Exploring chemical space in covalent and competitive glycosidase inhibitor design

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General Introduction

Glycoside hydrolases (glycosidases/GHs) are widely abundant enzymes in all kingdoms of life and are important biocatalysts that catalyze the hydrolysis of glycosidic linkages in oligo/polysaccharides, glycoproteins and glycolipids with tremendous efficiency.[1] Abnormal glycosidase activity is intimately associated with a variety of human diseases. Overexpression of heparanase, for example, is implicated in almost all cancers examined, and correlates with increased tumor size, tumor angiogenesis, enhanced metastasis and poor prognosis.[2] Specific inhibitors of glycosidases are of great value, not only because they can serve as useful biological tools to study the catalytic machinery, mechanism and itinerary of target enzymes by crystal structure analysis of (covalent) inhibitor-enzyme complexes,[3] but also because they may act as starting points for the development of therapeutic drugs for the treatment of glycosidase-mediated diseases.[4] Additionally, covalent mechanism-based inhibitors have been used as scaffolds for the development of activity-based probes (ABPs) which allow profiling of glycosidases in complex biological systems.[5] This dissertation describes the synthesis and biochemical evaluation of covalent inhibitors and ABPs for retaining α-amylases and lysosomal β-glucocerebrosidase (GBA), as well as the development of a panel of uronic acid-type 1-*N*-iminosugars as potential competitive heparanase inhibitors. This chapter introduces some mechanistic aspects of glycosidases, including the catalytic mechanisms employed by retaining and inverting glycosidases, the design of mechanism-based enzyme inhibitors and an overview of activity-based protein profiling workflows.

1.1 Classification of glycosidases

Glycosidases can be classified in several ways. The simplest classification is the one based on substrate specificity and is captured in the Enzyme Commission (EC) number for a given enzyme.[6] This classification, however, does not reflect the structural features of the enzymes and is not appropriate for enzymes that act on several substrates. In 1991, a new classification system based on amino acid sequence similarities was introduced,[7] which proved to be a highly valuable tool in understanding the structure/function relationships of glycosidases. To date, over 160 Glycoside Hydrolase (GH) families are categorized and are available in the Carbohydrate Active enZyme (CAZy) database (www.cazy.org).[8] Since sequence and protein fold similarities are directly related, glycosidases within the same GH family usually possess similar structural features and catalytic mechanisms.[9,10]

Glycosidases typically hydrolyze their substrates by employing either a retaining or inverting mechanism, as firstly described by Daniel E. Koshland, Jr in 1953.[11] Two major catalytic mechanisms employed by most glycosidases involve oxocarbenium ion-like transition

