulfated inhibitor **54** might be the most potent inhibitor in this series in line with known polysulfated inhibitors.[17–19] Synthesis of aziridine derivatives **56** and **57** with substitution on the aziridine nitrogen might be another possibility towards potent HPSE inhibitors.

Although inhibitor **50** has shown effect *in vivo*, the stability of the glycosidic linkage in the structure might be of concern. Enzymatic hydrolysis would release glucuronic acid configured cyclophellitol which is a covalent inhibitor of retaining exo-β-glucuronidases such as the human enzyme GUSB.[20] This enzymatic hydrolysis of the inhibitor might lead to lower efficacy

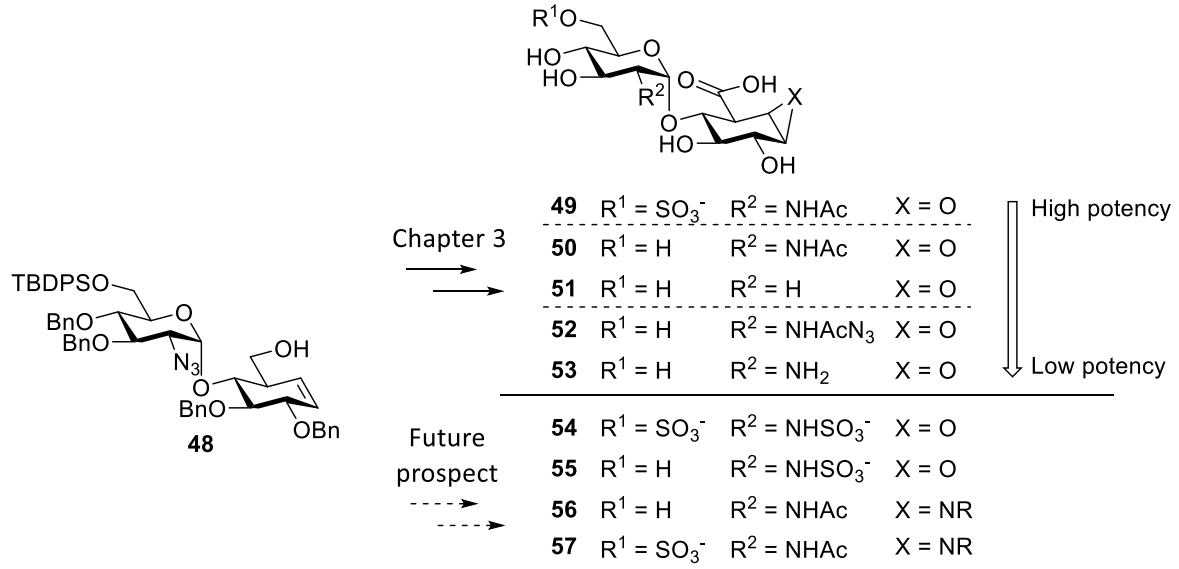
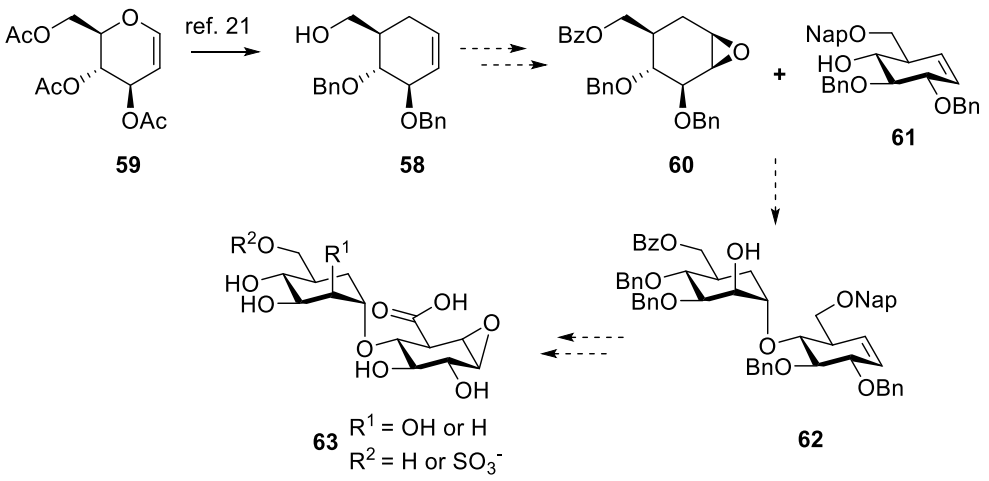


Figure 5.2| Covalent HPSE inhibitors synthesized in this thesis in order of potency as assessed by a competitive ABPP assay with recombinant HPSE and suggestions for possible inhibitors that may be accessible from **48**.

in vivo due to the lower effective concentration and to side effects by inhibition of off target enzymes by the degradation product. An attractive way to stabilize the glycosidic linkage is the replacement of the acetal functionality with an ether linkage by substitution of one of the two oxygen atoms with a methylene group (Scheme 5.7).

Carbaglucal **58**[21] prepared from tri-*O*-acetyl-D-glucal (**59**) could be transformed into properly protected epoxide **60**. Lewis acid catalyzed trans-diaxial opening of the epoxide with cyclophellitol alkene **61** could provide alcohol **62**. The inversion of the axial alcohol in **62** might be difficult due to elimination side reactions. However, since there are mannose configured HPSE inhibitors (mupafostat) it could be argued that this mannose configured compound might also be a sufficiently potent inhibitor. Another possibility is deoxygenation of the 2' alcohol since deoxygenated inhibitor **51** has shown no significant drop in potency compared to 2'NHAc substituted **50**. Naphthyl deprotection followed by oxidation and debenzoylation would allow selective 6'O-sulfation and hydrogenation may provide the set of carba-pseudodisaccharides (**63**).

The conformation of the glycosidic linkage is largely dictated by the exo-anomeric effect.[22] Overlap of one of the exocyclic oxygens lone pairs with the anti-bonding orbital of the polarized O5-C1 bond stabilizes the 'exo-conformation' and reduces the flexibility around the glycosidic bond. This conformational preference is not present in analogues where the acetal group is replaced by an ether such as carbasugars or C-glycosides.[23] Inhibitors bind in one specific conformation leading to an entropy penalty and consequently reduced potency for more flexible inhibitors. This may explain why some stabilized carbohydrate mimics have a lower inhibitor potency compared to the parent structures.[24,25]

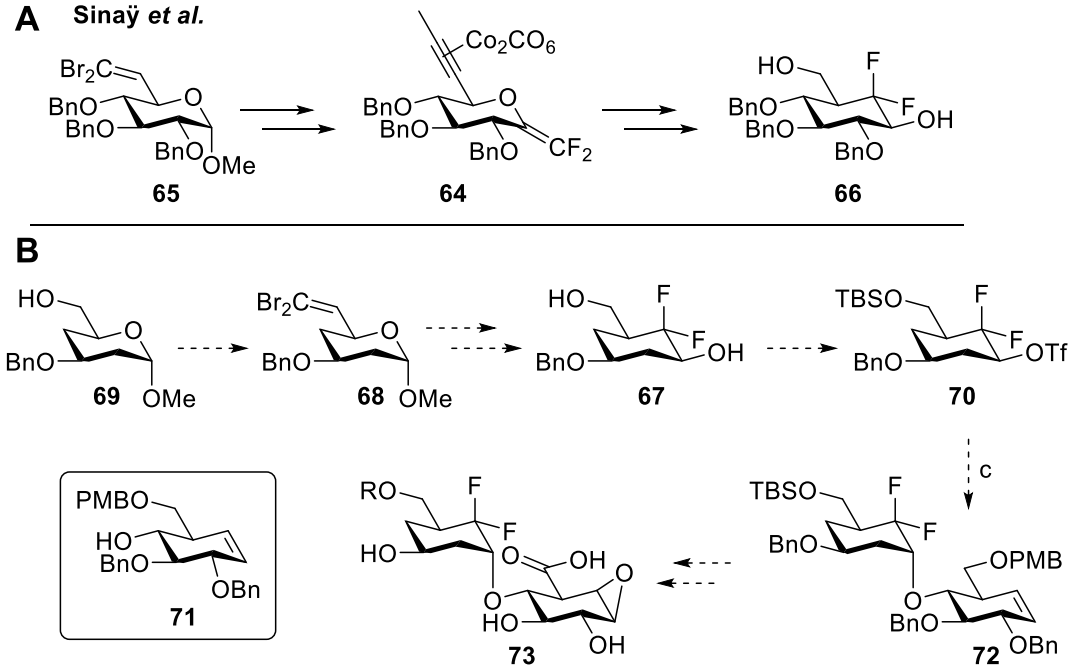


Scheme 5.7| Synthesis of carbasugar analogues of the HPSE inhibitors presented in this thesis.

A way to (partially) restore the exo-anomeric effect is the introduction of a gem-difluorocarbasugar.[26,27] A synthesis of gem-difluorocarba-β-D-glucose has been reported with the triisobutylaluminium (TIBAL) catalyzed sugar-to-carbocycle rearrangement of **64** as key step (Scheme 5.8A).[28] The alkyne-cobalt complex, obtained from dibromoalkene **65**, is electron donating and is needed to stabilize the positive charge during the rearrangement. Ozonolysis followed by stereoselective reduction provides diol **66**.

