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Cyclophellitol and its derivatives: synthesis and application as beta-glycosidase inhibitors

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Citation

Li, K. J. (2014, December 11). *Cyclophellitol and its derivatives: synthesis and application as beta-glycosidase inhibitors*. Retrieved from <https://hdl.handle.net/1887/30102>

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1

General Introduction and Outline

Glycosidases are involved in a wide range of fundamental biological processes and are essential enzymes in the production of biofuels. Malfunctioning of glycosidases has been linked to several lysosomal storage disorders. Numerous glycosidase inhibitors have been synthesized and isolated from nature and are extensively studied as potential therapeutics and as tools in mechanistic studies. In the recent past, activity-based protein profiling (ABPP) has been included in the toolbox to elucidate the role and function of glycosidases in a biological context. This Chapter aims to provide background information on the various studies described in this Thesis. The first part of this Chapter highlights some aspects of glycosidases. The second part highlights some non-covalent and covalent glycosidase inhibitors and their use as scaffold in the generation of activity-based glycosidase probes.

1.1 General Aspects of Glycosidases

Carbohydrates and glycoconjugates are the most diverse class of biomolecules occurring in nature. They are involved in the maintenance of cellular structural integrity, cell to cell interactions, communication processes and regulation of protein activity amongst others.¹⁻⁴ Metabolism of these carbohydrate structures is therefore crucial to the functioning of a given organism. Degradation of polysaccharides and glycoconjugates is a challenging task owing to the stability of glycosidic linkages in these structures. This is illustrated by the biopolymer cellulose with a half-life for spontaneous hydrolysis in the range of 5 million years.⁵ In nature, an elegant solution is provided to solve this problem in the form of enzymes known as glycosidases that enhance the hydrolysis rate by 10^{17} fold, resulting in a half-life of only milliseconds.⁵ With this kind of rate acceleration, glycosidases, also known as glycoside hydrolases (GH) are identified as highly proficient catalysts.

1.2 Classification of Glycosidases

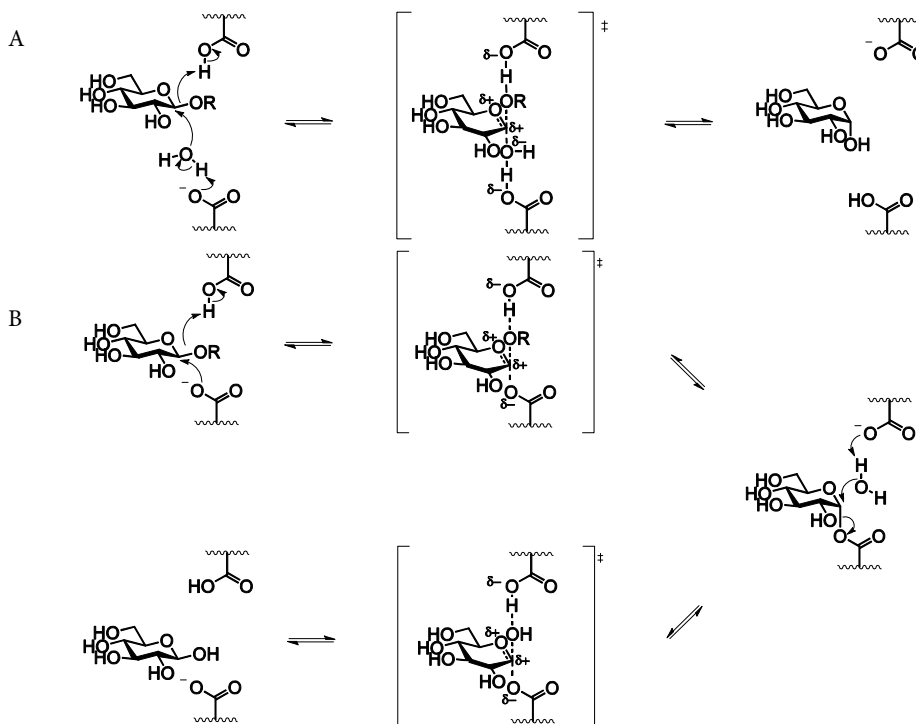
1.2.1 Substrate specificity, cleavage site and amino acid sequence

