# Inhibitors and probes targeting endo-glycosidases

Boer, C. de

# 1

# 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.

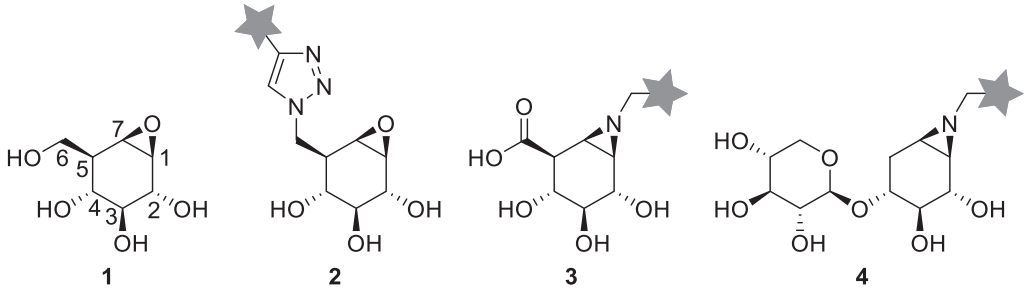


**Figure 1.1|** The structure of cyclophellitol and synthetic cyclophellitol derivatives. Stars denote various reporter moieties (biotin, fluorophore). For ease of comparison with parent glycosides carbon atoms in cyclophellitol and analogous compounds are numbered as if they were carbohydrates throughout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