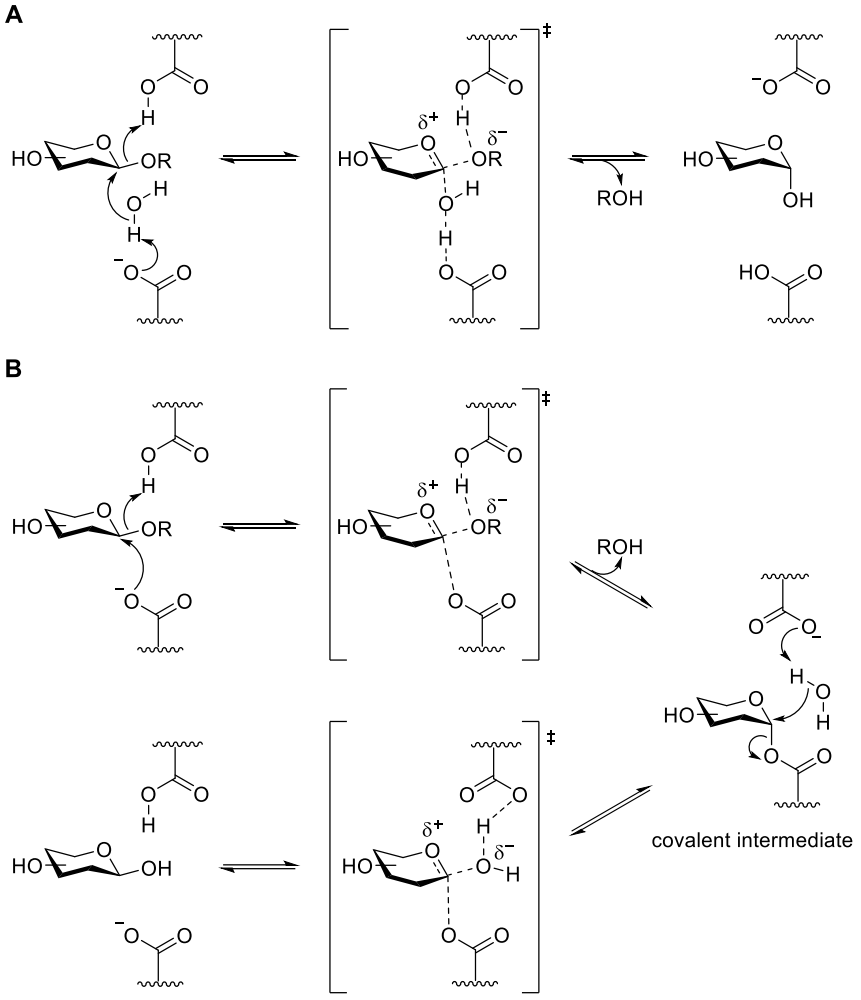

Figure 1.1. General mechanism of the hydrolysis of a β-glycoside employed by A) inverting glycosidases and B) retaining glycosidases.

states and a pair of carboxylic acids/carboxylates (aspartic acid/aspartate or glutamic acid/glutamate) as key residues within the glycosidase active site (Figure 1.1). In inverting glycosidases, one carboxylate residue acts as the catalytic base to deprotonate the incoming water molecule during its attack at the anomeric carbon. The other carboxylic acid then acts as the catalytic acid to protonate the aglycon oxygen atom, thereby assisting in its departure from the anomeric center (Figure 1.1A). The reaction completes in one step where the hydroxyl displaces the aglycon following a S_N2-type mechanism, releasing the hydrolyzed product with inversion of stereochemistry at the anomeric carbon.[12] In retaining glycosidases, S_N2-type displacement occurs twice involving two oxocarbenium ion-like transition states (classical Koshland double-displacement mechanism). During the first step, the catalytic nucleophile attacks the anomeric center while the catalytic acid/base residue protonates the leaving aglycon to form a covalent substrate-enzyme intermediate with inversion of stereochemistry at the anomeric position (Figure 1.1B). During the second step, the catalytic acid/base deprotonates a

water molecule, which subsequently displaces the anomeric carboxylate in a similar reaction sequence adopted by inverting glycosidases, releasing the enzyme and the hydrolyzed product with net retention of anomeric stereochemistry.[12]

Glycosidases are further classified into two groups based on the position of hydrolysis in the substrate (Figure 1.2). Exo-glycosidases usually hydrolyze their substrates at the non-reducing end of a polysaccharide chain. The active site of exo-glycosidases is typically pocket-shaped, providing space to accommodate only the terminal monosaccharide.[13] In contrast, endo-glycosidases cleave their substrates at internal positions of the polysaccharide chain. They have a wider cleft- or tunnel-shaped active site to accommodate multiple sugar residues, often at both sites of the scissile glycosidic linkage.[13]

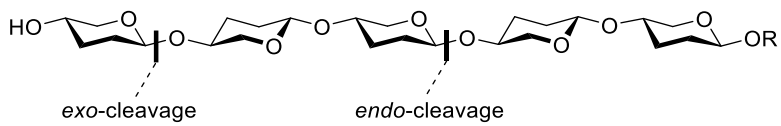

Figure 1.2. Exo- and endo-glycosidase cleavage site in the polysaccharide chain.

1.2 Covalent, irreversible, cyclophellitol-based glycosidase inhibitors

Glycosidases distort their sugar substrates during the catalytic process, and analyzing the conformational changes of substrates during the reaction itineraries they undergo can help considerably in the design of enzyme inhibitors.[3,14] For example, the glucopyranose in a β-glucoside preferably adopts a lowest energy chair ($^4C_1$) conformation in solution, but upon accommodation in the active site of a retaining β-glucosidase, it is distorted into a skew boat ($^1S_3$) conformation forming a Michaelis complex with the enzyme where the aglycon is pseudo-axially positioned to facilitate in-line attack by the catalytic nucleophile (Figure 1.3A).[15,16] During transition state formation the glucopyranose moiety of the substrate (also termed the glycon) is further distorted into a higher energy half-chair ($^4H_3$) conformation where the C5, O5, C1 and C2 atoms are coplanar, to accommodate the developing partial oxocarbenium double bond between O5 and C1. Following substitution of the aglycon, the substrate forms a covalent intermediate with the catalytic nucleophile, adopting a relaxed $^4C_1$ conformation. During the second half of the reaction the glycon conformation is further distorted in a reverse order, releasing the product with same conformation as the initial substrate. The same $^1S_3 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$ itinerary is also proposed for other retaining β-glycosidases including GH79 β-glucuronidases[17], GH10 β-xylanases[18] and GH2 β-galactosidases.[19] In contrast, many retaining α-glycosidases, such as GH31 α-glucosidases[20] and GH27 α-galactosidases,[21] employ a reverse $^4C_1 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^1S_3$ conformational itinerary (Figure 3B). It is less clear which conformational

