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Activity-based protein profiling of glucosidases, fucosidases and glucuronidases

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General introduction

Glycoside hydrolases (GHs), enzymes that catalyze the hydrolytic cleavage of glycosidic bonds, receive continuing interest both in fundamental and applied biology and biomedicine. Lysosomal storage disorders (LSDs) are caused by inborn metabolic errors due to deficiency in specific lysosomal enzymes, most commonly GHs. Diagnosis and treatment of LSDs require regular quantification of the active lysosomal enzymes in patient tissues. Activity-based protein profiling (ABPP) has emerged in the past decades as a powerful technique to study enzyme families in cell extracts and living tissues. Originally developed for serine hydrolases and cysteine proteases, various enzyme classes can be studied by means of ABPP today, including retaining GHs. The research described in this thesis focused on expanding the field of activity-based glycosidase profiling through the development and application of activity-based probes (ABPs) for several retaining GHs, namely β -glucosidases, α -L-fucosidases, α -glucosidases and β -glucuronidases. The first part of this chapter provides an overview of retaining and inverting β -glucosidases, including the catalytic mechanisms they employ in processing their substrates and the mechanism-based inhibitors of retaining β -glucosidases. The final part of this chapter presents a historical perspective on ABPs of retaining β -glucosidases as well as an outline of the contents of the following chapters.

1.1 β -Glucosidases

Glycoside hydrolases (GHs) are found in all kingdoms of life, and play important roles in a broad range of biological processes.¹⁻⁵ β -Glucosidases catalyze the hydrolysis of β -D-glucosidic linkages, with release of glucose (*exo*-glucosidase) or oligosaccharides featuring a glucose moiety at the reducing end (*endo*-glucosidases) as the result,⁶ this could also be expanded to other *exo*- and *endo*-glycosidases classification. Based on the stereochemical outcome of the hydrolysis reaction, β -glucosidases can be divided into retaining and inverting enzyme groups. The two catalytic mechanisms employed by β -glucosidases to achieve glycosidic bond cleavage are shown in Figure 1.

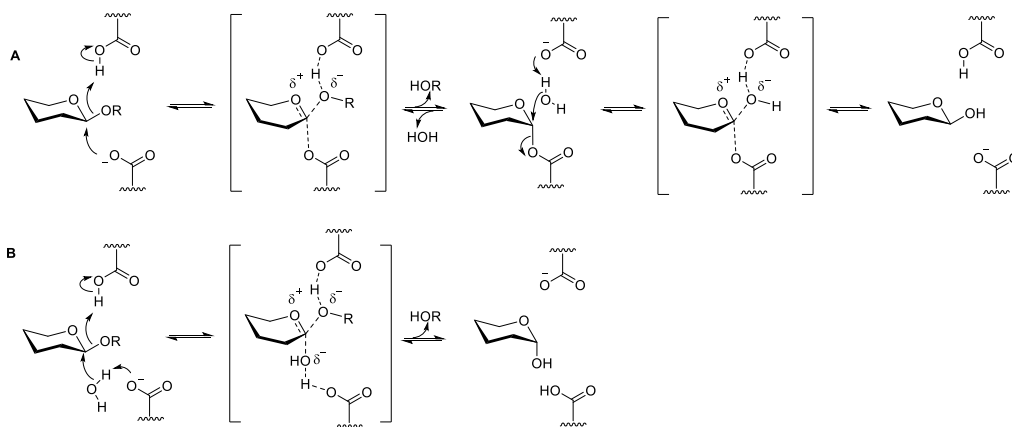


Figure 1. Generalized mechanisms for β -D-glucosides hydrolysis: A) retaining β -glucosidase and B) inverting β -glucosidase.

Retaining GHs process their substrates with overall retention of anomeric configuration whereas inverting GHs do so with inversion of configuration.⁷ In 1953, Daniel Koshland proposed that retaining glycosidases act by a double-displacement mechanism involving an enzyme or substrate nucleophile (two inversions resulting in net retention of the anomeric configuration).⁸ The catalytic machinery of a retaining β -glucosidase involves two catalytic carboxylates (Figure 1A): one acting as an acid-base catalyst and the other acting as a nucleophile. In the first step (glycosylation), the aglycon is protonated and expelled in a formal S_N2 process by the nucleophilic residue to yield a covalent glycosyl-enzyme intermediate. In the second step (deglycosylation), water enters the enzyme active site, gets activated by (partial) deprotonation by the catalytic acid/base carboxylate and substitutes in another formal S_N2 process the active site nucleophile in the glycosyl-enzyme adduct to yield with overall retention of configuration at the anomeric center of the hydrolyzed sugar. The active sites of inverting β -glucosidases are also composed of two carboxyl groups, with one acting as a general acid catalyst and the other as a general base catalyst (Figure 1B). Hydrolysis proceeds in a single step, in which the catalytic acid activates the aglycon while the catalytic

base activates the nucleophilic water molecule which displaces the aglycon via a single transition state with substantial oxocarbenium ion character.⁹