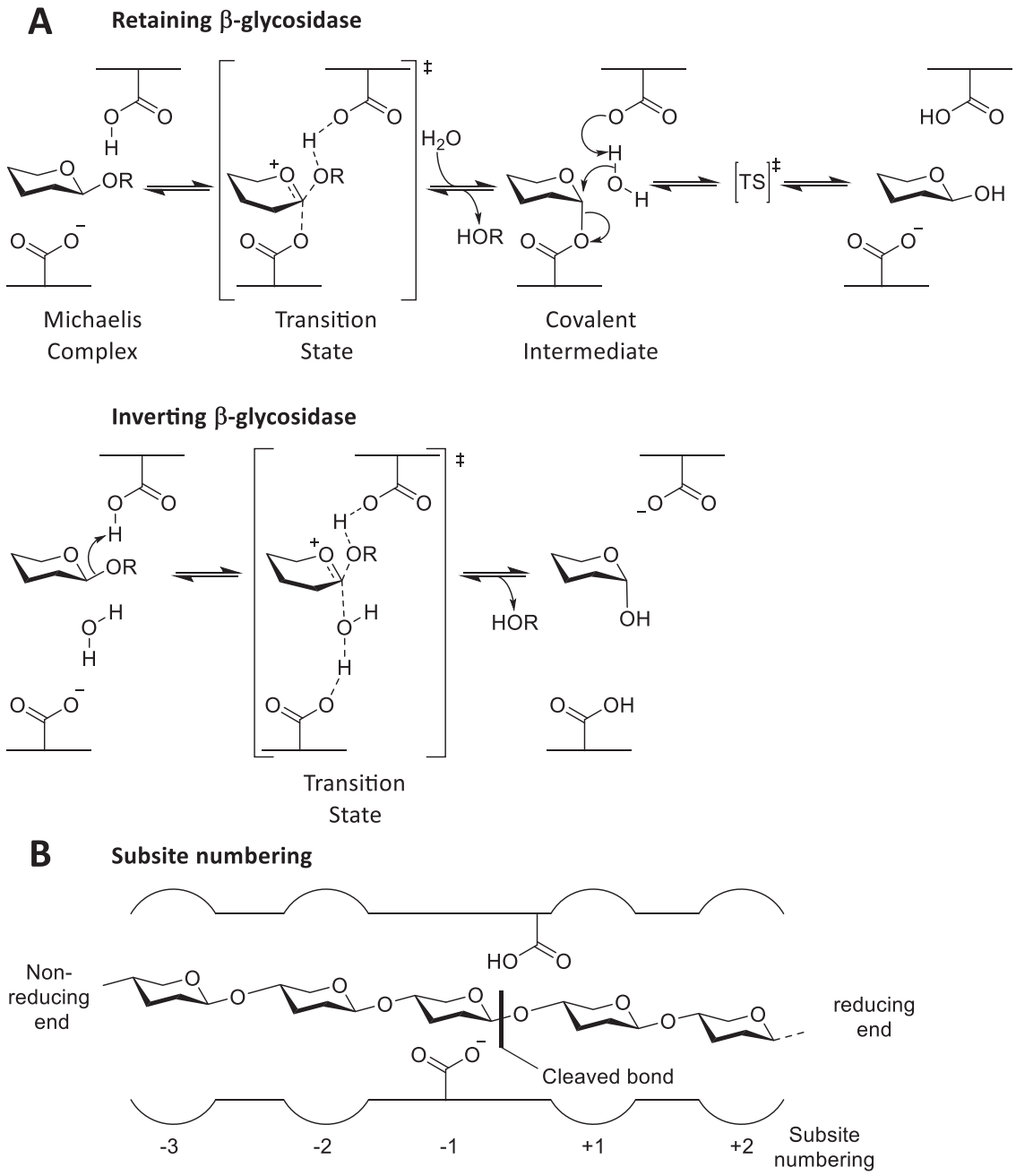


**Figure 1.2 | A**) The catalytic mechanism employed by most retaining and inverting glycosidases. **B**) The conventional numbering of glycosidase subsites.

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

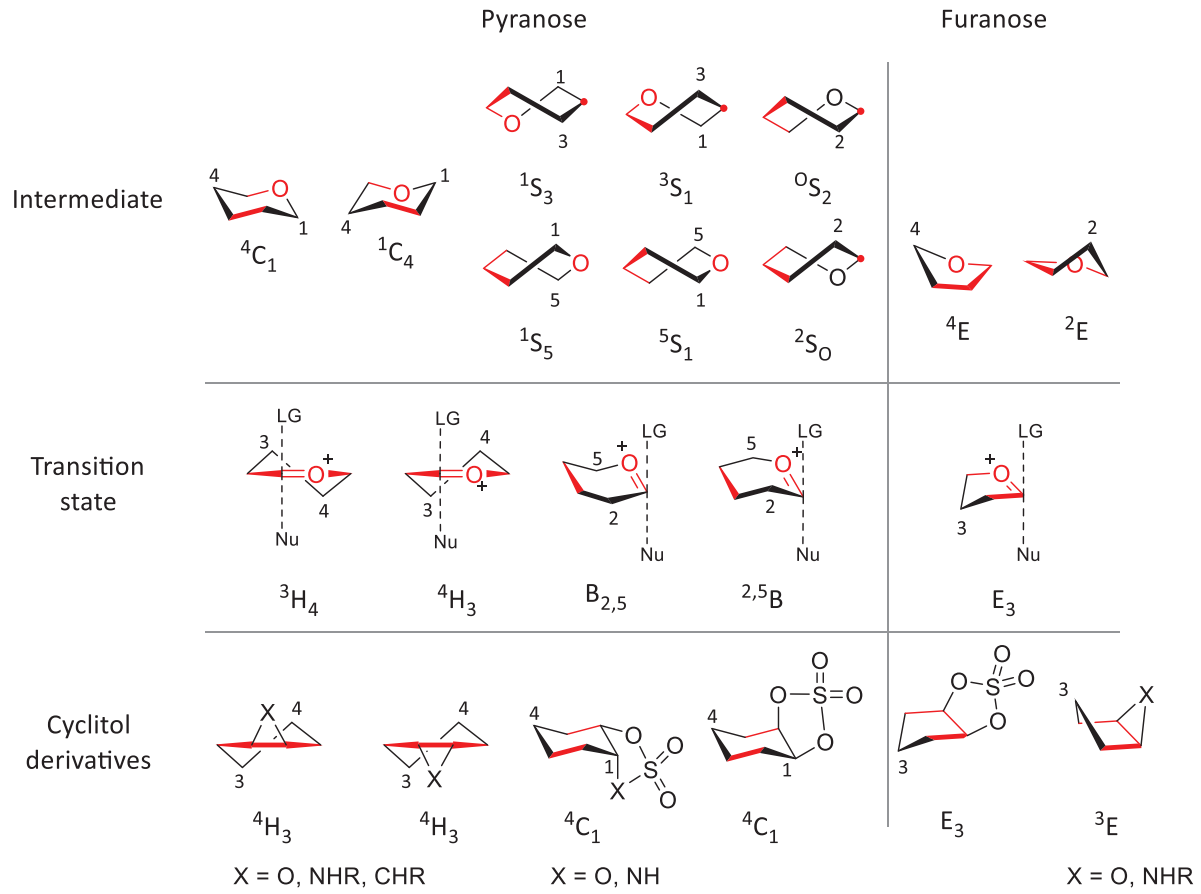


**Figure 1.3|** Conformations of pyranoses, furanose and cyclitol derivatives discussed in this chapter. Atoms and bonds in the same plane are red. Atoms above or below the plane are black and numbered. For transition states the general trajectory of the leaving group (LG) and catalytic nucleophile (Nu)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