The large array of glycosidases has been classified into different categories based on different criteria such as substrate specificity, catalytic mechanism, cleavage site of the glycosidic bond and amino acid sequence.⁶ Classification of glycosidases based on substrate specificity depends on the overall stereochemical configuration of glycosides. For instance, β -glucosidases prefer the catalysis of β -glucosides while α -galactose is the optimal substrate for α -galactosidases. Another criterion to classify glycosidases, in particular for those that are acting on polymeric substrates is the point of cleavage in a given chain. Glycosidases are referred to as exo, if they specifically act on the termini of polysaccharides, while endo-glycosidases promote internal hydrolysis of glycosidic linkages within a chain. Another important classification of carbohydrate processing enzymes was proposed by Henrissat *et al.* in 1991.^{7,8} Here, glycosidases are ordered into more than 120 different families based on amino acid sequence similarity and this classification forms the basis of the Carbohydrate Active Enzymes (CaZy) Database (available online at www.cazy.org). Glycosidases within a particular family share structural similarities and often their hydrolytic mechanisms are the same. In some cases, members from different families are grouped into glycosidase clans when they display similarities in their tertiary structure, catalytic residues and mechanism, while no homology in their amino acid sequence is observed.

1.2.2 Mechanism of hydrolysis

Glycosidases can be categorized into two major mechanistic classes based on the stereochemical outcome of the anomeric glycoside configuration. Inverting glycosidases process their substrates with net inversion of anomeric stereochemistry, whereas retaining glycosidases process their substrates with net retention.⁶ Their hydrolytic mechanisms were first postulated by Koshland in 1953.⁶ In both cases oxocarbenium ion-like transition states and two carboxylic acids (Asp or Glu) residues are involved. In inverting glycosidases, the two carboxylic acids residues are positioned approximately 10.5 Å apart. Glycosides are hydrolyzed via a direct displacement of the aglycon moiety by deprotonation of an incoming water molecule and concomitant attack on the anomeric carbon (Figure 1A). In contrast, the two catalytic residues in retaining glycosidases are typically situated 5.5 Å apart and as a consequence hydrolysis proceeds via a double displacement mechanism. In the first step, also termed glycosylation, a covalent enzyme-glycosyl intermediate with inversed configuration of the anomeric centre is formed with the active site nucleophile. This step is assisted by

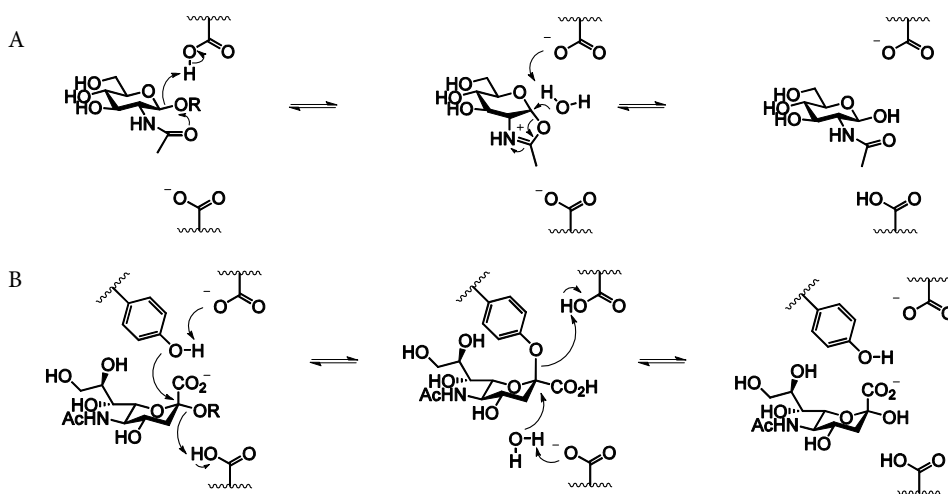
Figure 1. General mechanism of (A) inverting glycosidases and (B) retaining glycosidases.



the other catalytic residue that acts as a general acid by protonation of the interglycosidic oxygen. In the next (deglycosylation) step, the same amino acid residue now acts as a general base and deprotonates an incoming water molecule. Subsequently, the covalent glycosyl-enzyme intermediate is hydrolyzed and a glycan exhibiting the same stereochemical configuration as the initial substrate is liberated.

