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Cyclophellitol analogues for profiling of exo- and endo-glycosidases

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Chapter 1

Introduction and outline

Glycosidic bonds are one of the most stable linkages within natural biopolymers, with half-lives of starch and cellulose reaching approximately 5 million years.[1] Glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds in oligosaccharides[2], glycoproteins[3] and glycolipids[4], by increasing the hydrolysis rate by 10^{17}-fold.[1] Correct processing of glycans is essential to cellular function as glycans are involved in energy-storage, signaling pathways, folding, stability, activity and functioning of proteins. Glycosidases are generally highly specific towards the absolute configuration of their glycosidic substrate, and due to the structural diversity of glycans,[5] many distinct glycosidase families have evolved. To date, glycosidases are categorized in over 130 glycoside hydrolase (GH) families, based on structural similarities and amino acid sequence in their catalytic domain (see: *cazypedia.org*).[6] While research on glycosidases has successfully unraveled their function in various complex biological systems, the role and catalytic mechanism of many glycosidases remains unclear. Glycosidases can be studied using specific (covalent) inhibitors, which allow interrogation of their catalytic machinery, mechanism and itinerary from

X-ray crystallographic snapshots of (covalent) enzyme-inhibitor complexes. Additionally, covalent activity based probes (ABPs) allow profiling of glycosidases in biological settings, thereby enabling the study of glycosidase involvement in health and disease. This Thesis describes the synthesis and biochemical evaluation of such (covalent) glycosidase inhibitors and ABPs. Since glycosidases are highly specific towards their substrates, the design of such inhibitors requires knowledge of their function and mechanistic aspects. Some of these aspects are introduced in this Chapter.

1.1 Glycosidases in biomedicine and biotechnology

Aberrant glycosidase function often results in accumulation of its substrates, which is at the basis of a number of human diseases.[7] For example, impaired activity of lysosomal β-glucocerebrosidase (glucosylceramidase, GBA1), caused by inherited mutations in the gene encoding for this enzyme, results in accumulation of its substrate, glucosylceramide, in macrophages, inducing spleen and liver enlargement, skeletal disorders, anemia and other symptoms.[8] This lysosomal storage disorder is called Gaucher disease and current treatments are based on intravenous administration of recombinant GBA1 (enzyme replacement therapy[9]) or reducing glucosylceramide biosynthesis by inhibiting glucosylceramide synthase (substrate reduction therapy[10]). Recently, competitive GBA inhibitors have been evaluated as alternatives for the treatment of Gaucher disease.[11] This strategy, termed pharmacological chaperone therapy (PCT), relies on the administration of such competitive GBA inhibitors to assist folding of the nascent GBA polypeptide into its active conformation in the endoplasmic reticulum. Other lysosomal storage disorders include Pompe disease (α-glucosidase deficiency),[12] Fabry disease and Krabbe disease (deficiency in α- and β-galactocerebrosidase, respectively),[13,14] mannosidosis (deficiency in α- and β-mannosidases)[15,16] and mucopolysaccharidosis type III-B (deficiency in α-*N*-acetyl-glucosaminidase).[17]

Glycosidases are widely applied enzymes in biotechnology. For example, they are used for enzymatic “biobleaching” in the paper and pulp industries.[18] Glycosidases are also used in food production, as ingredients of detergents, in the production of textiles,[19] and in the production of biofuels (such as ethanol) from organic (waste) material.[20] In the latter application, polymeric plant material is hydrolysed by glycosidases to afford monomeric saccharides, which are subsequently fermented to produce combustible materials.

1.2 Mechanistic aspects of glycosidases

Glycosidases can be subdivided into two distinct classes, namely *exo*- and *endo*-glycosidases (Figure 1). *Exo*-glycosidases hydrolyse polysaccharides at the terminal non-reducing (in some cases, also reducing) end of the carbohydrate chain. Typically, their active site is shaped as a pocket, providing space to accommodate a monosaccharide.[21] In contrast, e*ndo*-glycosidases hydrolyse polysaccharide chains at internal positions. Their active site is either cleft- or tunnel shaped, and can accommodate multiple monosaccharide residues.[21] Glycosidase active sites can be dissected in multiple subsites (-*n* to +*n*) which interact with the polysaccharide substrate.[22] The -*n* subsite(s) accommodate the glycon moiety, whereas the aglycon is positioned in the +*n* subsite(s). The hydrolytic apparatus is situated at subsite -1. Generally, e*xo*-glycosidases have strong interactions with the monosaccharide occupying the -1 position, but less so with the aglycon at +1. The minimal substrate size of e*ndo*-glycosidases is a trisaccharide, hence *endo*-glycosidases have strong interactions at subsites -2 to +1, however, several subsites are generally involved in substrate binding. For example, the active site of cellobiohydrolase I from *Trichoderma reesei* spans up to 11 binding sites (-7 to +4).[23]