In a similar way diol **67** may be obtained from dibromide **68** which in turn could be accessible from alcohol **69**[29] (Scheme 5.8B). A silyl ether may be selectively introduced on the primary alcohol in diol **67** followed by generation of the secondary triflate (**70**). The triflate might be substituted with alcohol **71** to obtained **72**. Putative HPSE inhibitor **73** might be obtained via the reaction sequence describes for the inhibitors in chapter 3. Reduced hydrophilicity by removal of the 2' and 4' alcohol groups and hydrolytic stability with preservation of the optimal anomeric conformation might make **73** a potent HPSE inhibitor.

The rest of chapter 3 is dedicated to the development of selective ABPs for HPSE based on the developed covalent inhibitors. Epoxide **74** was synthesized as the first design of a potentially selective HPSE ABP (Figure 5.3). The molecule displayed diminished potency for HPSE (compared to **75**[20]) while maintaining potency for proHPSE. The reaction of proHPSE



Scheme 5.8| A) Sugar to carbocycle rearrangement approach to gem-difluorocarba-β-D-glucose. **B**) Proposed synthesis route towards deoxygenated gem-difluorocarbasugar analogues of HPSE inhibitors synthesized in this thesis.

with inhibitors might be insignificant since proHPSE has no reported enzymatic activity. However, it has been postulated that the beneficial effects of HPSE inhibitors may be the result of the reduction of intracellular HPSE activity by limiting cellular uptake of HPSE.[30] Inhibition of HPSE inside the cell is not directly possible since the most used HPSE inhibitors are not cell permeable. The irreversible 'inhibition' of proHPSE might be a unique opportunity to achieve intracellular HPSE inhibition. Because proHPSE is transported outside the cell during HPSE biosynthesis, the extracellular proHPSE could be irreversibly acylated with cyclophellitol derivatives. Internalization followed by activation by cathepsin L will yield intracellular inhibited HPSE.

Although **74** is not a potent ABP for HPSE the design of covalent HPSE and proHPSE inhibitors based on this monosaccharide mimetic approach remains attractive because the compounds based on this scaffold might have a more suitable physicochemical profile compared to the disaccharide mimics. Scaffolds **76** and **77** would allow the late stage

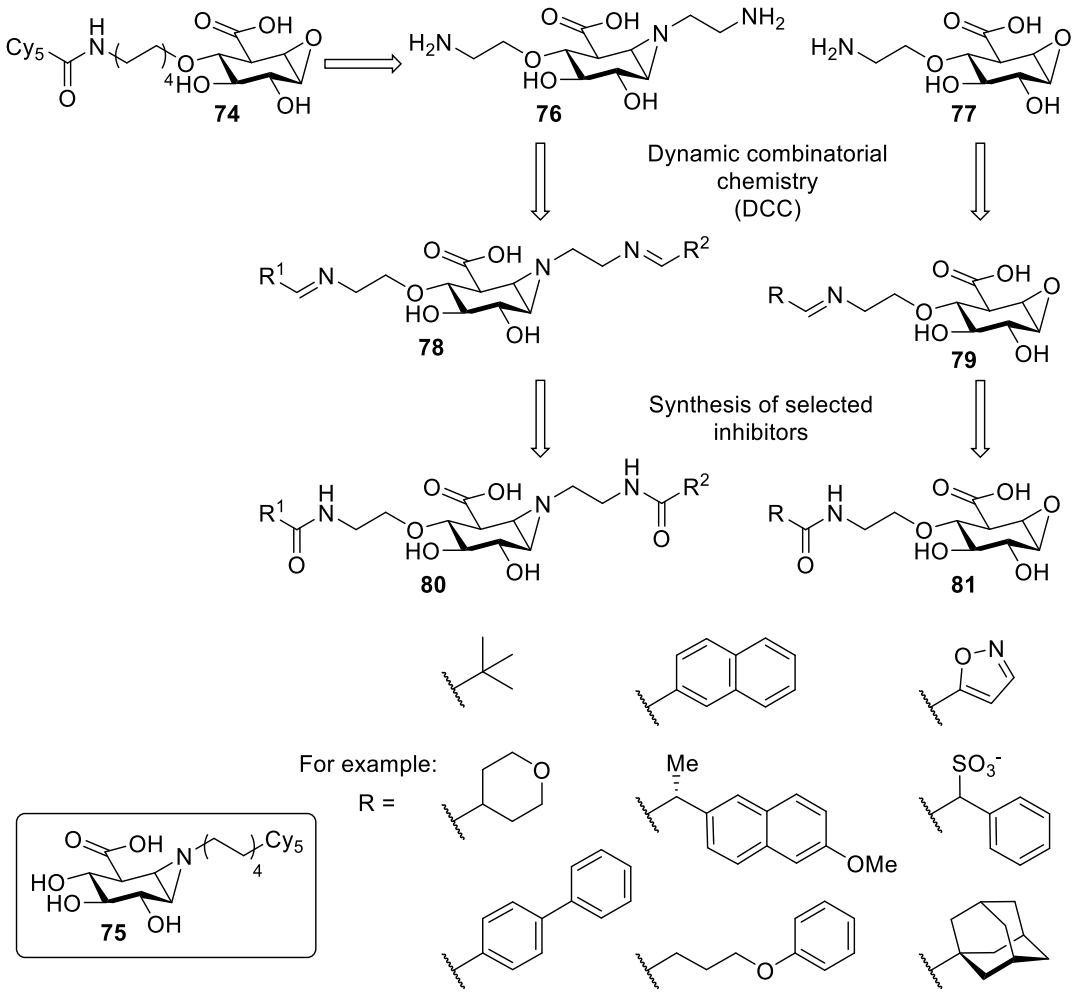


Figure 5.3| Potential irreversible proHPSE and HPSE inhibitor screening with Dynamic combinatorial chemistry followed by chemical synthesis of selected inhibitors.

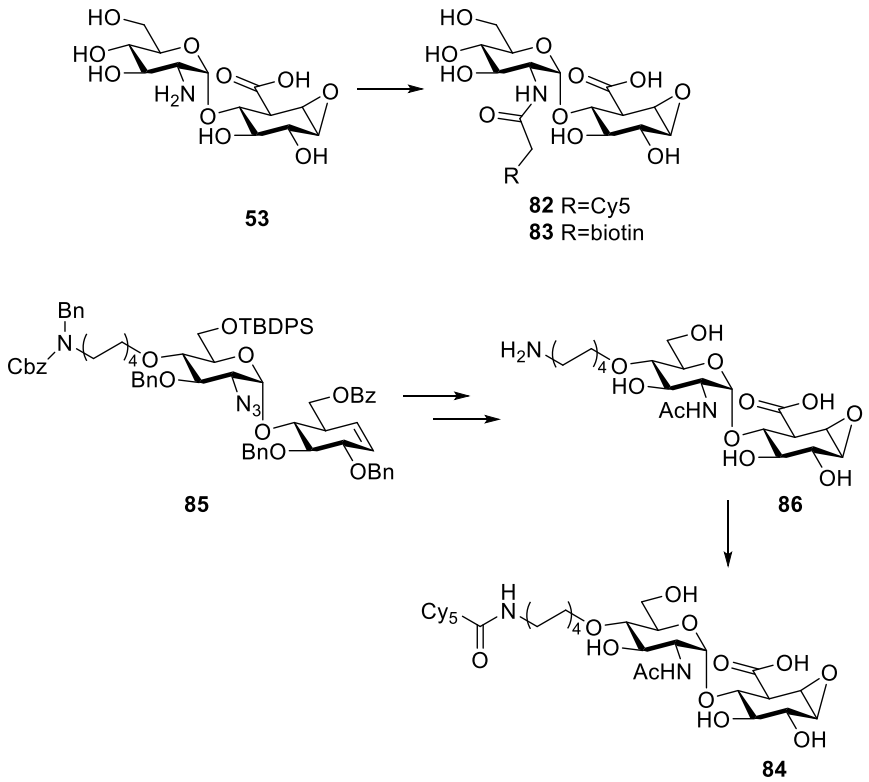
introduction of diverse substituents allowing the synthesis of a library to determine a structure activity relationship. The chemical synthesis and purification of a large enough library of derivatives would be challenging due to the limited availability of the cyclophellitol building block and the difficult purification of the products.

Dynamic combinatorial chemistry (DCC) might be a suitable approach to screen a large chemical space without the need to synthesize many individual derivatives.[31,32] For this approach two sets of fragments with compatible functional groups are mixed. The functional groups, in this case amines **76** and **77** and a set of aldehydes, will reversibly react and form a thermodynamic equilibrium of reaction products, in this case imines **78** and **79**. Addition of the target protein will increase the stability of binders to the protein and shift the equilibrium towards these products. Quenching of the reaction, in this case by reduction of the imine, and analysis of the product distribution may reveal inhibitors for the enzyme.

DCC methodology is normally applied to find reversible inhibitors. However, in this case the amine derivatives (**80** and **81**) are covalent inhibitors of the enzyme. The product will in this case be covalently attached to the enzyme so LC-MS analysis of the resulting products will not be possible. A suitable approach might be the direct detection of binding ligands by X-ray crystallography. [33] DCC could be performed on crystals of HPSE and proHPSE and the resulting electron density maps could directly reveal the relevant binding modes. Stable analogues of the discovered inhibitors could be synthesized and the potency for both HPSE and proHPSE could be determined by ABPP with broad spectrum β-glucuronidase ABP **75**.[20]

Putative HPSE ABPs based on the disaccharide inhibitors are also reported in chapter 3 (Scheme 5.9). Fluorescent and biotin conjugated inhibitors **82** and **83** with substitution on the 2'*N* position were synthesized by selective acylation of inhibitor **53**. **82** was however unable to label HPSE. The 4'O alkylated derivative **84** was prepared via **85** and **86** by a similar reaction sequence as used for the inhibitors. The second amine in the scaffold required orthogonal protection to the azide. The deprotection of the chosen NCbzBn protecting groups was however only partially compatible with the epoxide functionality in the molecule. In the future instead of the NCbzBn other protecting groups such as PMB could be considered to improve the yield of the deprotection step.