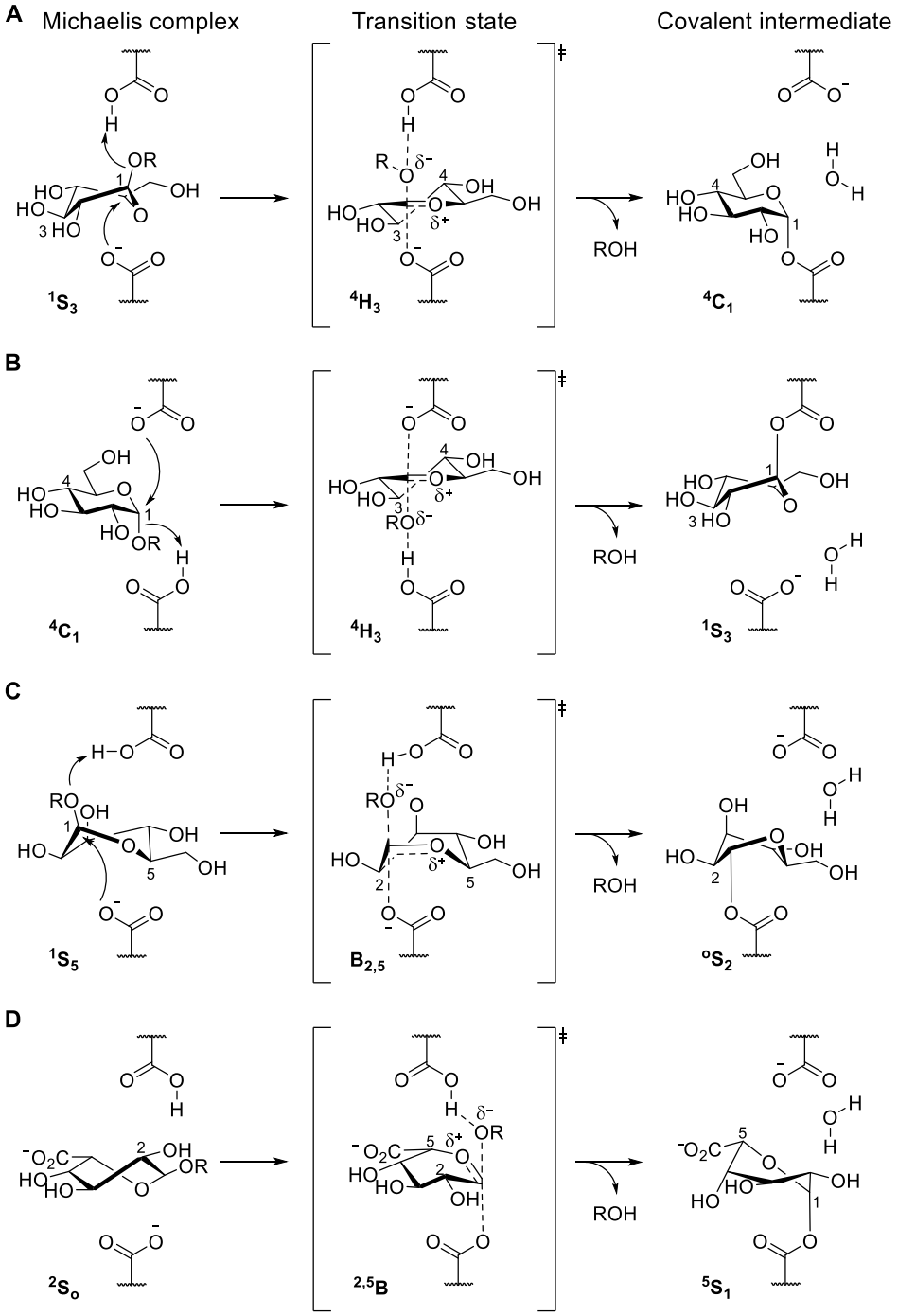

Figure 1.3. Different conformational itineraries of the substrate during hydrolysis (from initial substrate binding to covalent intermediate formation) by retaining β-D-glucosidase (A), α-D-glucosidase (B), β-D-mannosidase (C) and α-L-iduronidase (D).