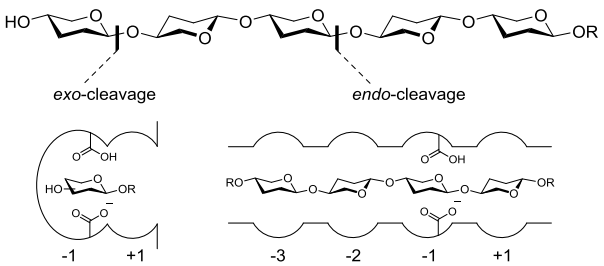

Figure 1 Glycosidases can be classified into *endo*- and *exo*-acting hydrolases. *Exo*-glycosidases generally cleave their glycan substrate at the non-reducing end of the polysaccharide chain. Their active site is pocket-shaped and contains only two subsites (-1 and +1). *Endo*-glycosidases cleave polysaccharides at internal positions, and their cleft or tunnel shaped active sites can be divided into multiple subsites.

Glycosidases can also be divided in two major classes based on the stereochemical outcome of the hydrolytic reaction. These classes employ distinct catalytic mechanisms as was first outlined by Daniel E. Koshland, Jr in 1953.[24] Inverting glycosidases hydrolyse their substrates with inversion of stereochemistry at the anomeric position (Figure 2). During this one-step, single displacement mechanism,

the reaction itinerary proceeds through an oxocarbenium-like transition state. The catalytic apparatus consists of a base and acid residue, most often a carboxylate and a carboxylic acid, thus aspartic acid and/or glutamic acid (Asp/Glu) residues. The catalytic residues are located 6-12 Å apart, providing space for the substrate and a water molecule. When the substrate is correctly coordinated in the active site, the catalytic base deprotonates the water molecule, which subsequently displaces the substrate's aglycon moiety. The aglycon is displaced with protic assistance of the catalytic acid/base residue.[25–27]

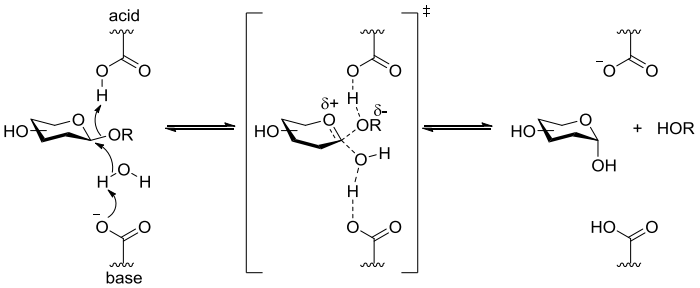

Figure 2 Catalytic reaction mechanism of the hydrolysis of a β-glycoside employed by inverting glycosidases, using a one-step single displacement mechanism.

Retaining glycosidases employ a two-step, double displacement mechanism to hydrolyse their substrates (Figure 3). The active site contains a catalytic nucleophile (Asp/Glu) and an acid/base residue (Asp/Glu), situated approximately 5.5 Å apart. During the first step (glycosylation), the substrate undergoes nucleophilic attack at the anomeric centre, while the aglycon is protonated by the catalytic acid/base. An oxocarbenium-like transition state emerges, the aglycon is expelled and a covalent intermediate is formed between the substrate and the enzyme. During the second step (deglycosylation), the catalytic base deprotonates a water molecule, which then attacks the anomeric centre of the enzyme-substrate complex. Following a second transition state, the covalent enzyme-substrate bond is cleaved and the hydrolysed substrate is released, with retention of stereochemistry at the anomeric position.