84 did show labeling of HPSE. The labeling was evaluated in different lysates and was found to be more selective than labeling with broad spectrum β-glucuronidase probe **75**. The ability



Scheme 5.9| Synthesis of HPSE inhibitor derivatives carrying fluorescent reporter moieties on different positions.

to selectively visualize HPSE activity may provide insight in HPSE related pathology and allow for efficient screening of HPSE inhibitors.

PslG

The chemical synthesis of a putative two step ABP for PslG (**87**, Figure 5.4) is described in *chapter 4*. PslG is reported in the literature as an endo-mannosidase expressed by *Pseudomonas aeruginosa* to hydrolyze the polysaccharide Psl, a constituent of *Pseudomonas Aeruginosa* derived biofilms. Covalent attachment of **87** with recombinant PslG could not be observed by X-ray crystallography and mass spectrometry.

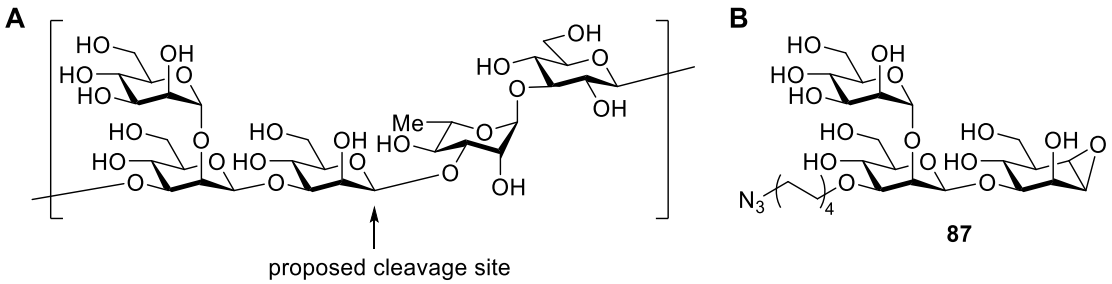
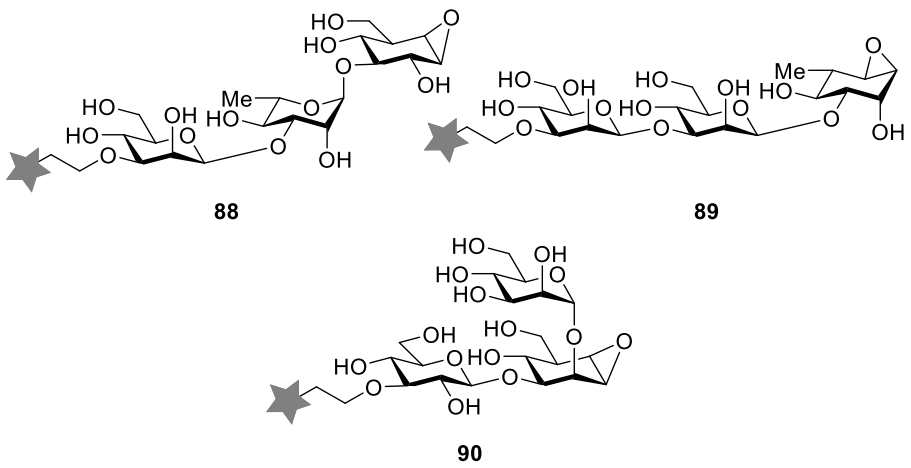


Figure 5.4| A) Repeating pentamer of the Psl polysaccharide and the proposed cleavage site of PslG. **B**) Synthesized activity-based probe based on the proposed catalytic activity of PslG.



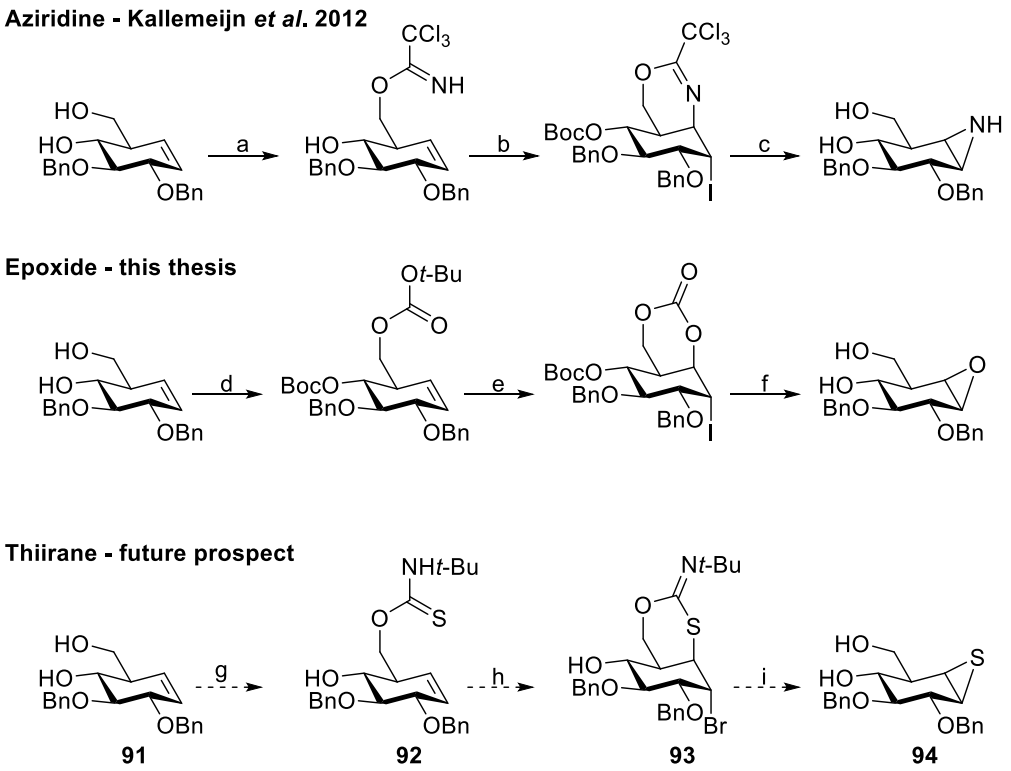
88 **89** **90**

Figure 5.5| Three possible activity-based probes for PslG depending on it classification as a β-glucosidase **88**, an α-L-rhamnosidase **89** or a β-mannosidase **90**. Star denotes various reporter groups.

To confirm the proposed specificity of PslG synthetic or isolated Psl fragments should be reacted with PslG and the reaction products should be analyzed by NMR or MS methods. ABPs could be generated based on the newly found cleavage point. Several examples of potential PslG probes are depicted in figure 5.5.

Efficient (late stage) transformation of an alkene into a reactive warhead is important for the successful synthesis of increasingly complex cyclophellitol-based ABPs. Aziridines[34] and epoxides have been stereospecifically introduced on the cyclophellitol scaffold by intramolecular iodocyclization reactions (Scheme 5.10). The main advantage of this approach is the complete stereo control which is not always achieved with other methods such as epoxide formation using peracid reagents (chapter 3). The methodology may be extended to the stereoselective synthesis of thiiranes to stimulate the inclusion of these warheads in covalent inhibitor and activity-based probe development projects.

Primary alcohol **91** may be reacted with an isothiocyanate[35] or thiocarbamoyl chloride to produce thiocarbamate **92**. Bromocyclization might produce bromide **93** which might be transformed into thiirane **94** by reaction with ammonium hydroxide.[36]

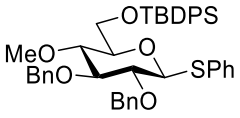


Scheme 5.10| Reagents and conditions used in iodocyclization approaches for stereoselective aziridine and epoxide formation: **a**) CCl_3CN, DBU, DCM, 0°C. **b**) I_2, $NaHCO_3$, H_2O. **c**) i. HCl, MeOH; ii. HCl, dioxane 60°C; iii. $NaHCO_3$, MeOH, 60% over 5 steps. **d**) Boc_2O, DMAP, THF. **e**) NIS, AcOH. **f**) K_2CO_3, MeOH, 75% over 3 steps. Proposed conditions for stereoselective thiirane synthesis: **g**) *t*-BuNCS, pyr.[35] **h**) Br_2, DMF.[36] **i**) NH_3, H_2O.[36]

5.1 Experimental

General experimental procedures are shown in the experimental section of chapter 2.