itinerary the GH13 α-amylases use. Structural characterization of the covalent intermediate complexes of human pancreatic α-amylase (HPA) trapped using two different compounds – an *in-situ* generated 5-fluoro-idosyl trisaccharide (MeG2-5Fido) and a chemoenzymatically synthesized maltobiose 1,6-*epi*-cyclophellitol (both by the Withers' group) – revealed that the sugar in the -1 subsite adopts a $^4C_1$ chair conformation.[22,23] Although a computational analysis

of the conformational itineraries for a GH13 α-amylase has yet to be done, a detailed analysis of a homologous GH13 amylosucrase from *Neisseria polysaccharea* proposes a $^{4}C_{1} \rightarrow [^{4}H_{3}]^{\ddagger} \rightarrow {}^{4}C_{1}$ trajectory for the glycosylation step,[24] consistent with the observed conformation of the inhibitors bound in the active site of HPA.

There are also other glycosidase families which process their substrates following a completely different reaction itinerary.[3,14] For example, hydrolysis of a β-mannopyranoside by retaining β-mannosidases involves the formation of the Michaelis complex in an $^{1}S_{5}$ conformation (Figure 1.3C). Following a boat conformation ($B_{2,5}$) in the transition state, the covalent β-mannoside-enzyme complex is formed in a $^{o}S_{2}$ conformation. Similarly, GH38 α-mannosidases follow a reverse $^{o}S_{2} \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^{1}S_{5}$ conformational pathway for the glycosylation step. For GH39 α-L-iduronidases the conformational change from Michaelis complex to transition state and covalent intermediate has been reported to follow a $^{2}S_{o} \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^{5}S_{1}$ itinerary (Figure 1.3D).

Covalent, irreversible glycosidase inactivators abrogate the enzyme activity through the formation of a covalent bond between the enzyme and a reactive functionality on the inactivator.[25,26] This covalent attachment of the inactivator either blocks access to the enzyme active site or modifies an active site residue that is crucial for catalysis, thus leading to loss of enzyme activity. An important class of covalent retaining glycosidase inactivators are cyclitol epoxides such as conduritol B epoxide[27] (CBE, **1**, Figure 1.4A). CBE irreversibly inhibits β-glucosidases and (with lower potency) α-glucosidases in a mechanism-based manner.[28,29] The reactivity of CBE towards both β- and α-glucosidases can be attributed to its inherent C_{2}-symmetry in its structure, making it both a β- and an α-glucopyranose mimetic.[30] Cyclophellitol (**2**, Figure 1.4A), a natural product isolated from *Phellinus sp.*[31] and now synthetically available,[32,33] is, by virtue of its hydroxymethyl substituent, a better β-glucopyranose mimic and inhibits retaining β-glucosidases with much higher potency than CBE (lacking the C6 methylene compared to **2**).[34-36] It exhibits considerably improved selectivity towards β-glucosidases over α-glucosidases. Substitution of the epoxide oxygen with a nitrogen atom gives cyclophellitol aziridine (**3**, Figure 1.4A), which is an even more potent inhibitor of β-glucosidases.[37,38] Both compounds **2** and **3** adopt a $^{4}H_{3}$ half chair conformation, thereby mimicking the transition state conformation adopted by retaining β-glucosidase substrates during their hydrolysis.[39] Upon binding in the enzyme active site, the strained epoxide/aziridine ring is opened by nucleophilic attack at the 'anomeric carbon' by the catalytic nucleophile, forming a covalent inactivator-enzyme adduct (Figure 1.4B). Compared to the acylal linkage