Not all glycosidases catalyze the hydrolytic cleavage of glycosidic bonds conform the classical Koshland retaining or inverting mechanism. For instance, *N*-acetyl- β -hexosaminidases and some hyaluronidases hydrolyze their substrates via an oxazoline intermediate by utilizing the *N*-acetyl group at C2 as an intramolecular nucleophile (Figure 2A).^{9,10} Furthermore, a tyrosine residue in some retaining sialidases has been identified as the nucleophile, which nucleophilicity was enhanced by a neighbouring basic amino acid residue (Figure 2B).^{11,12} The resulting covalent sialidase-substrate adduct is then readily hydrolyzed. Finally, some distinct hydration and/or elimination mechanisms are known as the catalytic process to cleave glycoside linkages. For instance, glycosidases from the GH4 and GH109 family employ a hydration-elimination mechanism, involving both NAD^+ as co-factor and a divalent metal ion (e.g. Mn^{2+} , Ni^{2+} , Co^{2+} and Fe^{2+}).¹³ On the other hand, α -1,4-glucan lyases (GH31) carry out an elimination mechanism that involves the formation of an anhydrofructose.¹⁴ For a more detailed overview of these distinct glycosidase mechanisms, a recently published review by Jongkees *et al.* can be consulted.¹⁵

Figure 2. (A) Substrate-assisted mechanism of *N*-acetylhexosaminidases and (B) Hydrolysis by sialidases with tyrosine as the catalytic nucleophile.



1.3 Gaucher Disease

Malfunctioning of specific glycosidases is linked to a variety of lysosomal storage disorders.¹⁶⁻¹⁸ Of particular interest for this Thesis is the impaired activity of glucocerebrosidase (GBA), which results in Gaucher disease. GBA, a retaining β -exoglucosidase, is a key enzyme in the catabolism of glucosylceramide.¹⁹ Recently, it became apparent that GBA deficiency is also a major risk factor for the manifestation of Parkinson's disease.²⁰⁻²² Gaucher disease is the most prevalent lysosomal storage disorder with an incidence about 1:60000 in the general population, increasing to 1:1000 in the Jewish population. Point mutations in the GBA gene result in severe storage of its substrate glucosylceramide in macrophages forming Gaucher cells and thereby inducing a variety of clinical symptoms (for instance anaemia, hepatosplenomegaly and thrombocytopenia).^{23,24} The symptoms observed in patients vary from mild to severe and are closely related to the residual GBA activity in lysosomes. For instance, lethal skin permeability issues are caused by complete GBA deficiency, while type I Gaucher patients develop visceral related issues or remain asymptomatic. Next to the type I variant, more severe forms of Gaucher disease exists (type II and III) in which neurological symptoms are involved that, in case of type II results in lethality in the first year of life.²⁵⁻²⁷

To date, two therapies are available to treat Gaucher disease: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). The former is based on the pioneering work by Brady and co-workers in the 1970-80s.^{28,29} This strategy involves the chronic intravenous administration of GBA to patients. In the early stage, the administered GBA was isolated from human placenta which in 1990s was replaced by a recombinant version (Imiglucerase; Cerezyme).³⁰ Drawbacks of this treatment are its high costs and the restrictive patient group. Only type I patients are effectively treated as the enzyme cannot cross the blood-brain barrier to ease the neurological symptoms. Substrate reduction therapy relies on downregulation of the influx of glucosylceramide to lysosomes by inhibiting the enzyme that is responsible for the biosynthesis of glucosylceramide - glucosylceramide synthase.³¹⁻³⁴ Inhibition of this enzyme is achieved by oral administration of *N*-butyldeoxynojirimycin, which was approved as an orphan drug in 2002 under the trade name Zavesca or Miglustat. Studies have shown that this compound was able to pass the blood-brain barrier and therefore suitable to treat Gaucher patients with neurological symptoms.³⁴ A third treatment for Gaucher disease, termed chaperone mediated therapy (CMT) is currently in development.^{25, 35} Key point in this therapy is increasing the amount of properly folded GBA that reaches the lysosome by administration of active site-directed inhibitors (designed as chemical

chaperones). Many point mutations in the GBA gene are associated with improper or retarded GBA enzyme folding in the endoplasmic reticulum (ER). As a result, the misfolded enzymes cannot traverse to the lysosome and are degraded instead by the ubiquitin-proteasome system. The concept of using chemical chaperones to stabilize the protein folding is based on the observation that galactostatin was able to stabilize a mutated form of α -galactosidase and thereby enhancing the overall enzyme activity.³⁶ This finding initiated the search for chemical chaperones for GBA and deoxynojirimycin-type inhibitors (for instance isofagomine) are commonly considered as potential chemical chaperones.^{37,38} Recently, fluoroglucosides bearing anomeric phosphorus-based groups were proposed as an alternative chaperone class.³⁹