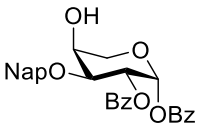
Phenyl 2,3-di-*O*-benzyl-4-*O*-methyl-6-*O*-*t*-butyl-diphenylsilyl-1-thio-β-D-glucopyranoside (10)



Alcohol **11**[7] (0.800 g, 1.16 mmol) was dissolved in THF (2.32 ml). MeI (0.43 ml, 6.95 mmol) and NaH (60% in mineral oil, 0.278 g, 6.95 mmol) were added and the reaction was stirred for 3 hours. The reaction was quenched with MeOH. Water was added and the product was extracted with Et_2O (2x). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. Column chromatography (Et_2O/pentane, 0/1 -> 1/9, v/v) afforded the product (0.820 g, 1.16 mmol, quant.).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 – 7.72 (m, 4H), 7.60 – 7.56 (m, 2H), 7.44 – 7.27 (m, 16H), 7.22 – 7.17 (m, 3H), 4.89 – 4.81 (m, 3H), 4.72 (d, *J* = 10.3 Hz, 1H), 4.65 (d, *J* = 9.6 Hz, 1H), 3.93 (qd, *J* = 11.4, 2.7 Hz, 2H), 3.65 – 3.56 (m, 4H), 3.55 – 3.45 (m, 2H), 3.29 (ddd, *J* = 9.5, 3.5, 1.9 Hz, 1H), 1.09 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.5, 138.4, 136.0, 135.8, 134.3, 133.7, 133.2, 131.8, 129.8, 129.7, 129.0, 128.6, 128.6, 128.3, 128.3, 128.0, 127.9, 127.8, 127.4, 87.5, 87.0, 80.7, 80.1, 79.2, 76.1, 75.5, 62.8, 60.9, 27.0, 19.5. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{43}H_{48}O_5SSiNa$ 727.2884, found 727.2878.

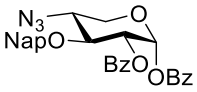
1,2-di-*O*-benzoyl-3-*O*-(2-naphthylmethyl)-L-arabinopyranose (16)



Diol **15**[8] (0.90 g, 2.5 mmol) was dissolved in toluene (25 ml, 0.1 M). Bu_2SnO (0.75 g, 3.0 mmol) was added and the mixture was heated to 90°C and stirred for 1 hour. The mixture was allowed to cool and was subsequently concentrated under reduced pressure. The crude product was dissolved in DMF (20 ml, 0.1 M) and 2-naphthylene bromide (0.66 g, 3.0 mmol), and cesium fluoride (0.61 g, 4.0 mmol) were added and the mixture was stirred overnight at rt. The reaction mixture was then poured into water (200 ml) and the product was extracted with Et_2O (3 x 70 ml). The combined organic layers were washed with brine, then dried over $MgSO_4$, filtered and concentrated. Column chromatography (pentane/EtOAc, 5/1-> 2/1, v/v) afforded the product as an oil (0.69 g, 1,38 mmol, 55%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.93 – 7.28 (m, 19H), 6.60 (d, *J* = 3.5 Hz, 1H), 5.79 (dd, *J* = 9.6, 3.5 Hz, 1H), 4.89 (q, *J* = 12.2 Hz, 2H), 4.30 – 4.18 (m, 2H), 4.03 (m, 2H), 2.77 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 165.3, 164.5, 134.3, 133.3, 133.1, 132.9, 132.9, 129.6, 129.5, 129.3, 129.2, 128.3, 128.2, 127.7, 127.6, 126.8, 126.1, 126.0, 125.6, 91.3, 74.1, 72.0, 68.9, 66.5, 64.1, 60.2, 14.0. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{30}H_{26}O_7Na$ 521.1571, found 521.1567.

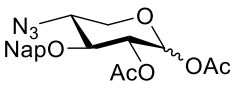
1,2-di-*O*-benzoyl-3-*O*-(2-naphthylmethyl)-4-deoxy-azido-α-D-xylopyranose (17)



Alcohol **16** (6.2 g, 12.4 mmol) was co-evaporated with toluene and was subsequently dissolved in dry DCM (120 ml, 0.1 M), then pyridine (1.5 ml, 18.6 mmol) was added and the mixture was cooled to -20 ^{0}C. Tf_2O (2.5 ml, 14.9 mmol) was added dropwise and the reaction was stirred for 1 hour at -20 ^{0}C. The reaction was diluted with DCM (250 ml), then washed with H_2O (3 x 120 ml), then dried over $MgSO_4$, filtered and concentrated at rt. The crude was then dissolved in DMF (31 ml) and sodium azide (8.06 g, 124 mmol) was added, the mixture was stirred overnight at rt. The reaction was diluted with H_2O (300 ml), then extracted with Et_2O (3 x 100 ml), and the combined organic layers were washed with brine, then dried over $MgSO_4$, filtered and concentrated. Column chromatography (pentane/EtOAc, 8/1, v/v) afforded the product as an oil (5.8 g, 10.6 mmol, 90%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 – 7.97 (m, 2H), 7.89 – 7.84 (m, 2H), 7.77 – 7.73 (m, 1H), 7.69 – 7.60 (m, 4H), 7.53 – 7.29 (m, 8H), 6.58 (d, *J* = 3.5 Hz, 1H), 5.43 (dd, *J* = 9.7, 3.6 Hz, 1H), 5.09 – 4.92 (m, 2H), 4.18 (t, *J* = 9.3 Hz, 1H), 3.99 – 3.87 (m, 2H), 3.76 – 3.69 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 165.4, 164.6, 134.7, 133.9, 133.6, 133.3, 133.2, 129.9, 129.8, 129.3, 129.1, 128.8, 128.6, 128.5, 128.0, 127.8, 127.3, 126.2, 126.2, 126.2, 90.9, 78.2, 75.6, 72.3, 62.4, 61.1. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{30}H_{25}N_3O_6Na$ 546.1636, found 546.1633.

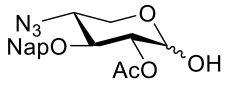
1,2-di-*O*-acetyl-3-*O*-(2-naphthylmethyl)-4-deoxy-azido-D-xylopyranose (18)



17 (3.3 g, 6.2 mmol) was dissolved in DCM/MeOH (1/1, v/v, 30 ml), then NaOMe solution in MeOH was added (0.28 ml, 1.2 mmol) and stirred overnight. The reaction was quenched with $Et_3N{\cdot}HCl$ (0.3 g, 1.9 mmol), after which the mixture was concentrated and co-evaporated with dioxane (3x). The crude was then dissolved in pyridine/Ac_2O (2:1, v/v, 30 ml) and stirred overnight at rt. The reaction was quenched with H_2O (4 ml) at 0 ^{0}C and the diluted with EtOAc (120 ml). The mixture was then washed with HCl (1M aq. 3x 40 ml), $NaHCO_3$ (sat. aq. 3x 40 ml) and brine, dried over $MgSO_4$, filtered and concentrated. Column chromatography (pentane/EtOAc, 9/1, v/v) afforded the product as an oil (2.2 g, 5.2 mmol, 88% over 2 steps).

For α isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 – 7.75 (m, 4H), 7.52 – 7.41 (m, 3H), 6.23 (d, *J* = 3.6 Hz, 1H), 5.06 – 4.81 (m, 3H), 3.91 – 3.81 (m, 2H), 3.77 – 3.67 (m, 1H), 3.66 – 3.53 (m, 1H), 2.14 (s, 3H), 1.94 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.8, 169.5, 169.2, 135.1, 134.8, 133.3, 133.2, 128.5, 128.4, 128.1, 128.0, 127.8, 127.0, 126.7, 126.4, 126.4, 126.3, 126.2, 125.9, 125.7, 90.0, 78.2, 75.5, 71.8, 62.1, 60.9, 21.0, 20.7. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{20}H_{21}N_3O_6Na$ 422.1323, found 422.1327.

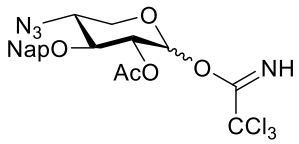
2-*O*-acetyl-3-*O*-(2-naphthylmethyl)-4-deoxy-azido-D-xylopyranose (19)



Ester **18** (2.2 g, 5.4 mmol) was dissolved in THF (11 ml, 0.5 M), then piperidine (0.6 ml, 5.9 mmol) was added and the mixture was stirred overnight at rt. The mixture was diluted with EtOAc (120 ml) and washed with HCl (1 M aq. 3x 40 ml) and brine, then dried over $MgSO_4$, filtered and concentrated. Column chromatography (pentane/EtOAc, 3/1, v/v) over silica afforded the product as a white solid (1.3 g, 3.6 mmol, 68%, α/β 3/1).

Major isomer: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 – 7.76 (m, 4H), 7.50 – 7.40 (m, 3H), 5.36 (d, *J* = 3.5 Hz, 1H), 4.99 (t, *J* = 11.3 Hz, 1H), 4.93 – 4.87 (m, 1H), 4.85 (dd, *J* = 9.5, 3.5 Hz, 1H), 3.94 (t, *J* = 9.2 Hz, 1H), 3.78 – 3.70 (m, 2H), 3.67 – 3.59 (m, 1H), 2.95 (s, 1H), 2.01 (d, *J* = 8.7 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.4, 135.3, 133.4, 133.2, 128.5, 128.4, 128.1, 127.9, 127.8, 127.0, 126.8, 126.4, 126.3, 126.3, 126.2, 125.9, 125.9, 90.9, 77.8, 75.4, 73.6, 61.2, 60.1, 21.0. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{18}H_{19}N_3O_5Na$ 380.1217, found 380.1215.