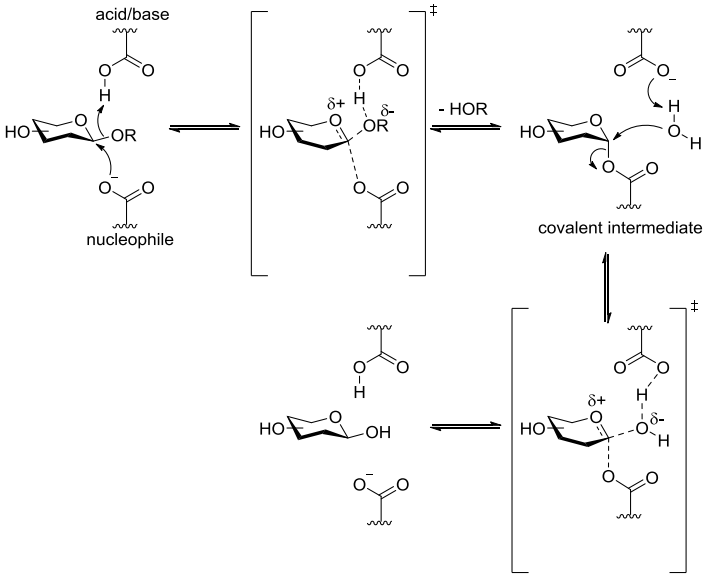

Figure 3 Catalytic reaction mechanism of the hydrolysis of a β-glycoside employed by retaining glycosidases, using a two-step double displacement mechanism. The reaction pathway involves a covalent enzyme-substrate intermediate, which is subsequently hydrolyzed.

Most glycosidases can be categorized in these two classes, however there are exceptions. For instance, 2-*N*-acetyl substrates are hydrolysed by GH families 18, 20, 25, 56, 84 and 85 via neighbouring group participation.[28–31] These enzymes lack a catalytic nucleophile; instead, the 2-acetamido group acts as such and intramolecularly displaces the aglycon. Therefore, while the enzymatic itinerary follows a two-step double displacement mechanism, no covalent intermediate is formed with the substrate. Other enzyme classes include: sialidases,[32,33] which utilize a tyrosine residue as catalytic nucleophile; myrosinases,[34] which lack a catalytic acid and employ an external base; and NAD-dependent glycosidases,[35,36] which employ an oxidation-elimination-reduction pathway. Recently, it was proposed that GH99 *endo*-mannosidases follow a two-step, double displacement mechanism with neighbouring group participation of OH-2, forming an 1,2-anhydro-epoxide which is subsequently hydrolyzed.[37–39]

The classical Koshland hydrolytic reaction mechanism is widely appreciated by the scientific community; typically schematically represented by placement of the catalytic residues at the top and bottom face of the substrate anomeric centre. In

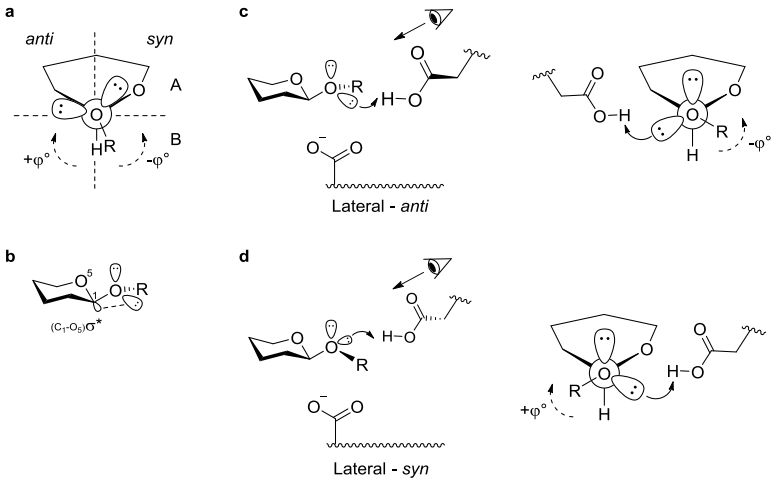

Figure 4 Semi-lateral protonation of the aglycon by the catalytic acid/base residue. (a) The spacial environment of a glycoside can be divided into two half-spaces (*syn* and *anti*) which are separated by a plane defined by the anomeric C-H bond. The catalytic acid/base in the majority of glycosidases resides in the *anti* half-space. A plane perpendicular to the anomeric C-H bond defines the A or B half-spaces. For β-glycosidases the catalytic acid/base resides in the A half-space, whereas for α-glycosidases it is situated in the B half-space. (b) The exo-anomeric effect in β-glycosides. (c) Substrate protonation by an *anti*-protonator. (d) Substrate protonation by a *syn*-protonator.

reality, while the catalytic nucleophile residue is indeed placed below or above the anomeric centre, this does not hold true for the catalytic acid/base. As first postulated by Vasella and co-workers[40] and later confirmed by X-ray crystallographic snapshots of many enzyme-inhibitor complexes,[41] the catalytic acid/base is positioned semi-lateral ('half-space' A for β-glycosidases, 'half-space' B for α-glycosidases) from the aglycon oxygen, and is situated in the *syn* (near the endocyclic oxygen) or *anti* (opposite the endocyclic oxygen) half-space (Figure 4a).