1.2 Mechanism-based inhibitors and activity-based probes of retaining β -glucosidases

Mechanism-based inhibitors are molecules that react with an enzyme, normally within the active site and following the mechanism employed by the enzyme in substrate processing. When a mechanism-based inhibitor is brought into contact with a target enzyme, a covalent bond between the reactants (enzyme and inhibitor) is formed that is stable over time, thus leading to permanent inactivation of the enzyme. Mechanism-based enzyme inhibition can be achieved most easily when an enzyme processes its substrate through the intermediate formation of a covalent intermediate. From this point of view, retaining β -glucosidases appear more susceptible to mechanism-based inhibition than inverting β -glucosidases.¹⁰ Cyclophellitol (**1**)¹⁰ and cyclophellitol aziridine (**2**)¹¹ are typical examples of mechanism-based inhibitors against retaining β -glucosidases (Figure 2A). They are activated by protonation by the general acid/base catalytic residue and next opened by the nucleophilic carboxylic acid to yield a covalent enzyme-substrate adduct. Compared to the acylal (O-C-O-C=O) linkage that emerges during β -glucoside processing (see Figure 1A), the resulting ester linkage is considerably more stable and the retaining β -glucosidase is effectively and irreversibly inhibited.

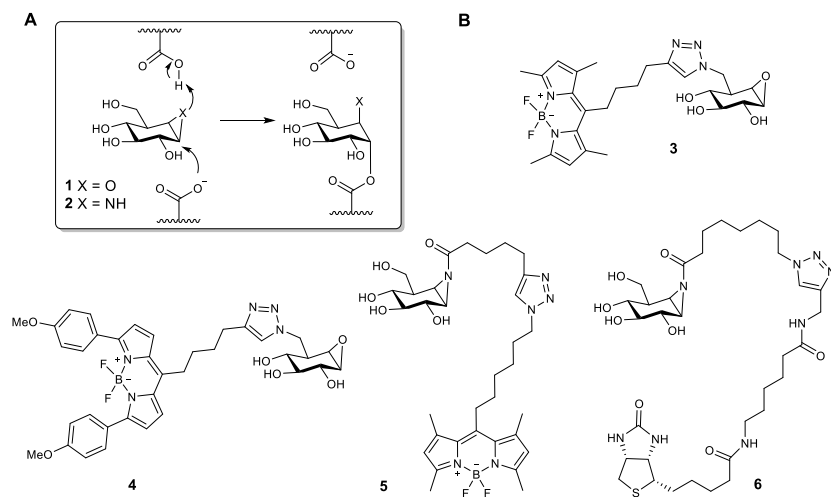


Figure 2. A) Proposed mechanism of retaining β -glucosidases inhibition by cyclophellitol (**1**) and cyclophellitol aziridine (**2**). B) Examples of ABPs based on cyclophellitol and cyclophellitol aziridine scaffolds.

Activity-based protein profiling (ABPP) has emerged as a useful technology to study GHs activities in various surroundings. ABPP, pioneered by the Cravatt laboratory for the study of

serine hydrolase families,¹² utilizes activity-based probes (ABPs), compounds designed to specifically react in a covalent and irreversible fashion with an enzyme or a class of enzymes and that are equipped with a reporter molecule (fluorophore, biotin, bioorthogonal group) for detection and/or identification of the covalently captured enzymes (Figure 3). An ABP normally contains a reactive moiety (or 'warhead') that can form a covalent bond with the enzyme (family) of interest, but is sufficiently inert to survive in cell extracts or living cells when not in contact with the enzyme target(s). A spacer links the reactive moiety to a reporter group (tag), so that the latter does not interfere with binding the enzyme. The third essential structural element, the tag group, commonly exists of a fluorescent group (for instance, BODIPY, rhodamine or fluorescein) for visualization in gel or in living cells or a biotin group for affinity enrichment, purification and subsequent mass spectrometry detection.

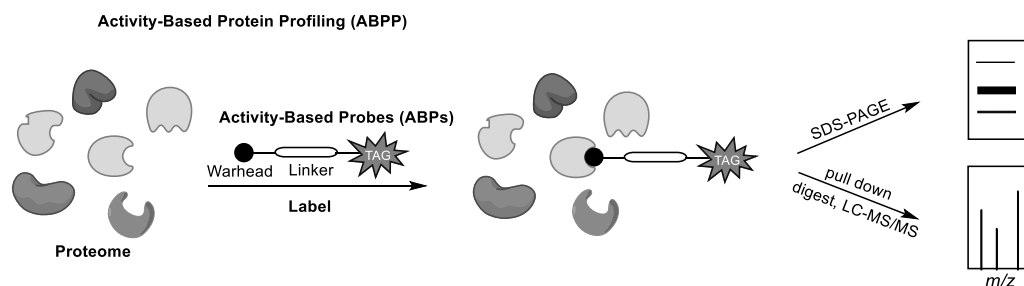


Figure 3. In a typical ABPP experiment, a proteome is treated with the ABP. Key to the success of the probe is the covalent attachment to the enzyme(s) of interest. Depending on the tag (reporter) group, the labeled enzyme(s) can then be either directly visualized with SDS-PAGE by fluorescent scanning or can be purified by streptavidin pull down, digested with trypsin followed by analysis of the resulting peptides by mass spectrometry