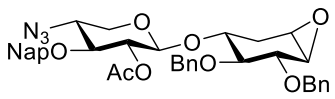
2-*O*-acetyl-3-*O*-(2-naphthylmethyl)-4-deoxy-azido-D-xylopyranosyl 2,2,2-trichloroacetimidate (13)



Lactol **19** (0.12 g, 0.32 mmol) was dissolved in DCM (1.6 ml, 0.2 M), then CCl_3CN (64 µL, 0.64 mmol) and K_2CO_3 (88 mg, 0.64 mmol) were added and the mixture was stirred overnight at rt. The mixture was filtered over celite and concentrated. Column chromatography (pentane/EtOAc, 5/1, v/v) afforded the product as an oil (0.15 g, 0.29 mmol, 90%, 1/2, α/β).

β: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (s, 1H), 7.88 – 7.77 (m, 4H), 7.52 – 7.44 (m, 3H), 5.86 (d, *J* = 5.8 Hz, 1H), 5.31 – 5.25 (m, 1H), 4.96 – 4.89 (m, 2H), 4.28 – 4.23 (m, 1H), 3.75 – 3.71 (m, 2H), 3.58 – 3.51 (m, 1H), 1.98 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 161.2, 134.7, 128.5, 128.1, 127.9, 127.1, 127.0, 126.4, 126.3, 126.1, 125.9, 96.0, 78.0, 74.2, 69.5, 63.2, 59.1, 20.9.

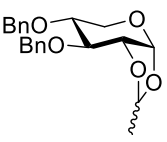
4-*O*-(2-*O*-acetyl-3-*O*-(2-naphthylmethyl)-4-deoxy-azido-D-xylopyranosyl)-2,3-di-*O*-benzyl-xylocyclophellitol (12)



Donor **13** (0.13 g, 0.26 mmol) and acceptor **14** (61 mg, 0.19 mmol) were combined and co-evaporated with toluene. The mixture was dissolved in dry DCM (2 ml), 3Å molecular sieves were added and the mixture was stirred for 1 hour before cooling to -30°C. TMSOTf (9 µL, 0.05 mmol) was added and the mixture was stirred for 1 hour at -30 ^{0}C. The reaction was quenched with Et_3N (12 µL), diluted with DCM (50 ml), washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (pentane/EtOAc, 6/1 -> 4/1, v/v) afforded the product as a white solid (77 mg, 0.12 mmol, 61%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 – 7.73 (m, 4H), 7.50 – 7.39 (m, 3H), 7.39 – 7.22 (m, 12H), 5.00 – 4.88 (m, 3H), 4.81 (d, *J* = 11.6 Hz, 1H), 4.74 – 4.63 (m, 3H), 4.42 (d, *J* = 6.7 Hz, 1H), 3.99 (dd, *J* = 12.0, 4.9 Hz, 1H), 3.82 – 3.70 (m, 2H), 3.65 – 3.57 (m, 1H), 3.53 (t, *J* = 8.3 Hz, 1H), 3.38 (dd, *J* = 10.1, 7.7 Hz, 1H), 3.26 – 3.21 (m, 1H), 3.14 – 3.05 (m, 2H), 2.49 (m, 1H), 1.95 (s, 3H), 1.71 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.5, 138.9, 137.9, 134.9, 133.3, 133.2, 128.7, 128.64, 128.57, 128.42, 128.39, 128.31, 128.25, 128.14, 128.09, 128.06, 127.97, 127.8, 127.5, 127.0, 126.3, 126.23, 126.19, 126.0, 99.4, 82.6, 80.0, 79.5, 75.3, 73.7, 73.5, 72.6, 63.0, 60.4, 54.0, 53.4, 30.0, 21.0. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{38}H_{39}N_3O_8Na$ 688.2629, found 688.2625.

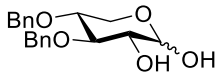
3,4-di-*O*-benzyl-1,2-*O*-ethylidene-α-D-xylopyranose (29)



1,2,3,4-Tetra-*O*-acetyl-D-xylopyranose (**27**) (8.60 g, 27,0 mmol) was dissolved in DCM (180 ml, 0.15 M) and cooled to 0°C. HBr (33% in AcOH, 11 ml) was added and the mixture was stirred for 2 hours. TLC (2/1, pent/EtOAc) indicated complete consumption of the starting material and the mixture was diluted with DCM and washed subsequently with H_2O, $NaHCO_3$ (2x) and brine. The organic phase was dried with $MgSO_4$ and concentrated *in vacuo* at 30°C.

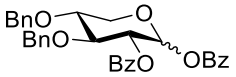
The crude bromide (**28**) was dissolved in MeCN (135 ml, 0.2 M). TBAI (6.0 g, 16.2 mmol) and $NaBH_4$ (1.23 g, 32.4 mmol) were added and the mixture was stirred overnight. The reaction was quenched with water and extracted with EtOAc (3x). The combined organic layers were washed with HCl (1M, 2x), $NaHCO_3$ and brine, dried with $MgSO_4$ and concentrated *in vacuo.*
The crude product was dissolved in MeOH (90 ml, 0.3 M), NaOMe (5.4 M in MeOH, 1.1 ml) was added and the mixture was stirred at rt until TLC (2/1, pent/EtOAc) indicated full conversion of starting material into a polar product. The mixture was neutralized with amberlite (H^+), filtered and concentrated under reduced pressure.
DMF (10 ml) was added and the solution was coevaporated with toluene (3x). DMF (135 ml, 0.2 M), TBAI (0.87 g, 2.7 mmol) and BnBr (12,84 ml, 108,1 mmol) were added and the mixture was cooled to 0°C before adding NaH (60% in mineral oil, 3.78 g, 94,6 mmol). The mixture was stirred overnight before it was quenched with MeOH. Et_2O and H_2O were added. The layers were separated and the aqueous layer was extracted with Et_2O (3x). The combined organic layers were washed with H_2O (2x) and brine (2x), dried with $MgSO_4$, filtered and concentrated *in vacuo*. The products were purified by column chromatography (Et_2O/pentane, 1/9 -> 1/4, v/v) yielding the products as a colorless oil as a 3/1 mixture of diastereomers (4.27 g, 11.9 mmol, 44%).
Mayor isomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.26 (m, 10H), 5.45 – 5.40 (m, 1H), 5.09 (q, *J* = 4.9 Hz, 1H), 4.67 – 4.52 (m, 4H), 4.06 – 4.02 (m, 1H), 3.88 (dd, *J* = 4.0, 3.0 Hz, 1H), 3.82 – 3.78 (m, 1H), 3.77 – 3.64 (m, 2H), 1.47 (d, *J* = 4.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 138.1, 137.8, 128.6, 128.6, 128.5, 128.5, 128.0, 128.0, 127.9, 127.9, 127.9, 100.8, 97.0, 78.3, 76.4, 74.8, 72.2, 71.9, 60.2, 20.3. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{21}H_{24}O_5Na$ 379.1516, found 379.1513.

3,4-di-*O*-benzyl-D-xylopyranose (30)



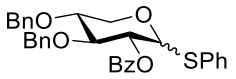
29 (2.57 g, 7.21 mmol) was dissolved in dioxane (36 ml, 0.2 M). H_2SO_4 (0.5 M aq, 36 ml) was added and the mixture was stirred at 75°C overnight. TLC (9/1, v/v, pent/EtOAc) indicated consumption of starting material. H_2O was added and the mixture was extracted with EtOAc (2x). The combined organic layers were washed with $NaHCO_3$ (aq. sat.) and brine, dried with $MgSO_4$, filtered and concentrated under reduced pressure. This yielded the product which was used without further purification (2.24 g, 6.81 mmol).
Mayor isomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.27 (m, 10H), 4.98 – 4.91 (m, 1H), 4.73 – 4.62 (m, 3H), 4.54 (d, *J* = 11.7 Hz, 1H), 4.04 – 3.97 (m, 1H), 3.91 – 3.82 (m, 2H), 3.81 – 3.73 (m, 1H), 3.68 – 3.62 (m, 1H), 3.50 – 3.45 (m, 1H), 3.34 (d, *J* = 10.3 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 137.8, 137.2, 128.8, 128.7, 128.7, 128.3, 128.2, 128.2, 128.1, 127.9, 127.8, 92.5, 76.1, 74.1, 73.1, 71.7, 69.8, 62.5. HRMS (ESI) m/z: $[M+Na]^+$ calc for $C_{19}H_{22}O_5Na$ 353.1359, found 353.1354.

1,2-di-*O*-benzoyl-3,4-di-*O*-benzyl-D-xylopyranose (31)



Crude diol **30** (2.25 g, 6.81 mmol) was dissolved in DCM (13.6 ml, 0.5 M). The solution was cooled to 0°C. Pyridine (2.74 ml, 34.1 mmol) and BzCl (2.77 ml, 23.8 mmol) were added add and the reaction was stirred overnight. H_2O was added and the mixture was extracted with EtOAc (2x). The combined organic layers were washed with $NaHCO_3$ (aq. sat.) and brine, dried with $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (Et_2O/pentane, 1/9 -> 1/4 , v/v) yielded the product (2.62 g, 4.86 mmol, 67% over 2 steps, α/β 5/4).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 – 8.02 (m, 6H), 8.00 – 7.96 (m, 2H), 7.70 – 7.64 (m, 1H), 7.62 – 7.50 (m, 5H), 7.46 – 7.36 (m, 15H), 7.33 – 7.23 (m, 10H), 6.64 (d, *J* = 3.6 Hz, 1H), 6.13 (d, *J* = 6.3 Hz, 1H), 5.56 (dd, *J* = 7.5, 6.3 Hz, 1H), 5.44 (dd, *J* = 9.8, 3.6 Hz, 1H), 4.99 (d, *J* = 11.2 Hz, 1H), 4.94 – 4.70 (m, 7H), 4.32 – 4.19 (m, 2H), 4.04 – 3.85 (m, 5H), 3.73 (dd, *J* = 11.9, 7.9 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.5, 165.4, 165.0, 164.7, 138.1, 137.8, 137.8, 133.7, 133.7, 133.4, 130.2, 130.2, 129.9, 129.9, 129.8, 129.5, 129.5, 129.4, 129.0, 128.7, 128.7, 128.6, 128.5, 128.5, 128.4, 128.2, 128.1, 128.1, 128.0, 127.8, 127.8, 93.0, 90.8, 79.0, 78.7, 77.6, 76.3, 75.5, 74.3, 74.0, 72.9, 72.0, 70.6, 63.5, 62.6.