The *syn* or *anti* positioning of the catalytic acid/base is conserved within GH families, and roughly 70% of all families are *anti*-protonators. The lowest energy conformation in β-glycosides involves the glycosidic anomeric bond rotated in a minus-synclinal angle (-ϕ°) to allow hyperconjugative overlap of its antiperiplanar oxygen lone-pair orbital with the C1-O5 antibonding (σ*) orbital (Figure 4b), a phenomenon called the exo-anomeric effect.[42] In *anti*-protonating β-glycosidases, the catalytic acid/base is situated in the *anti*-A quadrant and protonates this antiperiplanar glycosidic oxygen lone-pair orbital (Figure 4c). This cancels out the stabilizing exo-anomeric effect,

thereby lowering the activation barrier for hydrolysis. *Syn*-protonating β-glycosidases display their catalytic acid/base in the *syn*-A quadrant (Figure 4d). Protonation of the aglycon requires a minus-anticlinal (+ϕ°) rotation of the glycosidic bond, thereby cancelling out the exo-anomeric effect as well.

As depicted in Figure 2 and 3, the mechanistic reaction itineraries for inverting and retaining glycosidases follow different steps involving substrate distortions towards the transition state(s). Such conformational distortions were initially proposed for hen egg white lysozyme,[43] and eventually found to be glycosidase-specific.[44,45] For example, upon substrate entrance into the active site, most retaining β-glucosidases distort their substrates into a $^{1}S_{3}$ conformation (Michaelis complex), followed by a $^{4}H_{3}$ transition state conformation (Figure 5a).[46–48] Following substitution of the aglycon, the resulting covalent intermediate occupies a relaxed $^{4}C_{1}$ conformation, which is then further hydrolysed. While many reaction itineraries of other glycosidase classes are yet unknown, this same $^{1}S_{3}\rightarrow[^{4}H_{3}]^{\ddagger}\rightarrow{}^{4}C_{1}$ itinerary is also proposed for other glycosidase families including GH2 β-galactosidases[49,50], GH10 *endo*-xylanases[51,52] and GH39 xylosidases.[53] In contrast, many α-glucosidases[54,55] and α-galactosidases[56] follow a 'reversed' $^{4}C_{1}\rightarrow[^{4}H_{3}]^{\ddagger}\rightarrow{}^{1}S_{3}$ conformational pathway (Figure 5b).

Hydrolysis of α-mannopyranosides by retaining α-mannosidases involves substitution of the aglycon to furnish a covalent β-mannoside-enzyme complex. The construction of β-glycosidic linkages from α-mannosides is a well-known challenge for synthetic organic chemists.[57] Substitution of α-mannoside donors towards β-mannopyranosides is hampered by the steric hindrance of O2 with the incoming nucleophile. Furthermore, α-mannosides are stabilized by the anomeric effect and the opposing [C1,O1 – C2,O2] dipoles so that conformational distortion is disfavoured. Lastly, introduction of a glycosidic β-linkage in mannopyranosides induces the Δ2 effect[58]; a destabilizing alignment of the [C1,O(1,5) – C2,O2] dipoles. Nature has provided an effective way of introducing glycosidic β-linkages in mannopyranosides. By forming a Michaelis complex with the substrate in the $^{0}S_{2}$ conformation, the aglycon is arranged in a pseudo axial position, and inline attack of the carboxylate is facilitated (Figure 5c).[59,60] Following a $B_{2,5}$ transition state, the covalent intermediate is formed in the $^{1}S_{5}$ conformation which reduces the destabilizing Δ2 effect. In analogy, β-mannosidases follow the 'reversed' itinerary, employing $^{1}S_{5}\rightarrow[B_{2,5}]^{\ddagger}\rightarrow{}^{0}S_{2}$ substrate conformations (Figure 5d).[61–63]