ABPs are generally designed with most ease when dealing with enzymes that, during processing, form a covalent intermediate with their substrate. Based on this theory, mechanism-based retaining β -glucosidase inhibitors, cyclophellitol (**1**) and cyclophellitol aziridine (**2**), have been used as scaffolds for ABP development. These ABPs include cyclophellitol derivatives and cyclophellitol aziridines derivatives modified with either a BODIPY or a biotin.¹³ The first-generation of cyclophellitol-inspired retaining β -glucosidase ABPs are compounds **3** and **4** (Figure 2B). Both ABPs **3** and **4**, though having a large BODIPY moiety appeared to be quite potent and efficient inhibitors of glucocerebrosidase (GBA), inhibiting this enzyme much more potently than the parent compound, cyclophellitol **1**.¹⁴ These probes were applied to visualize active GBA molecules in various mouse tissue extracts and in living cells. More recently, cyclophellitol aziridine **2** was employed for the design of second generation retaining β -glucosidase ABPs, such as fluorescent probe **5** and biotin probe **6**.¹⁵ These probes, with the epoxide substituted for aziridine allowing the reporter groups to be introduced on the aziridine nitrogen proved to be more potent towards a range of retaining

β -glucosidases. They were shown to label in murine tissue extracts, apart from GBA, the non-lysosomal glycosylceramidase (GBA2), the cytosolic β -glucosidase (GBA3) and intestinal lactase/phorizin hydrolase (LPH), as well as numerous plant retaining GHs such as myrosinases, β -glucosidases, β -galactosidases and β -xylosidases in *Nicotiana benthamiana*.¹⁶

1.3 Activity-based retaining glycosidase probes based on cyclophellitol aziridine

Retaining β -glucosidases are one class of glycoside hydrolases (GHs), which are often classified on the basis of their amino acid sequence according to the CAZy system (www.cazy.org), a database of Carbohydrate-Active enZYmes (CAZymes).¹⁷ GHs in the same CAZy family often share similarities in their catalytic mechanism.¹⁸ For instance, glycosyl hydrolase family 1 (GH1) contains enzymes that possess a classical $(\alpha/\beta)_8$ triosephosphate isomerase (TIM) barrel fold and employ a Koshland double-displacement mechanism in their substrate turnover.¹⁹ Cyclophellitol aziridine is a rather potent and selective mechanism-based retaining β -glucosidase inhibitor, and is readily modified into a retaining β -glucosidase ABP through acylation or alkylation of the aziridine nitrogen with functional tag groups.¹³ The research described in this thesis aimed to demonstrate that the cyclitol aziridine scaffold allows for the development of ABPs targeting other retaining glycoside hydrolases, specifically, α -L-fucosidases, α -glucosidases and β -glucuronidases. The following paragraph outlines the contents of this thesis, which essentially describes the feasibility of configurational and functional analogues of cyclophellitol aziridine as effective and selective in-class retaining glycosidase activity-based probes.

1.4 Aim and outline of thesis

The design of configurational cyclophellitol aziridine isomers as starting points for the development of ABPs targeting different retaining GHs requires suitable routes of synthesis for their preparation. Existing literature on cyclophellitol aziridine synthesis is scarce, but has grown in recent years. **Chapter 2** provides a concise overview of the existing routes for synthesis of cyclophellitol aziridine isomers. **Chapter 3** describes the synthesis of cyclophellitol aziridine based α -L-fucosidase ABPs and L-fuconojirimycin inhibitors, as well as *in vitro* and *in vivo* profiling of active GH29 α -L-fucosidases in mammalian tissue. A comparative study between *N*-acyl aziridine and *N*-alkyl aziridine ABPs for β -glucosidases and α -L-fucosidases is described in **Chapter 4**. **Chapter 5** provides the development of α -glucoside cyclophellitol aziridine ABPs for GH31 α -glucosidases. Cyclophellitol aziridine ABPs for β -glucuronidases are the subject of the studies described in **Chapter 6** (synthesis) and **Chapter 7** (biological studies). In **Chapter 6**, the preparation of both *N*-acyl and *N*-alkyl aziridine isomers of β -glucuronide and related ABPs for β -glucuronidases are described. **Chapter 7** shows the probes from **Chapter 6** to be able to modify both GH2 lysosomal β -glucuronidase (an *exo*-glycosidase) and GH79 heparanase (an *endo*-glycosidases). **Chapter 8** gives a summary of the research described in this thesis and suggests some future prospects.

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