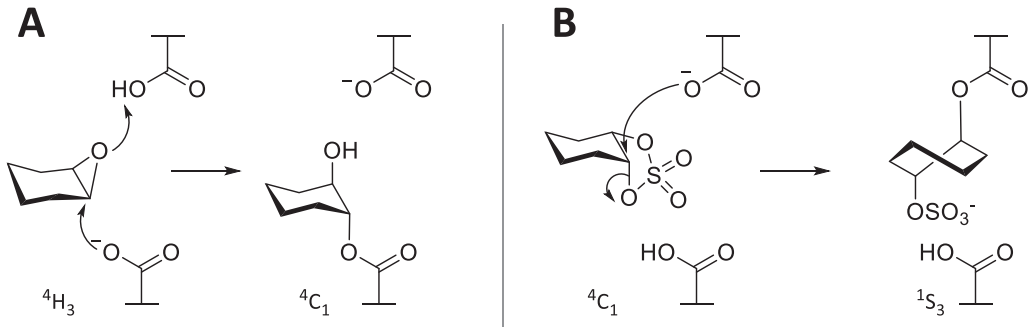


**Figure 1.4| A**) Mechanism of inactivation of a retaining β-glycosidase by a cyclitol epoxide derivative. **B**) Mechanism of inactivation of a retaining α-glycosidase by a cyclitol cyclic sulfate.

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.