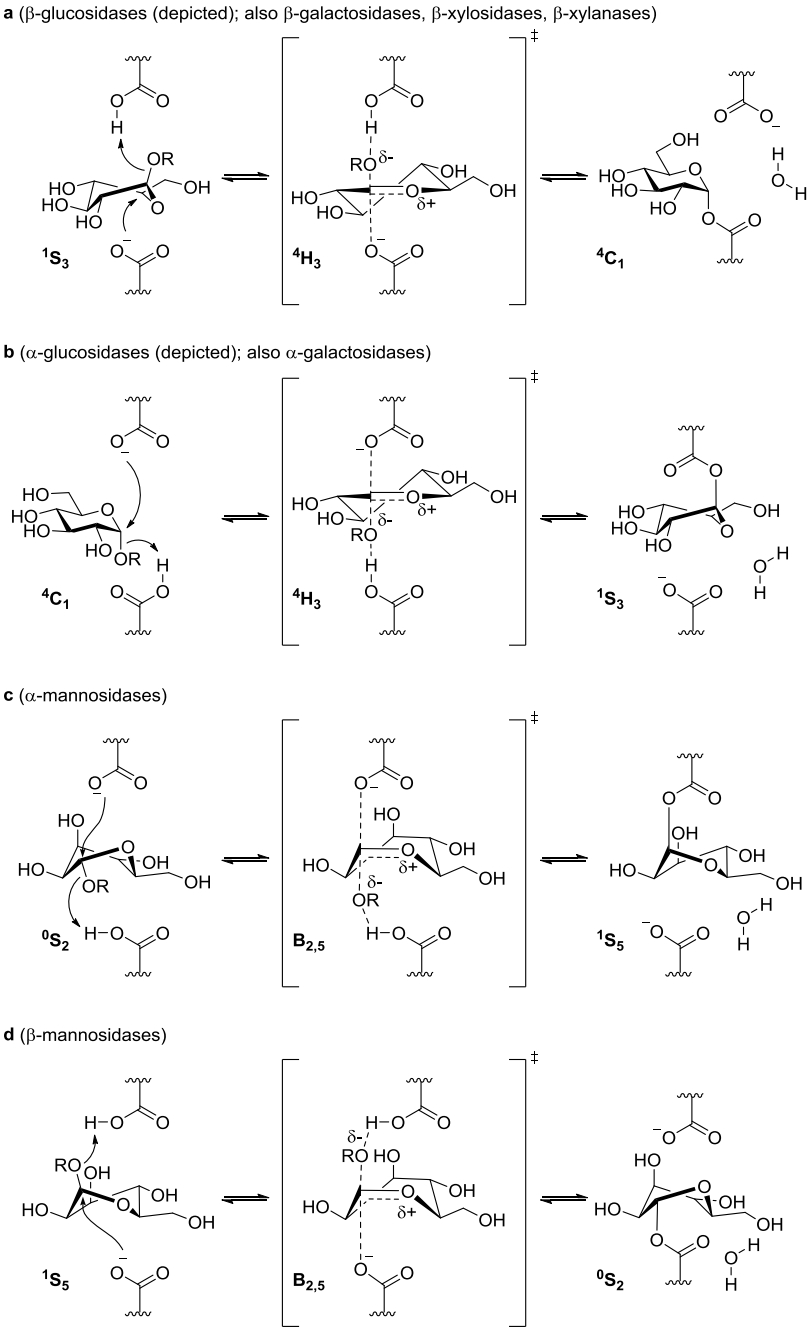

Figure 5 Different conformational itineraries of the substrate during processing by retaining α- and β-glycosidases. The hydrolysis of the covalent intermediates is omitted for clarity.

1.3 Activity-based protein profiling

Enzymes that form a covalent bond with their substrate during catalysis are amenable to activity-based protein profiling (ABPP).[64,65] ABPP commences with incubation of a relevant biological sample with an activity-based probe (ABP), which reacts selectively, covalently and irreversibly with the enzyme (class) of interest. With this technique, generally only active enzymes are targeted. An ABP generally consists of a recognition element, which ensures recognition by the target enzyme selectively, and an electrophile (warhead), which reacts covalently and irreversibly with the catalytic machinery of functional enzymes. Furthermore, the ABP is equipped with a reporter (fluorescent or affinity) tag, which enables quantitative and qualitative read-out of the biochemical assay (Figure 6a).