1.4 Industrial Relevance of Glycosidases

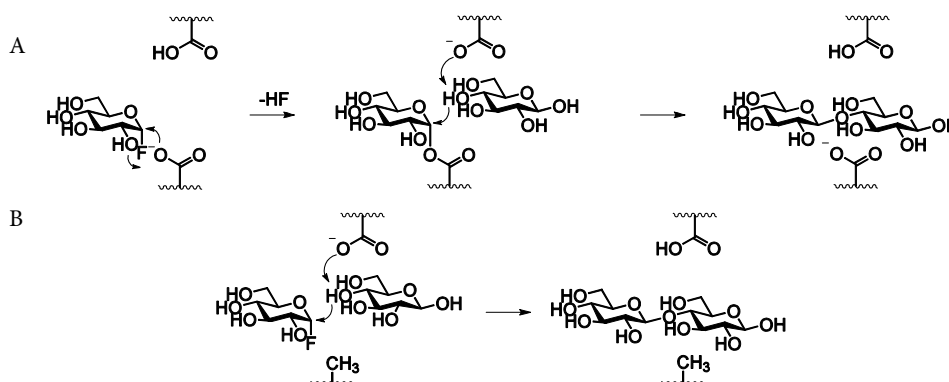
Xylanases and cellulases are the most commonly exploited glycosidases in the industry. They are produced by a vast array of bacteria and fungi such as *Clostridium thermocellum* and *Hypocrea jecorina*. These enzymes have found application in a wide variety of industrial branches.⁴⁰ For instance, xylanases are used in the pulp- and paper industry for pre-bleaching the kraft pulp with the advantage that less harsh chemical conditions are needed, thereby lowering environmental pollution and production costs. On the other hand, cellulases are applied in the wine industry to increase the aroma in wine or in the textile industry to remove excess dye from denim fabric. Many other applications in food, animal feed, laundry and detergent industry have been described in literature.⁴⁰ Of particular interest for this Thesis is the application of cellulases in industrial production of bio-ethanol. This process requires the synergistic action of both endo- and exocellulases to efficiently degrade cellulose, a linear 1,4- β -glucose polysaccharide. The resulting end-product is the disaccharide cellobiose, which is consecutively converted into D-glucose by a β -glucosidase and fermented to generate ethanol.⁴⁰⁻⁴³

1.5 Application of Glycosidases in the Assembly of Oligosaccharides and Glycoconjugates

Polysaccharides and glycoconjugates are indispensable biopolymers in nature. A vast array of methodologies has been developed to produce these molecules, though not always with the same efficiency. Isolation of carbohydrate polymers from their natural biological sources tends to be troublesome and low-yielding. Chemical synthesis of oligosaccharides is a powerful strategy, but requires elaborate protection-and

deprotection strategies to achieve optimal stereo- and regioselective control of the glycosidic bond formation.⁴⁴ This is often accompanied with low yield as a combined result of difficult purification steps and product loss during each step of the multistep synthesis. An alternative to the mentioned chemical method is enzymatic synthesis with the prominent advantage of regio- and stereospecific control of the glycosylation reaction. In nature, glycosyltransferases are responsible for the assembly of polysaccharides and glycoconjugates.⁴⁵ These enzymes facilitate the transfer of a given glycan moiety from an activated nucleotide-phosphoglycosyl donor to a suitable acceptor. The use of glycosyltransferases has been limited owing to poor availability of the enzymes and utilization of expensive phosphoglycosyl donors.⁴⁶

Figure 3. Transglycosylation reactions with activated GlcF by (A) wild-type retaining β -glucosidase and (B) glycosynthase.