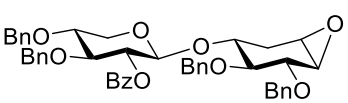
Phenyl 2-O-benzoyl-3,4-di-O-benzyl-1-thio-D-xylopyranoside (21)



Benzoyl ester **31** (1.07 g, 1.99 mmol) was dissolved in DCM (9.9 ml, 0.1 M). Thiophenol (0.22 ml, 2.19 mmol) was added and the mixture was cooled to 0°C. $BF_3{\cdot}Et_2O$ (0.05 ml, 0.40 mmol) was added and the mixture was slowly warmed to room temperature overnight. The next day the mixture was cooled to 0°C and more $BF_3{\cdot}Et_2O$ (0.05 ml, 0.40 mmol) was added and the mixture was stirred at 0°C for 5 hours. Thiophenol (0.10 ml and $BF_3{\cdot}Et_2O$ (0.05 ml, 0.40 mmol) were added and the solution was warmed to room temperature overnight. The solution was diluted with DCM and quenched with Et_3N. The solution was washed with NaOH (1M, aq. 4x) and brine, dried over $MgSO_4$ filtered and the volatiles were removed under reduced pressure. Column chromatography (Et_2O/pentane, 1/19 -> 3/17, v/v) provided the product as an anomeric mixture (0.830 g, 1.57 mmol, 79%, 1/2 α/β).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 – 8.07 (m, 1H), 8.06 – 8.03 (m, 2H), 7.64 – 7.57 (m, 2H), 7.51 – 7.41 (m, 7H), 7.36 – 7.18 (m, 23H), 5.85 (d, *J* = 5.2 Hz, 1H), 5.34 – 5.27 (m, 2H), 4.95 (d, *J* = 8.0 Hz, 1H), 4.90 – 4.84 (m, 1H), 4.80 – 4.62 (m, 6H), 4.26 (dd, *J* = 11.8, 4.5 Hz, 1H), 4.17 (dd, *J* = 11.6, 9.8 Hz, 1H), 4.04 (dd, *J* = 9.2, 8.1 Hz, 1H), 3.86 – 3.79 (m, 2H), 3.77 – 3.70 (m, 2H), 3.53 – 3.45 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.8, 165.4, 138.0, 137.9, 133.8, 133.5, 133.3, 132.3, 131.9, 130.1, 130.0, 130.0, 129.7, 129.1, 129.0, 128.6, 128.5, 128.5, 128.4, 128.1, 128.1, 128.0, 128.0, 128.0, 127.9, 127.8, 127.5, 87.0, 86.2, 80.4, 79.0, 77.6, 76.9, 75.3, 74.6, 73.5, 73.1, 73.1, 71.6, 66.1, 61.7. HRMS (ESI) m/z: $[M+NH_4]^+$ calc for $C_{32}H_{34}O_5SN$ 544.2152, found 544.2151.

4-*O*-(2-*O*-benzoyl-3,4-di-*O*-benzyl-β-D-xylopyranosyl)-2,3-di-*O*-benzyl-xylocyclophellitol (22)

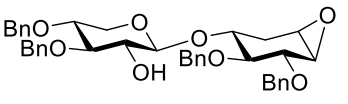


Thioglycoside donor **21** (87 mg, 0.17 mmol) and acceptor **14** (49 mg, 0.15 mmol) were co-evaporated with toluene. DCM (1.0 ml) and NIS (40 mg, 0.18 mmol) were added and the solution was cooled to -70°C. TMSOTf (0.015 mmol, 0.1 ml of 0.15 M solution in DCM) was added and the mixture was warmed to -50°C over 30 minutes. Et_3N (0.05 ml) was added and solution was diluted with EtOAc and washed with $NaHCO_3$ (aq. sat.), $Na_2S_2O_3$ (aq. 1 M) and brine, dried over $MgSO_4$, filtered and the volatiles were removed under reduced pressure.

Column chromatography (Et_2O/pentane, 1/9 -> 3/7 ,v/v) afforded the product (81 mg, 0.11 mmol, 73%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 – 7.98 (m, 2H), 7.60 – 7.53 (m, 1H), 7.43 (t, *J* = 7.8 Hz, 2H), 7.38 – 7.22 (m, 15H), 7.14 (tdd, *J* = 9.6, 4.5, 1.8 Hz, 5H), 5.22 – 5.17 (m, 1H), 4.95 (d, *J* = 10.9 Hz, 1H), 4.77 (d, *J* = 11.5 Hz, 1H), 4.71 – 4.54 (m, 7H), 3.97 (dd, *J* = 12.0, 3.9 Hz, 1H), 3.80 – 3.68 (m, 4H), 3.33 (dd, *J* = 10.1, 7.7 Hz, 1H), 3.26 – 3.18 (m, 1H), 3.09 (d, *J* = 3.4 Hz, 1H), 3.04 (d, *J* = 3.6 Hz, 1H), 2.41 (ddd, *J* = 14.6, 5.3, 2.4 Hz, 1H), 1.56 (ddd, *J* = 14.6, 10.5, 1.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3, 138.9, 138.0, 138.0, 137.9, 133.2, 130.0, 129.8, 128.6, 128.5, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 127.9, 127.9, 127.9, 127.7, 127.4, 99.7, 82.5, 80.3, 79.4, 77.5, 75.3, 74.4, 73.8, 73.4, 73.2, 73.2, 63.3, 54.0, 53.4, 29.9. HRMS (ESI) m/z: $[M+NH_4]^+$ calc for $C_{46}H_{50}O_9N$ 760.3480, found 760.3477.

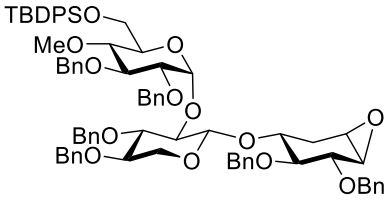
4-*O*-(3,4-di-*O*-benzyl-β-D-xylopyranosyl)-2,3-di-*O*-benzyl-xylocyclophellitol (23)



Benzoyl ester **22** (79 mg, 0.11 mmol) was dissolved in DCM/MeOH (1.0 ml, 7/3, v/v). NaOMe (5.4 M, 0.01 ml) was added and the solution was stirred overnight. The reaction was quenched with NH_4Cl and the volatiles were removed under reduced pressure. Column chromatography (EtOAc/pentane, 1/4 -> 3/7, v/v) provided the product (53 mg, 0.083 mmol, 78%).

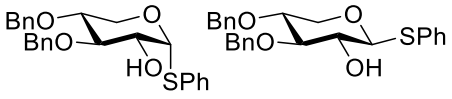
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 21H), 4.87 (d, *J* = 11.1 Hz, 1H), 4.79 (s, 2H), 4.77 (s, 0H), 4.73 – 4.64 (m, 3H), 4.60 (d, *J* = 11.7 Hz, 1H), 4.44 – 4.39 (m, 1H), 4.00 – 3.94 (m, 1H), 3.88 – 3.79 (m, 2H), 3.57 – 3.50 (m, 3H), 3.42 (dd, *J* = 10.2, 7.7 Hz, 1H), 3.29 – 3.18 (m, 2H), 3.13 (d, *J* = 3.6 Hz, 1H), 2.87 (s, 1H), 2.63 (ddd, *J* = 14.6, 5.3, 2.4 Hz, 1H), 1.82 (ddd, *J* = 14.6, 10.4, 1.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.6, 138.0, 137.8, 128.6, 128.6, 128.6, 128.3, 128.3, 128.1, 128.1, 128.0, 128.0, 127.9, 127.6, 100.7, 82.4, 81.6, 79.8, 76.9, 75.1, 74.5, 73.4, 73.0, 72.0, 70.9, 62.9, 53.9, 53.7, 30.6. HRMS (ESI) m/z: $[M+NH_4]^+$ calc for $C_{39}H_{46}O_8N$ 656.3218, found 656.3213.