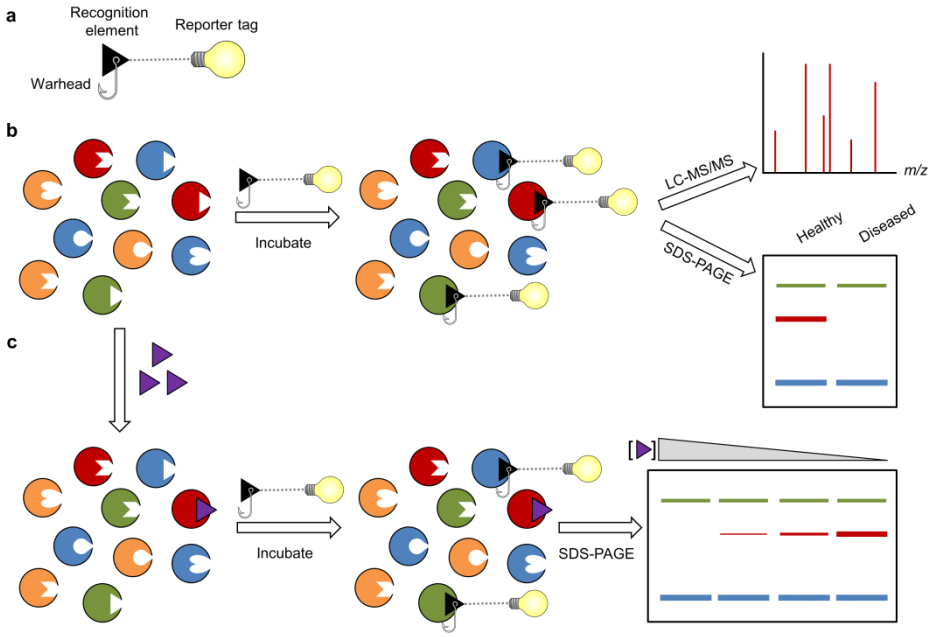

Figure 6 Schematic overview of activity-based protein profiling (ABPP) techniques. (a) The activity-based probe consists of a recognition element containing an electrophilic trap for irreversible reaction with the target enzyme, and a reporter tag, which enables analysis of the experiment. (b) Incubation of a complex biological sample with the probe results in binding with the target enzymes, which can then be identified by proteomics or analyzed by SDS-PAGE in a comparative ABPP study. (c) In a competitive ABPP assay, the complex biological sample is incubated with a range of inhibitor concentrations, and then post-labeled by the probe. Only the fraction of non-inhibited target enzymes will bind to the probe.

For example, probes that are equipped with a biotin affinity tag are used to enrich the enzyme(s) that reacted with the ABP, via affinity purification using streptavidin immobilized on beads. The enriched protein(s) are subsequently removed from the beads using proteolysis (usually trypsinolysis) and the resulting peptides analysed by LC-MS/MS and matched against protein sequence databases to identify the target protein (Figure 6b).[66] Alternatively, active enzymes of interest can be visualized by probes containing a fluorescent reporter tag, for instance a BODIPY, cyanine or TAMRA fluorophore. Following sample incubation and irreversible inactivation of the target enzyme(s) with the probe, the complex protein mixture is denatured and then analysed by SDS-PAGE. Using fluorescent imaging, the protein band(s) with corresponding molecular weight correlating to the targeted enzyme are visualized. Precursor enzymes, inactive or malfunctioning enzymes are generally not targeted by this technique and the band intensity can be linked to enzyme expression levels. Thus, ABPs can be used to correlate enzyme concentration to tissues, or as diagnostic tools for investigating active enzyme levels in patients *versus* healthy individuals (comparative ABPP[67–69]). Furthermore, fluorescent ABPs are used in competition assays, where the enzyme of interest is pre-incubated with different inhibitor concentrations followed by labelling with the ABP (competitive ABPP,[70,71] Figure 6c). With such assays, inhibitory potency is visualized which corresponds to the decrease in fluorescent signal. When the ABP used targets multiple enzyme classes, off-target activity of the inhibitor can be visualized and identified. Enzymes that are currently studied by ABPP include serine hydrolases,[72–74] cysteine hydrolases,[75–77] proteasomes,[78–80] kinases[81–83] and also glycosidases.[84–86]