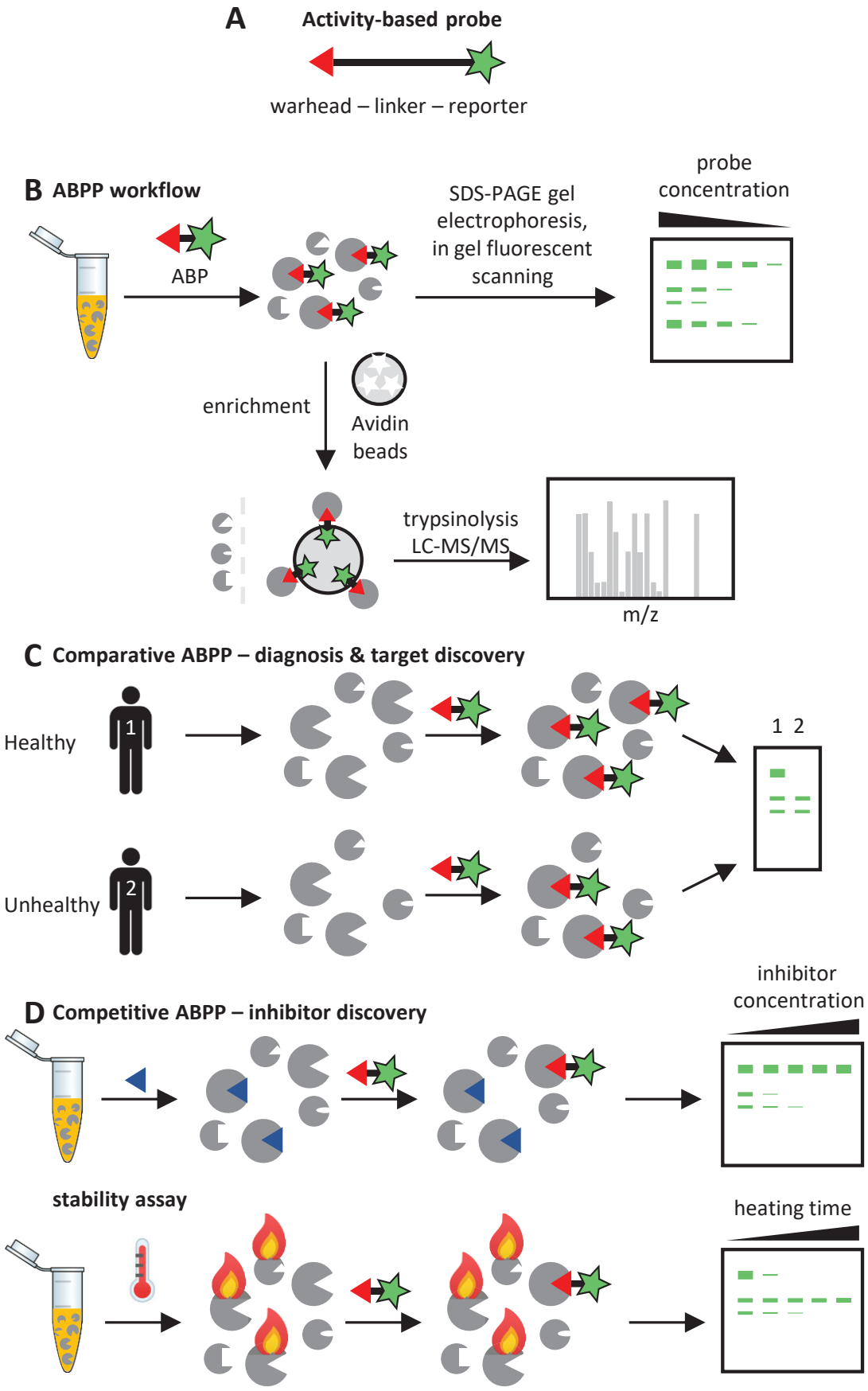


**Figure 1.5|** General structure of an activity-based probe and a schematic representation of several activity-based protein profiling experiments.