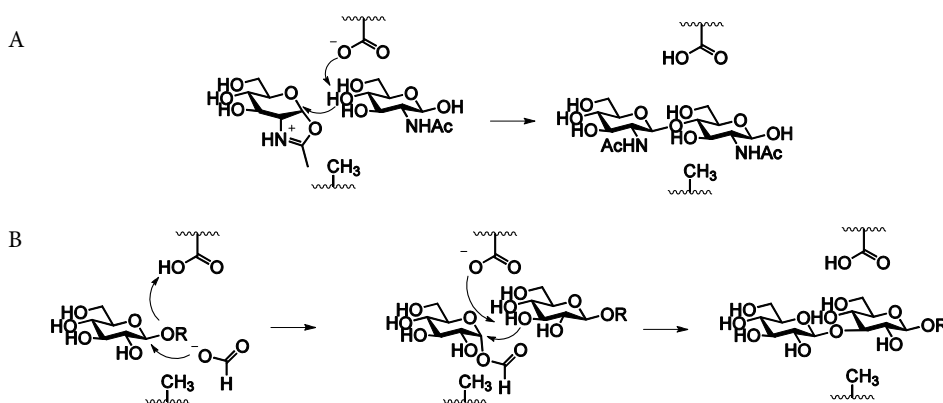


Retaining glycosidases form an alternative for the assembly of glycopolymers. This class of enzymes normally hydrolyzes glycosides via a double-displacement mechanism as discussed earlier in section 1.2.2. Simple reversal of the equilibrium by using high substrate concentration is difficult as the reaction runs in water (55 M), which promotes hydrolysis of the transglycosylated product.⁴⁷ A second method is the use of activated donors (glycosyl fluorides and arylglycosides) to favour the generation of a stable glycosyl-enzyme intermediate (Figure 3A).⁴⁸ Interception of this stable adduct by an acceptor other than water leads to efficient transglycosylation. This method is far from ideal since the transglycosylated product can be hydrolyzed as the enzyme is still hydrolytically active. The Withers laboratory solved this problem in 1998 by the development of glycosynthases.⁴⁹ Glycosynthases are engineered hydrolytically inactive glycosidases with preserved transglycosylation ability. Substitution of the general nucleophile in the *Agrobacterium sp.* β -glucosidase/galactosidase by a nonnucleophilic

amino acid yielded the Glu358Ala mutant with no detectable hydrolytic activity, while the rest of the active site, including the general acid/base catalyst was kept intact. In the presence of this glycosynthase, a multitude of arylglycosides was produced with either α -galactosyl fluoride (GalF) or α -glucosyl fluoride (GlcF) as activated donor and arylglycosides as acceptor. A remarkable difference in the number of glycosyl transfers was observed with the given glycosyl fluorides: single and multiple glycosyl transfers respectively in the presence of GalF and GlcF. In the latter case, the initial transglycosylated product was able to act as an acceptor in the ensuing glycosyl transfer reaction.

Most glycosynthases are derived from retaining glycosidases, but examples derived from inverting glycosidases and glycosidases employing a substrate-assisted hydrolysis have also been developed. Inverting glycosidases have been converted into glycosynthases by mutating the general base residue. Efficient transglycosylation without ensuing hydrolysis was then achieved by using an activated donor, similar as for glycosynthases derived from retaining glycosidases.⁵⁰ Related transglycosylations can be achieved with glycosidases employing substrate-assisted catalysis with an activated oxazoline glycan donors as illustrated in Figure 4A.⁵¹ A different glycosynthase approach was described by Moracci *et al.* who used sodium formate as external nucleophile in the presence of an activated arylglycosyl donor that features an anomeric configuration inherent to the natural substrate. Figure 4B shows the *in situ* formation of a formyl-glucoside intermediate, which is subsequently intercepted by a glucosyl acceptor.⁵²

Figure 4. Reaction mechanism of various glycosynthases. (A) Substrate-assisted catalysis and (B) External nucleophile-mediated catalysis.



After the first demonstration of glycosynthases as an alternative for the construction of oligosaccharides, many efforts were undertaken to increase the efficiency of glycosynthases and broaden their substrate repertoire. This resulted in a set of glycosynthases each with their own catalytic activity and specificity that have been exploited in the assembly of various glycopolymers, glycosphingolipids and glycoproteins. For instance, directed evolution of AbgGlu358Ala resulted in the Abg2F6 mutant (Ala19Thr, Glu352Gly, Gln248Arg and Met407Val) with a 1000 fold enhanced catalytic efficiency and with an expanded repertoire of activated donors (α -mannosyl fluoride and α -xylosyl fluoride).⁵³ Depending on the glycosynthase, α -glucuronyl fluorides⁵⁴ and β -fucosyl azides⁵⁵ can also be employed as donor, while the repertoire of acceptors can vary from sphingosine⁵⁶ and sphingosine-derivatives⁵⁷ to iminosugars⁵⁸, but also to resin-bound glycosyl acceptors.⁵⁹

Enzymatic synthesis has already contributed to a significant progress in the synthesis of oligosaccharides and glycoconjugates and there is room for further studies. So far, glycosidases from only 17 out of the 120 GH families have been transformed into glycosynthases. This indicates that the repertoire of glycosynthases can be expanded to produce glycopolymers with different linkages, which are still not feasible with the current set of glycosynthases.