1.4 Covalent, irreversible, cyclophellitol-based glycosidase probes

An important class of covalent retaining glycosidase inhibitors are the cyclitol-epoxides.[87] For example, conduritol B epoxide (CBE, **1**, Figure 7a), first described by Legler,[88] is a potent inactivator of lysosomal β-glucocerebrosidase (GBA1)[89] and other glucosidases.[90] The cyclitol core partially mimics the glucopyranoside substrate and is recognized as such. Following coordination in the active site, the catalytic nucleophile performs a nucleophilic attack on the oxirane at the anomeric position, and the nucleophilic displacement is aided by protonation of the ring oxygen by the catalytic acid/base, resulting in a covalent, irreversible complex (Figure 7b). However, CBE is not β-glucosidase selective and also inhibits α-glucosidases due to its C_2-symmetry, albeit with lower potency.[91] Cyclophellitol (**2**, Figure 7a), isolated as a natural product from *Phellinus* sp.[92] and available via various synthetic routes,[93,94] is also a covalent

irreversible β-glucosidase inactivator. It is structurally related to CBE, but contains a hydroxymethylene functionality at C5 (instead of hydroxyl for CBE), is selective for β-glucosidases over α-glucosidases and displays higher potency due to its higher natural substrate resemblance.[95] Subsequently it was found that the close structural analogue cyclophellitol aziridine[96] (**3**, Figure 7a) displayed even higher potency towards β-glucosidases.[97]

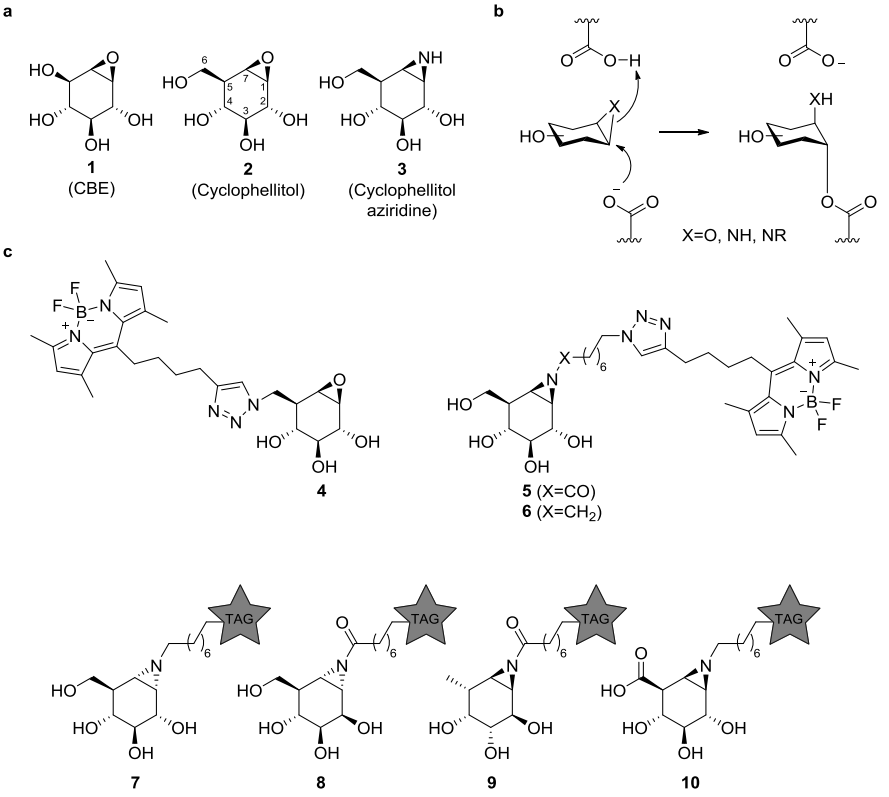

Figure 7 (a) Covalent irreversible inhibitors of β-glucosidases: conduritol B epoxide (**1**), cyclophellitol (**2**) and cyclophellitol aziridine (**3**). (b) Mechanism of glycosidase inactivation by cyclitol epoxides and aziridines. After opening of the electrophilic trap, a covalent adduct is formed which is stable towards hydrolysis. (c) Cyclophellitol equipped with a fluorescent tag at C6 (**4**) is a potent probe for GBA1. Cyclophellitol aziridine acylated (**5**) or alkylated (**6**) with a fluorescent tag are potent probes for the mammalian retaining β-glucosidases, GBA1, GBA2, GBA3 and LPH. Specific ABPs for α-glucosidases (**7**),[98] α-galactosidases (**8**),[99] α-fucosidases (**9**)[68] and β-glucoronidases (**10**)[100] have also been developed.