### *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.

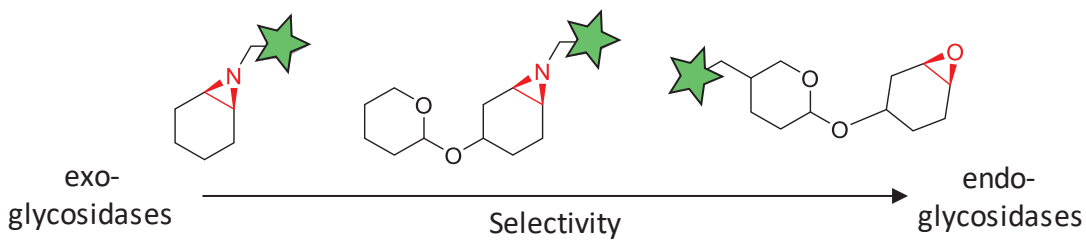


**Figure 1.6|** Design of selective endo-glycosidase inhibitors based on previous scaffolds.

## References


1. Atsumi, S.; Umezawa, K.; Iinuma, H.; Naganawa, H.; Nakamura, H.; Iitaka, Y.; Takeuchi, T. Production, Isolation and Structure Determination of a Novel β-Glucosidase Inhibitor, Cyclophellitol, from Phellinus Sp. *J. Antibiot.* **1990**, *43* (1), 49–53.
2. Withers, S. G.; Umezawa, K. Cyclophellitol: A Naturally Occurring Mechanism-Based Inactivator of Beta-Glucosidases. *Biochem. Biophys. Res. Commun.* **1991**, *177* (1), 532–537.
3. Gloster, T. M.; Madsen, R.; Davies, G. J. Structural Basis for Cyclophellitol Inhibition of a β-Glucosidase. *Org. Biomol. Chem.* **2007**, *5* (3), 444–446.
4. Wolfenden, R.; Lu, X.; Young, G. Spontaneous Hydrolysis of Glycosides. *J. Am. Chem. Soc.* **1998**, *120* (27), 6814–6815.
5. Witte, M. D.; Kallemeijn, W. W.; Aten, J.; Li, K.-Y.; Strijland, A.; Donker-Koopman, W. E.; van den Nieuwendijk, A. M. C. H.; Bleijlevens, B.; Kramer, G.; Florea, B. I.; et al. Ultrasensitive in Situ Visualization of Active Glucocerebrosidase Molecules. *Nat. Chem. Biol.* **2010**, *6* (12), 907-913.
6. Artola, M.; Kuo, C.-L.; Lelieveld, L. T.; Rowland, R. J.; van der Marel, G. A.; Codée, J. D. C.; Boot, R. G.; Davies, G. J.; Aerts, J. M. F. G.; Overkleeft, H. S. Functionalized Cyclophellitols Are Selective Glucocerebrosidase Inhibitors and Induce a Bona Fide Neuropathic Gaucher Model in Zebrafish. *J. Am. Chem. Soc.* **2019**, *141* (10), 4214-4218.
7. Wu, L.; Jiang, J.; Jin, Y.; Kallemeijn, W. W.; Kuo, C.-L.; Artola, M.; Dai, W.; van Elk, C.; van Eijk, M.; van der Marel, G. A.; et al. Activity-Based Probes for Functional Interrogation of Retaining β-Glucuronidases. *Nat. Chem. Biol.* **2017**, *13* (8), 867-873.
8. Jariwala, P. B.; Pellock, S. J.; Goldfarb, D.; Cloer, E. W.; Artola, M.; Simpson, J. B.; Bhatt, A. P.; Walton, W. G.; Roberts, L. R.; Major, M. B.; et al. Discovering the Microbial Enzymes Driving Drug Toxicity with Activity-Based Protein Profiling. *ACS Chem. Biol.* **2020**, *15* (1), 217-225.
9. Ben Bdira, F.; Jiang, J.; Kallemeijn, W.; de Haan, A.; Florea, B. I.; Bleijlevens, B.; Boot, R.; Overkleeft, H. S.; Aerts, J. M.; Ubbink, M. Hydrophobic Interactions Contribute to Conformational Stabilization of Endoglycoceramidase II by Mechanism-Based Probes. *Biochemistry* **2016**, *55* (34), 4823-4835.
10. 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.; et al. Dynamic and Functional Profiling of Xylan-Degrading Enzymes in Aspergillus Secretomes Using Activity-Based Probes. *ACS Cent Sci* **2019**, *5* (6), 1067-1078.
11. McGregor, N.; Artola, M.; Nin-Hill, A.; Linzel, D.; Haon, M.; Reijngoud, J.; Ram, A. F. J.; Rosso, M.-N.; van der Marel, G. A.; Codée, J. D. C.; et al. Rational Design of Mechanism-Based Inhibitors and Activity-Based Probes for the Identification of Retaining α-L-Arabinofuranosidases. *J. Am. Chem. Soc.* **2020**, *142* (10), 4648-4662.
12. Rempel, B. P.; Withers, S. G. Covalent Inhibitors of Glycosidases and Their Applications in Biochemistry and Biology. *Glycobiology* **2008**, *18* (8), 570-586.
13. Wu, L.; Armstrong, Z.; Schröder, S. P.; de Boer, C.; Artola, M.; Aerts, J. M. F. G.; Overkleeft, H. S.; Davies, G. J. An Overview of Activity-Based Probes for Glycosidases. *Curr. Opin. Chem. Biol.* **2019**, *53*, 25-36.
14. Henrissat, B.; Davies, G. Structural and Sequence-Based Classification of Glycoside Hydrolases. *Curr. Opin. Struct. Biol.* **1997**, *7* (5), 637-644.
15. Henrissat, B.; Callebaut, I.; Fabrega, S.; Lehn, P.; Mornon, J. P.; Davies, G. Conserved Catalytic Machinery and the Prediction of a Common Fold for Several Families of Glycosyl Hydrolases. *Proc. Natl. Acad. Sci.* **1995**, *92* (15), 7090-7094.
16. Gebler, J.; Gilkes, N. R.; Claeyssens, M.; Wilson, D. B.; Béguin, P.; Wakarchuk, W. W.; Kilburn, D. G.; Miller, R. C.; Warren, R. A.; Withers, S. G. Stereoselective Hydrolysis Catalyzed by Related Beta-1,4-Glucanases and Beta-1,4-Xylanases. *J. Biol. Chem.* **1992**, *267* (18), 12559-12561.
17. Lombard, V.; Golaconda Ramulu, H.; Drula, E.; Coutinho, P. M.; Henrissat, B. The Carbohydrate-Active Enzymes Database (CAZy) in 2013. *Nucleic Acids Res.* **2014**, *42*, 490-495.
18. Ndeh, D.; Rogowski, A.; Cartmell, A.; Luis, A. S.; Baslé, A.; Gray, J.; Venditto, I.; Briggs, J.; Zhang, X.; Labourel, A.; et al. Complex Pectin Metabolism by Gut Bacteria Reveals Novel Catalytic Functions. *Nature* **2017**, *544* (7648), 65–70.
19. Davies, G. J.; Wilson, K. S.; Henrissat, B. Nomenclature for Sugar-Binding Subsites in Glycosyl Hydrolases. *The Biochemical journal*. **1997**, *321*, 557–559.
20. Koshland, D. E. Stereochemistry and the Mechanism of Enzymatic Reactions. *Biol. Rev.* **1953**, *28* (4), 416-436.
21. Bissaro, B.; Monsan, P.; Fauré, R.; O'Donohue, M. J. Glycosynthesis in a Waterworld: New Insight into the Molecular Basis of Transglycosylation in Retaining Glycoside Hydrolases. *Biochem. J.* **2015**, *467* (1), 17-