1.6 Glycosidase Inhibitors and Activity-Based Probes

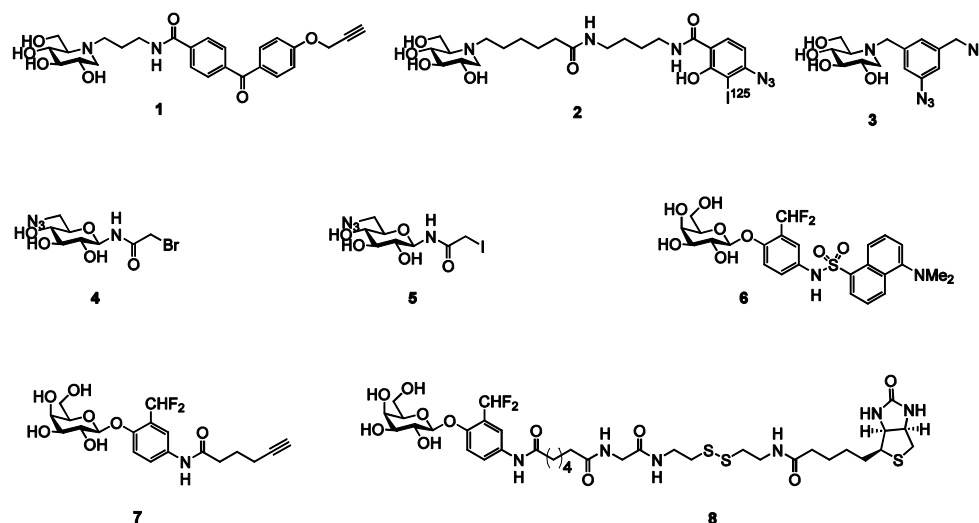
Given the multifunctional potential of glycosidases in various biological and industrial areas, numerous efforts have been made to develop inhibitors to elucidate the functions and mechanistic pathways of glycosidases. Inhibition by these compounds proceeds, either in a reversible or irreversible fashion. The former, also known as non-covalent inhibitors are of particular interest for medicinal chemistry as they show great therapeutic value in the treatment of lysosomal storage disorders, diabetes and viral infections.^{60,61} Some of these glycosidase inhibitors such as Acarbose and Miglustat (Zavesca) indeed found their way to the drug market.

Irreversible glycosidase inhibitors bind covalently to the enzyme, thereby eliminating its catalytic activity. This class of molecules has proven to be indispensable in providing structural and mechanistic details of glycosidases.^{62,63} Moreover, they serve as an ideal starting point for the development of activity-based probes (ABPs).⁶⁴ ABPs are active-site directed covalent inhibitors functionalized with a reporter group or ligation handle, which allow profiling of active enzymes in a complex proteome.⁶⁵ This method is well developed for serine hydrolases and numerous protease families⁶⁶, but proved to be

quite challenging for, in particular exoglycosidases owing to their pocket-shaped active site architecture. However, by careful examination of the active site structure and by rational design, numerous ABPs have been developed for glycosidases.⁶⁴ In the next section some glycosidase ABPs will be highlighted.

A prerequisite for profiling enzymes is the formation of a specific and covalent linkage with the enzyme of interest, which can be accomplished by different classes of glycosidase ABPs. For instance, photoaffinity probes (natural substrates or reversible inhibitors equipped with a photoactivatable group) enable the profiling of inverting glycosidases. Upon irradiation with UV-light, the photoactivatable group is transformed into a reactive intermediate enabling covalent attachment of the probe to the enzyme. Several inverting glycosidase photoaffinity probes have been designed utilizing the non-covalent iminosugar 1-deoxynojirimycin as scaffold as depicted in Figure 5 (1-3).⁶⁷

Figure 5. Structures of photoaffinity probes 1-3, affinity probes 4, 5 and quinone methide probes 6-8.