4-*O*-(2-O-(2,3-di-*O*-benzyl-4-*O*-methyl-6-*O*-*t*-butyl-diphenylsilyl-α-D-glucopyranosyl)3,4-di-*O*-benzyl-β-D-xylopyranosyl)-2,3-di-*O*-benzyl-xylocyclophellitol (24)



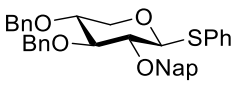
Donor **10** (67 mg, 0.095 mmol) TTBP (78 mg, 0.32 mmol) and Ph_2SO (19 mg, 0.094 mmol) were co-evaporated with toluene and dissolved in DCM (0.5 ml). Et_2O (0.5 ml) and 3Å molecular sieves were added and the mixture was stirred for 1 hour. The solution was cooled to -65°C and Tf_2O (0.07 ml of a 1.2 M solution in DCM, 0.081 mmol) was added. The reaction was allowed to warm to -50°C over 25 minutes and was cooled to -70°C. Acceptor **23** (40 mg, 0.063 mmol) was added as a solution in DCM (0.4 ml). The mixture was allowed to warm to -30°C and was quenched with Et_3N.
The solution was filtered, dilute with DCM and washed with $NaHCO_3$ (aq. sat.), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (EtOAc/pentane, 1/19 -> 3/17, v/v) afforded the product (24 mg, 0.019 mmol, 30%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 – 7.62 (m, 4H), 7.45 – 7.14 (m, 36H), 5.51 (d, *J* = 3.6 Hz, 1H), 4.98 – 4.86 (m, 5H), 4.77 – 4.68 (m, 5H), 4.64 (d, *J* = 11.6 Hz, 1H), 4.55 (d, *J* = 11.6 Hz, 1H), 4.50 (d, *J* = 7.0 Hz, 1H), 4.08 – 3.98 (m, 2H), 3.88 (ddd, *J* = 14.1, 11.1, 5.1 Hz, 2H), 3.79 (d, *J* = 7.6 Hz, 1H), 3.69 – 3.52 (m, 10H), 3.40 (dd, *J* = 11.6, 2.9 Hz, 1H), 3.35 (dd, *J* = 10.2, 7.6 Hz, 1H), 3.12 (dd, *J* = 11.7, 9.3 Hz, 1H), 3.04 (d, *J* = 3.6 Hz, 1H), 2.78 (dd, *J* = 3.5, 2.1 Hz, 1H), 2.45 (ddd, *J* = 14.5, 5.3, 2.4 Hz, 1H), 1.37 – 1.35 (m, 1H), 1.02 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.9, 138.7, 138.1, 138.1, 138.1, 135.9, 135.7, 133.9, 133.6, 129.6, 129.5, 128.7, 128.6, 128.6, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.6, 127.5, 100.3, 96.3, 82.5, 82.2, 81.4, 80.5, 79.8, 79.6, 79.2, 75.8, 75.7, 75.4, 74.7, 74.3, 73.4, 73.3, 71.2, 71.2, 63.8, 62.4, 60.6, 53.9, 53.4, 29.8, 28.9, 27.0, 19.5. HRMS (ESI) m/z: $[M+NH_4]^+$ calc for $C_{76}H_{88}O_{13}SiN$ 1250.6019, found 1250.6016.

Phenyl 3,4-di-*O*-benzyl-1-thio-α/β-D-xylopyranoside (45)



Thioglycoside **21** (354 mg, 0.672 mmol, α/β 1/2) was dissolved in MeOH/DCM (7 ml, 6/1, v/v). A catalytic amount of NaOMe was added and the mixture was stirred for 2 days. The reaction was quenched with AcOH and the volatiles were removed under reduced pressure. The crude was dissolved in a minimal amount of toluene and loaded on a silica column. Elution (Et_2O/pentane, 1/9 -> 1/4, v/v) provided the product as two separate isomers: α (200 mg, 0.473 mmol 70%) and β (90 mg, 0.213 mmol 32%).
α: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 – 7.47 (m, 2H), 7.36 – 7.20 (m, 13H), 5.29 (d, *J* = 2.6 Hz, 1H), 4.65 (d, *J* = 12.1 Hz, 3H), 4.57 (d, *J* = 11.8 Hz, 1H), 4.10 (ddd, *J* = 12.3, 4.7, 1.0 Hz, 1H), 3.94 (dddd, *J* = 9.9, 5.4, 2.7, 0.9 Hz, 1H), 3.85 (dd, *J* = 12.4, 2.7 Hz, 1H), 3.78 (td, *J* = 5.2, 1.0 Hz, 1H), 3.52 (tdd, *J* = 4.8, 2.7, 0.9 Hz, 1H), 3.44 (d, *J* = 9.8 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 137.8, 137.3, 135.1, 131.0, 129.0, 129.0, 128.6, 128.1, 128.1, 127.9, 127.8, 127.1, 88.0, 76.8, 74.5, 73.4, 71.9, 70.8, 64.2.
β: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 – 7.47 (m, 2H), 7.38 – 7.21 (m, 13H), 4.91 (d, *J* = 6.0 Hz, 1H), 4.82 (d, *J* = 11.6 Hz, 1H), 4.73 (d, *J* = 11.6 Hz, 1H), 4.61 (s, 2H), 4.27 (dd, *J* = 11.9, 3.3 Hz, 1H), 3.71 (q, *J* = 6.1 Hz, 1H), 3.62 (t, *J* = 6.2 Hz, 1H), 3.55 (td, *J* = 6.4, 3.3 Hz, 1H), 3.49 (dd, *J* = 11.9, 6.6 Hz, 1H), 3.29 (d, *J* = 6.3 Hz, 1H).^{13}C NMR (126 MHz, $CDCl_3$) δ 138.1, 137.6, 134.2, 131.9, 129.0, 128.6, 128.5, 128.1, 127.9, 127.9, 127.8, 127.6, 89.0, 79.4, 75.9, 73.9, 72.4, 70.9, 63.6. HRMS (ESI) m/z: $[M+NH_4]^+$ calculated for $C_{25}H_{30}O_4S$ 440.1890, found 440.1888.

Phenyl 2-*O*-(2-naphthylmethyl)-3,4-di-*O*-benzyl-1-thio-α/β-D-xylopyranoside (46)



Alcohol **45** (192 mg, 0.454 mmol, only β) was dissolved in DMF (2.3 ml, 0.2 M). NapBr (151 mg, 0.682 mmol), TBAI (17 mg, 0.045 mmol) and NaH (27 mg, 0.682 mmol) were added and the solution was stirred overnight. The mixture was quenched and diluted with water and extracted with Et_2O (3x). The combined organic layers were washed with water (3x) and brine, dried over $MgSO_4$, filtered and concentrated under reduced

pressure. Column chromatography (Et_2O/pentane, 1/19 -> 1/9, v/v) provided the product (138 mg, 0.245 mmol, 55%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 – 7.75 (m, 4H), 7.55 – 7.51 (m, 3H), 7.50 – 7.44 (m, 2H), 7.35 – 7.26 (m, 13H), 5.01 (d, *J* = 10.6 Hz, 1H), 4.94 – 4.89 (m, 2H), 4.85 (d, *J* = 11.0 Hz, 1H), 4.75 – 4.69 (m, 2H), 4.64 (d, *J* = 11.6 Hz, 1H), 4.10 – 4.05 (m, 1H), 3.71 – 3.62 (m, 2H), 3.53 – 3.47 (m, 1H), 3.27 (dd, *J* = 11.5, 9.3 Hz, 1H).^{13}C NMR (126 MHz, $CDCl_3$) δ 138.6, 138.2, 135.7, 133.9, 133.4, 133.2, 132.1, 129.1, 128.6, 128.6, 128.3, 128.1, 128.1, 128.0, 127.9, 127.8, 127.7, 127.0, 126.4, 126.2, 126.0, 88.6, 85.5, 80.6, 77.9, 75.8, 75.7, 73.4, 67.6.HRMS (ESI) m/z: $[M+Na]^+$ calculated for $C_{36}H_{34}O_4SNa$ 585.2070, found 585.2069.

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Nederlandse samenvatting

Glycosidases zijn enzymen die de hydrolyse van een glycosidische binding katalyseren. De enzymen kunnen onder andere geclassificeerd worden als endo- en exo-glycosidases. De exo-varianten hydrolyseren de eindstandige koolhydraat van een langere keten, terwijl de endo-varianten activiteit op de gehele keten kunnen hebben. Glycosidases spelen een belangrijke rol in uiteenlopende processen in levende systemen en zijn dus overal in de natuur te vinden.

Natuurlijk voorkomende glycosidases kunnen toegepast worden om op een milde en selectieve wijze glycosiden af te breken, bijvoorbeeld om producten te maken uit biomassa of om schadelijke biofilms op te lossen. Ook kunnen in sommige gevallen mensen met bepaalde aandoeningen gebaat zijn bij de remming van een glycosidase. Vanwege al deze toepassingen wordt er veel onderzoek gedaan naar het vinden, karakteriseren en remmen van glycosidases met uiteenlopende specificiteit.

In dit proefschrift staat de chemische synthese en de biologische toepassing van irreversibel covalente remmers van endo-glycosidases beschreven. De structuur van de ontworpen en gesynthetiseerde moleculen is opgebouwd rond de in de natuur gevonden enzymremmer cyclophellitol. De structuur van cyclophellitol wordt door de glycosidase herkent als een koolhydraat, maar in tegenstelling tot een koolhydraat bevat cyclophellitol een epoxide die fungeert als electrofiele val. Hierdoor kan het enzym wel een covalente interactie met de remmer aangaan maar deze niet meer hydrolyseren en wordt de activiteit van het enzym blijvend geremd. De covalente remmers kunnen uitgerust worden met een label waardoor de enzymen die covalent aan de remmers gebonden zijn gedetecteerd kunnen worden. Dit soort moleculen, die selectief enzymen met een bepaalde activiteit detecteerbaar kunnen maken, staan in de Engelstalige vakliteratuur bekend als ‘activity-based probes’ (ABPs). Tot op heden zijn de meeste ABPs ontwikkeld voor exo-glycosidases. In dit proefschrift ligt de nadruk op de ontwikkeling van ABPs en covalente remmers van verschillende endo-glycosidases.