35.
22. Artola, M.; Wu, L.; Ferraz, M. J.; Kuo, C.-L.; Raich, L.; Breen, I. Z.; Offen, W. A.; Codée, J. D. C.; van der Marel, G. A.; Rovira, C.; et al. 1,6-Cyclophellitol Cyclosulfates: A New Class of Irreversible Glycosidase Inhibitor. *ACS Cent. Sci.* **2017**, *3* (7), 784-793.
23. Artola, M.; Hedberg, C.; Rowland, R. J.; Raich, L.; Kytidou, K.; Wu, L.; Schaaf, A.; Ferraz, M. J.; van der Marel, G. A.; Codée, J. D. C.; et al. α-D-Gal-Cyclophellitol Cyclosulfamidate Is a Michaelis Complex Analog That Stabilizes Therapeutic Lysosomal α-Galactosidase A in Fabry Disease. *Chem. Sci.* **2019**, *10* (40), 9233–9243.
24. Heightman, T. D.; Vasella, A. T. Recent Insights into Inhibition, Structure, and Mechanism of Configuration-Retaining Glycosidases. *Angew. Chemie. Int. Ed.* **1999**, *38* (6), 750-770.
25. Beenakker, T. J. M.; Wander, D. P. A.; Offen, W. A.; Artola, M.; Raich, L.; Ferraz, M. J.; Li, K.-Y.; Houben, J. H. P. M.; van Rijssel, E. R.; Hansen, T.; et al. Carba-Cyclophellitols Are Neutral Retaining-Glucosidase Inhibitors. *J. Am. Chem. Soc.* **2017**, *139* (19), 6534-6537.
26. Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. Activity-Based Protein Profiling: From Enzyme Chemistry to Proteomic Chemistry. *Annu. Rev. Biochem.* **2008**, *77*, 383-414.
27. Willems, L. I.; Beenakker, T. J. M.; Murray, B.; Gagestein, B.; van den Elst, H.; van Rijssel, E. R.; Codée, J. D. C.; Kallemeijn, W. W.; Aerts, J. M. F. G.; van der Marel, G. A.; et al. Synthesis of α- and β-Galactopyranose-Configured Isomers of Cyclophellitol and Cyclophellitol Aziridine. *Eur. J. Org. Chem.* **2014**, *2014* (27), 6044-6056.
28. Armstrong, Z.; Kuo, C. L.; Lahav D.; Liu, B.; Johnson, R.; Beenakker T. J. M.; de Boer, C.; Wong C. S.; van Rijssel E. R., Debets M. F.; et al. Manno-epi-cyclophellitols Enable Activity-Based Protein Profiling of Human α-Mannosidases and Discovery of New Golgi Mannosidase II Inhibitors. *J. Am. Chem. Soc.* **2020**, DOI: 10.1021/jacs.0c03880
29. Jiang, J.; Kallemeijn, W. W.; Wright, D. W.; van den Nieuwendijk, A. M. C. H.; Rohde, V. C.; Folch, E. C.; van den Elst, H.; Florea, B. I.; Scheij, S.; Donker-Koopman, W. E.; et al. In Vitro and in Vivo Comparative and Competitive Activity-Based Protein Profiling of GH29 α-L-Fucosidases. *Chem. Sci.* **2015**, *6* (5), 2782-2789.
30. Artola, M.; Kuo, C.-L.; McMahon, S. A.; Oehler, V.; Hansen, T.; van der Lienden, M.; He, X.; van den Elst, H.; Florea, B. I.; Kermode, A. R.; et al. New Irreversible α-L-Iduronidase Inhibitors and Activity-Based Probes. *Chem. Eur. J.* **2018**, *24* (71), 19081-19088.
31. Ardèvol, A.; Rovira, C. Reaction Mechanisms in Carbohydrate-Active Enzymes: Glycoside Hydrolases and Glycosyltransferases. Insights from Ab Initio Quantum Mechanics/Molecular Mechanics Dynamic Simulations. *J. Am. Chem. Soc.* **2015**, *137* (24), 7528-7547.
32. Speciale, G.; Thompson, A. J.; Davies, G. J.; Williams, S. J. Dissecting Conformational Contributions to Glycosidase Catalysis and Inhibition. *Curr. Opin. Struct. Biol.* **2014**, *28*, 1-13.
33. Davies, G. J.; Planas, A.; Rovira, C. Conformational Analyses of the Reaction Coordinate of Glycosidases. *Acc. Chem. Res.* **2012**, *45* (2), 308-316.