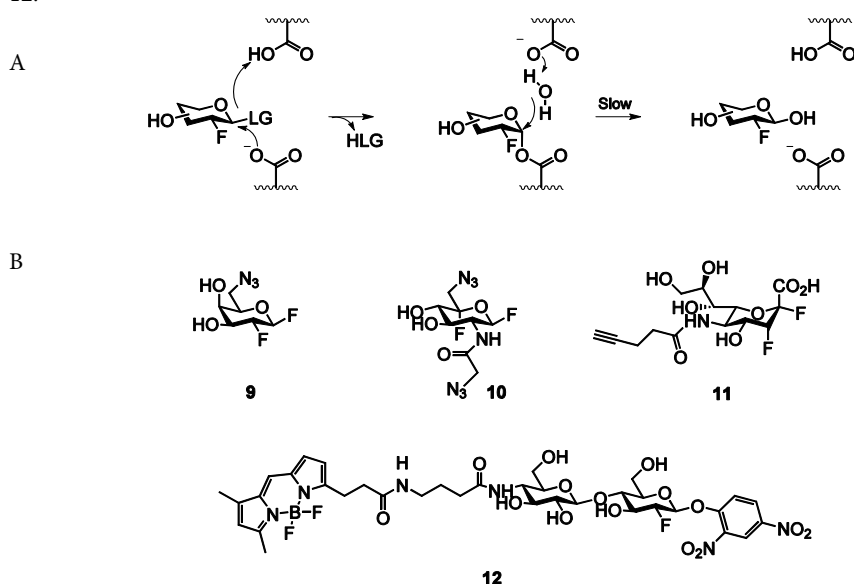


Alternatively, affinity probes such as *N*-halogenated-acetylglucosylamines 4 and 5 proved to be effective in labeling both inverting and retaining glycosidases.⁶⁸ A different approach to label both classes of glycosidases is the use of quinone methides probes such as 6-8⁶⁹, also referred to as suicide substrates. Upon enzymatic cleavage a highly reactive quinone methide is liberated that can react with any nucleophilic amino acid residue. Although these probes have shown some success in labeling of β -galactosidases^{69a} and a neuraminidase of *V. cholera*^{69b}, they proved to be suboptimal for

proteomic purposes. After cleavage, the reactive quinone methide loses its specific affinity for the targeted enzyme, resulting in indiscriminate labeling of enzymes in the given sample.

Two major classes of ABPs have been created for retaining glycosidases using fluorinated glycosides and cyclitol epoxides as scaffold.^{64a} These two sets of probes are chemically inert until they are activated by the catalytic diad of glycosidases. Fluorinated glycosides such as 2-deoxy-2-fluoro, 5-fluoro and 2-deoxy-2,2-difluoroglycosides were first introduced by the Withers laboratory as inhibitors of glycosidases.⁷⁰ During the catalytic hydrolysis mechanism, fluorinated glycosides are able to destabilize both oxocarbenium transition states in the double displacement mechanism of retaining glycosidases, resulting in a slowed-down formation and breakdown of the glycosyl-enzyme intermediate (Figure 6A). Incorporation of an activated anomeric leaving group accelerates the glycosylation step and hence the covalent glycosyl-enzyme adduct is accumulated, allowing sequential mechanistic, structural and biochemical studies. Transformation of these fluorinated inhibitors into their ABP congeners was first accomplished by Bertozzi and Vocadlo in 2004.⁷¹

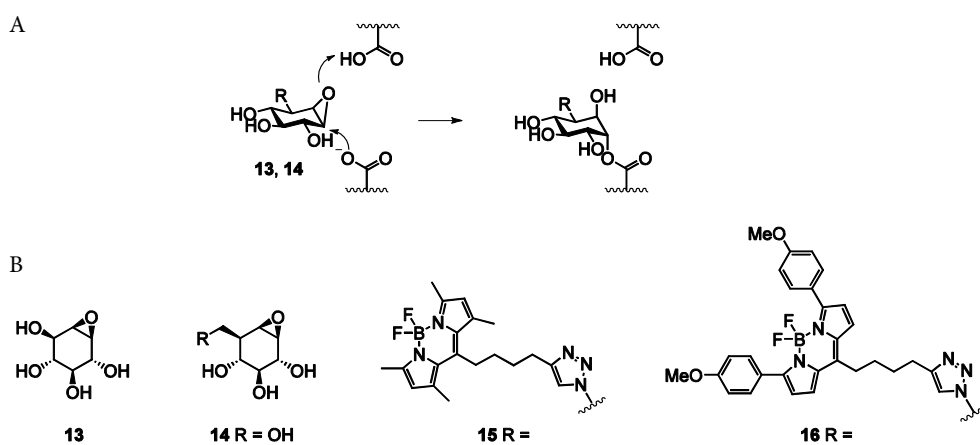
Figure 6. Fluorinated glycosides. (A) Mechanism of inhibition. (B) Examples of fluorinated ABPs **9**–**12**.