In hoofdstuk 1 wordt de voorkennis, opgedaan in voorgaande studies met cyclophellitol ABPs, samengevat aan de hand van voorbeelden van ontwerpstrategieën en toepassingen van op cyclophellitol gebaseerde ABPs. Het hoofdstuk beschrijft ook de huidige kennis over de mechanistische en conformationele aspecten van de reactie tussen cyclophellitol derivaten en verschillende klassen van — voornamelijk exo — glycosidases. Aan het eind van het

hoofdstuk wordt een overzicht gegeven van de meest gangbare experimenten die met een ABP uitgevoerd kunnen worden.

Hoofdstuk 2 richt zich op glycosidases die gebruikt kunnen worden om stugge biomassa om te zetten in makkelijk te verwerken bouwstenen. Om voldoende binding met de endo-glycosidase enzymen te bewerkstelligen moeten de ABPs een uitgebreider herkenningselement bevatten dan de bekende ABPs voor exo-glycosidases. In het hoofdstuk wordt de chemische synthese van ABPs voor cellulases, xyloglucanases en α-xylosidases beschreven. Deze ABPs kunnen de opsporing, karakterisatie en activiteitscontrole van de enzymen vergemakkelijken. De cellulase en xyloglucanase ABPs worden gesynthetiseerd via chemische glycosyleringen, onder pre-activatie condities, op cyclophellitol nucleofielen. De activiteit van de gesynthetiseerde endo-glycosidase ABPs bleek, in de enzymmengsels uitgescheiden door de schimmel *Aspergillus niger,* geen overlap te vertonen met exo-glycosidase ABPs. Dit nieuwe ABP ontwerp blijkt dus ABPs selectief voor endo-glycosidases op te kunnen leveren. Ook lieten de ABPs ontwikkeld voor cellulases en xyloglucanases verschil in specificiteit zien, wat erop kan wijzen dat met verschillend gesubstitueerde ABPs aparte subklassen van enzymen onderscheiden kunnen worden.

In hoofdstuk 3 worden drie verschillende ontwerpen voor ABPs en een set covalente remmers voor heparanase (HPSE) gesynthetiseerd. HPSE is de enige bekende endo-glycosidase in de extracellulaire matrix met activiteit voor heparansulfaat. Hoge HPSE activiteit wordt in verband gebracht met een verscheidenheid aan aandoeningen. De detectie en remming van HPSE activiteit zou dus kunnen bijdragen aan de diagnose en behandeling van verschillende ziekten. De remmers werden gesynthetiseerd uit één gezamenlijk tussenproduct en verschillen in substitutie op de -2 positie waardoor een structuur-activiteitsrelatie voor deze positie gemaakt kan worden. Het is gebleken dat sulfatering op de 6'O en acetylering of verwijdering van de 2'N positief effect hebben op de HPSE remming. Het installeren van een 2'amine of een 2' azido acetyl bleek geen potente remmers op te leveren. Twee van de remmers zijn effectief bevonden in verschillende HPSE inhibitie testen. Deze remmers kunnen gebruikt worden om het effect van HPSE inhibitie in detail te bestuderen en als beginpunt voor de ontwikkeling van een nieuwe klasse HPSE remmers voor medicinale toepassingen. ABPs met een label op de 2'N bleken, in lijn met de activiteit van de remmers, ook niet actief te zijn en cyclophellitol met een label op de 4O was niet selectief voor HPSE. Uiteindelijk bleek de disaccharide ABP met het label op de 4'O selectief voor HPSE.

In hoofdstuk 4 wordt de synthese beschreven van een azide gelabeld cyclophellitol derivaat gelijkend op de chemische structuur van de polysacharide, genaamd Psl. Deze polysaccharide wordt gevonden in de biofilms van de pathogene bacterie *Pseudomonas aeruginosa*. Het molecuul werd gesynthetiseerd door middel van een selectieve β-mannosylering op een mannose geconfigureerd cyclophellitol alkeen derivaat. Vervolgens werd het volledige molecuul ontschermd waarna een stereoselectieve epoxidering met een peroxycarbonzuur in loog het gewenste product opleverde. Het door dezelfde bacterie tot expressie gebrachte enzym PslG is in staat deze biofilms af te breken. De verwachting was dat de gesynthetiseerde ABP zou kunnen reageren met dit enzym. Dit was echter niet het geval, wat aanleiding zou kunnen geven om de karakterisatie van PslG te herzien. De ABP zou gebruikt kunnen worden om andere enzymen te vinden die met deze specificiteit Psl zouden kunnen afbreken.

Afsluitend staat in hoofdstuk 5 een samenvatting van de verkregen resultaten met aanbevelingen voor vervolgonderzoek.

List of publications

Mechanism-based heparanase inhibitors based on the cyclophellitol scaffold
de Boer, C.; Schröder, S. P.; Armstrong, Z.; Lit, V.; Ruijgrok, G.; Boyango, I.; Barash, U.; Kayal, Y.; Ilan, N.; Vlodavsky, I.; Wu, L.; Overkleeft, H. S. and Davies, G. J.
manuscript in preparation.

Activity-based protein profiling of retaining α-amylases in complex biological samples
Chen, Y.*; Armstrong, Z.*; Artola, M.; de Boer, C.; Florea B. I.; Aerts, J. M. F. G.; Overkleeft, H. S. and Davies, G. J.
manuscript in preparation.

On the origin of regioselectivity in palladium-catalyzed oxidation of glucosides
Wan, I. C.; Hamlin, T. A.; Eisink, N. N. H. M.; Marinus, N.; de Boer, C.; Vis, C.; Codée, J. D. C. Witte, M. D.; Minnaard, A. J. and Bickelhaupt, M. F.
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Glycosylated cyclophellitol-derived activity-based probes and inhibitors for cellulases
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An overview of activity-based probes for glycosidases
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Dynamic and functional profiling of xylan-degrading enzymes in aspergillus secretomes using activity-based probes
Schröder, S. P.*; de Boer, C.*; McGregor, N. G. S.; Rowland, R. J.; Moroz, O.; Blagova, E.; Reijngoud, J.; Arentshorst, M.; Osborn, D.; Morant, M. D.; Abbate, E.; Stringer, M. A.; Krogh, K. B. R. M.; Raich, L; Rovira, C.; Berrin, J.; van Wezel, G. P.; Ram, A. F. J.; Florea, B. I.; van der Marel, G. A.; Codée, J. D. C.; Wilson, K. S.; Wu, L.; Davies, G. J. and Overkleeft, H. S.
ACS Cent. Sci. **2019**, *5,* (6), 1067–1078.

The synthesis of cyclophellitol-aziridine and its configurational and functional isomers
Jiang, J.; Artola, M.; Beenakker, T. J. M.; Schröder, S. P.; Petracca, R.; de Boer, C.; Aerts, J. M. F. G.; van der Marel, G. A.; Codée, J. D. C. and Overkleeft, H. S.
Eur. J. Org. Chem. **2016**, 22, 3671–3678.

*equal contribution

Curriculum vitae

Casper de Boer was born in 1991 in Heemskerk, the Netherlands. He attended high school at the Kennemer College in Beverwijk. After graduating with the specialization 'Natuur en Techniek' in 2009, he started the bachelor Molecular Science and Technology at Leiden University and Delft University of Technology. His chemistry major was finalized with a thesis on the chemical synthesis of oligorhamnosides using new pivaloate-ester-like protecting groups. The thesis work was performed in the Bio-organic chemistry group under supervision of dr. Riccardo Castelli, dr. Jeroen Codée and prof. dr. Gijs van der Marel. During his minor he studied organic chemistry at the Eidgenössische Technische Hochschule in Zürich, Switzerland, in the context of the Erasmus exchange programme.

After obtaining his bachelor degree in 2012, he spend one year as a board member of the chemistry study association, Chemisch Dispuut Leiden (CDL). He continued his education with the master program Chemistry at Leiden University and he obtained his Master's degree in 2015 (cum laude). During the master program he participated in the Netherlands Research School for Chemical Biology honours program. He also performed an internship at the University of Groningen on the total synthesis of Taiwaniaquinoids under supervision of Jeffrey Buter in the group of prof. dr. Adriaan Minnaard. In the summer of 2014 he participated in the NWO student competition and was awarded a grant with two colleagues to perform a research project on the enzymatic reduction of CO_2. His master thesis, on the chemical synthesis of a fragment of the capsular polysaccharide of Enterococcus faecalis, was performed in the Bio-organic synthesis group at Leiden University under supervision of Qingju Zhang, dr. Jeroen Codée and prof. dr. Gijs van der Marel.

In 2015 he started the research described in this thesis in the same group under supervision of prof. dr. Overkleeft, prof. dr. Jeroen Codée and prof. dr. Gijs van der Marel in close collaboration with the group of prof. dr. Gideon Davies at the University of York, United Kingdom. Parts of the research were presented at NWO Chains 2016 and 2017 in Veldhoven, the Netherlands, the European Carbohydrate Symposium 2017 in Barcelona, Spain, and 2019 in Leiden, the Netherlands, the Annual ABPP meeting 2018 in Oxford, United Kingdom and 2019 in Leuven, Belgium, and at the Molecular Machines Nobel Prize Conference 2017, Groningen, The Netherlands.