Due to the covalent and irreversible mode of action that cyclophellitol employs towards β-glucosidases, its core structure was used for the generation of activity based probes. Endowing position C6 in cyclophellitol (**2**) with a fluorescent BODIPY tag afforded ABP **4** (Figure 7c), which proved to be a highly potent and selective probe for human GBA1 over the other three human β-glucosidases GBA2, GBA3 and lactase-phlorizin hydrolase (LPH).[101] In a subsequent study, the nitrogen in cyclophellitol aziridine (**3**) was equipped with a fluorescent tag affording *N*-acyl ABP **5**.[102] In this case, the reporter moiety occupies the space normally taken up by the natural substrate aglycon, and it was found that this probe labels all β-glucosidases GBA1, GBA2, GBA3 and LPH in mice with high potency. However, *N*-acyl ABP **5** was found to be somewhat unstable during synthesis, purification and handling. Later it was found that its more accessible *N*-alkylated counterpart (**6**) was equally effective in β-glucosidase labeling.[103] In analogy, specific ABPs based on the cyclophellitol template have been synthesized and evaluated for labeling of α-glucosidases (**7**),[98] α-galactosidases (**8**),[99] α-fucosidases (**9**)[68] and β-glucoronidases (**10**).[100]

1.5 Outline of this Thesis

To this day, all cyclophellitol-based inhibitors and ABPs have been close analogues of their natural substrate counterparts. As a result, these probes showed high selectivity towards their target glycosidases. While such probes are of high value for studying these specific enzyme classes, they impede the simultaneous profiling of glycosidases that process different substrate configurations with a single probe. The first Chapters in this Thesis focus on the structural derivatization of cyclophellitol-based probes with the aim of enabling inter-class labelling of glycosidases. **Chapter 2** describes the synthesis of cyclophellitol-based inhibitors and probes, which lack the hydroxymethylene functionality at C5, and their labelling is biochemically evaluated on complex biological samples. The synthesis of cyclophellitol-based probes which are lacking hydroxyl substituents at positions C2 and C4 are described in **Chapter 3**, as well as the biochemical evaluation of these probes towards inter-class labelling of α- and β-glucosidases, mannosidases and galactosidases in cell extracts. **Chapter 4** reports on the synthesis and biochemical evaluation of D-*lyxo* and D-*arabino*furanoside analogues of cyclophellitol aziridine. The synthesis of a novel reversible inhibitor, gluco-1*H*-imidazole, is described in Chapter **5** and its inhibitory activity and binding properties are investigated. To date, all cyclitol-epoxide/aziridine based probes are monomeric in structure and therefore only target *exo*-glycosidases. In the last Chapters of this Thesis, synthetic methodologies for the construction of *endo*-

glycosidase probes are described. **Chapter 6** reports on the introduction of a spiro-epoxide warhead onto a disaccharide moiety for the purpose of constructing probes for GH99 *endo*-α-mannosidases, and their labelling potency is evaluated. The construction of x*ylobiose*-cyclophellitol probes from *xylo*-cyclophellitol acceptors via direct glycosylation is described in **Chapter 7**, and the xylanase labelling potency is evaluated in the secretome of *Aspergillus niger*. Finally, **Chapter 8** provides a summary of this Thesis, followed by future prospects including the synthesis of multimeric irreversible probes and inhibitors for GH76 *endo*-α-mannosidase.

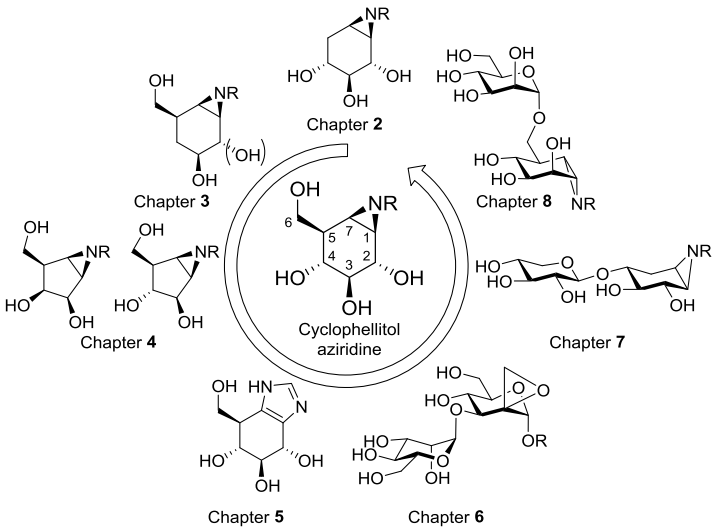

Figure 8 Global overview of the inhibitors and activity-based probes (based on the cyclophellitol aziridine scaffold, depicted in the middle) discussed in this Thesis.

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