Successful labeling of LacZ and five galactosidases from various GH families was achieved in this fashion with ABP **9** (Figure 6B). Further exploration with either an azide moiety as ligation handle or a reporter group directly appended to the fluorinated glycosides generated a set of fluorinated ABPs (**10-12**) for β -N-acetylhexosaminidase NagZ⁷², sialidases⁷³ and retaining endoglycosidases from *Cellulomonas fimi*⁷⁴.

As mentioned before, labeling by cyclitol epoxide derived ABPs are only feasible when activated by the catalytic machinery of glycosidases. These probes are based on Legler's pioneering work with the classical glucosidase inhibitor conduritol β -epoxide (CBE, **13**).^{75,76} This compound enables the inhibition of retaining α - and β -glucosidases by an acid-catalyzed nucleophilic attack on the pseudo-anomeric position of CBE. As a result, a stable and irreversible ester bond between the enzyme and CBE is generated (Figure 7A). A naturally occurring CBE-analogue is cyclophellitol **14** that was first isolated from the mushroom strain *Phellinus sp.* by Atsumi *et al.*⁷⁷ This compound shares similar inactivation mechanism as CBE, but proved to be a selective β -retaining glucosidase inhibitor with increased potency owing to the hydroxymethyl substituent at C5.⁷⁸⁻⁸⁰ Turning cyclophellitol **14** in its ABP counterpart was achieved by grafting a reporter group at the primary alcohol, resulting in the highly specific and potent probes **15** and **16** for glucocerebrosidase (GBA)(Figure 7B).⁸¹ ABPs **15** and **16** have proven their value in monitoring GBA levels *in vitro* and *in vivo* of healthy and Gaucher cells.

Figure 7. Cyclitol epoxides. (A) Mechanism of inhibition and (B) Structures of conduritol β -epoxide **13**, cyclophellitol **14** and cyclophellitol-derived ABPs **15** and **16**.



Aim and outline of Thesis

The remarkable ability of cyclophellitol **14** and cyclophellitol-derived ABPs **15** and **16** initiated the research described in this Thesis. The focus of this Thesis is on the development of cyclophellitol **14** and its derivatives as mechanism-based and competitive retaining β -glycosidase inhibitors as well as glycosidase proteomic profiling tools. **Chapter 2** gives an overview of the current developments in activity-based glycosidase profiling of in particular retaining β -exoglucosidases. Their design principles and applications in studies on glycosidase activities will be highlighted. In **Chapter 3**, the detailed synthesis and purification of cyclophellitol and its aziridine analogue, cyclophellitol aziridine is described. These molecules serve as template for the design of potential new cyclophellitol-derived ABPs and inhibitors. The synthesis and biological evaluation of a novel broad-spectrum cyclophellitol aziridine-based ABP is discussed in **Chapter 4**. **Chapter 5** deals with the synthesis of a small library of cyclophellitol derivatives as glycosidase inhibitors by modifying the epoxide warhead. Their inhibitory activities provide a guideline whether their corresponding ABPs are amenable for glycosidase profiling purposes. The research described in **Chapter 6** and **7** entails the development of cyclophellitol and cyclophellitol aziridine inhibitors and their corresponding ABPs for the cellulose-degrading machinery of fungus *H. jecorina* via a chemical or a chemo-enzymatic approach. **Chapter 8** details the synthesis and biological evaluation of a small library of exo-*N*-cyclic deoxynojirimycin analogues. **Chapter 9** gives a short summary of the research described in **Chapter 3** to **8** and provides some future prospects on research that may be considered based on the results presented in this Thesis.

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