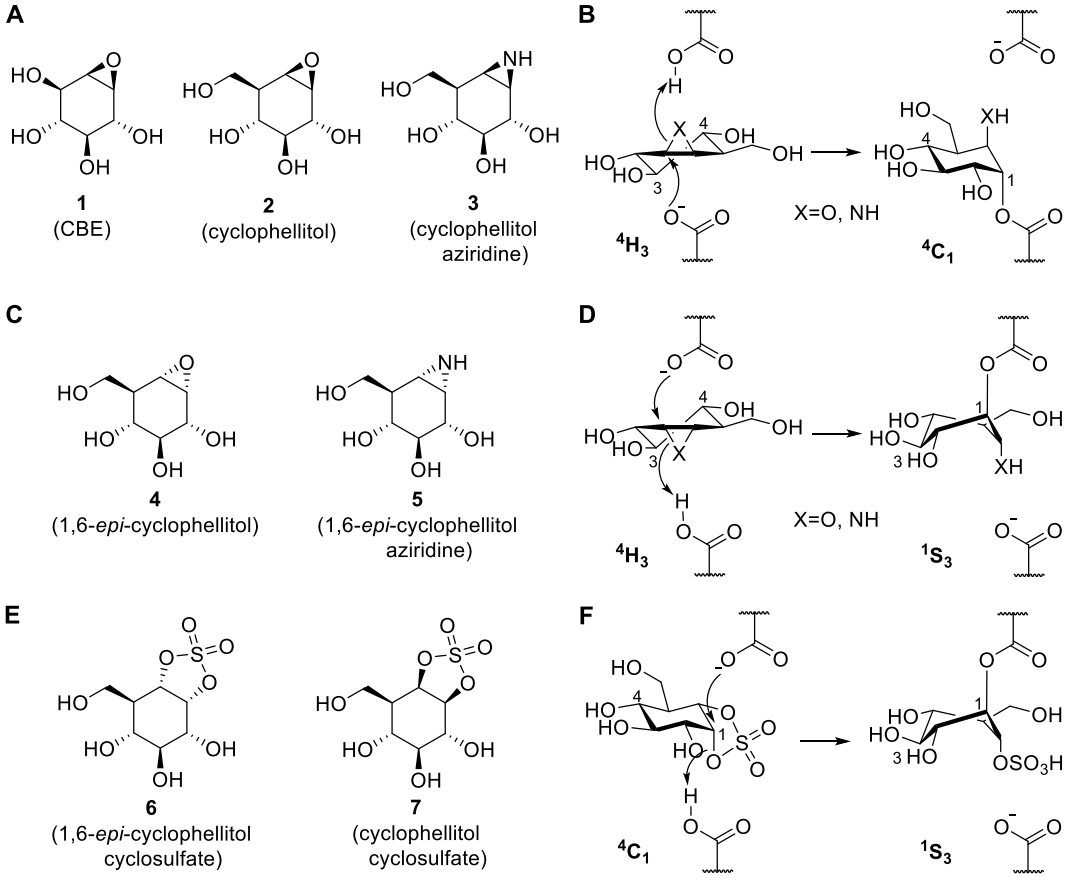

Figure 1.4. Covalent irreversible inhibitors of retaining β- and α-glucosidases and mechanisms of glucosidase inactivation. A) Structure of conduritol B epoxide (**1**), cyclophellitol (**2**), and cyclophellitol aziridine (**3**); B) Mechanism of retaining β-glucosidase inactivation by **2** and **3**; C) Structure of 1,6-*epi*-cyclophellitol (**4**) and 1,6-*epi*-cyclophellitol aziridine (**5**); D) Mechanism of retaining α-glucosidase inactivation by **4** and **5**; E) Structure of 1,6-*epi*-cyclophellitol cyclosulfate (**6**) and cyclophellitol cyclosulfate (**7**); F) Mechanism of retaining α-glucosidase inactivation by **6**.

formed during β-glucoside hydrolysis, the resulting ester linkage cannot undergo normal hydrolysis due to the absence of the endocyclic oxygen, and the enzyme is irreversibly inhibited. In analogy, α-glucopyranose-configured cyclitol epoxide and aziridine, termed 1,6-*epi*-cyclophellitol and 1,6-*epi*-cyclophellitol aziridine (**4** and **5**, Figure 1.4C)[40,41] are potent inhibitors of GH31 α-glucosidases by again mimicking the substrate $^4H_3$ transition state conformation (Figure 1.4D).[42] The scope of cyclophellitol-based covalent inhibitors was further expanded by the development of 1,6-*epi*-cyclophellitol cyclosulfate (**6**, Figure 1.4E) which is a rapid and potent irreversible inhibitor of α-glucosidases.[43] Compound **6** binds to the active site of α-glucosidases adopting a favored $^4C_1$ 'Michaelis complex like' conformation, which is perfectly poised for nucleophilic attack by the enzyme (Figure 1.4F). In contrast, the β-glucose-configured cyclophellitol cyclosulfate (**7**, Figure 1.4E) which also adopts a $^4C_1$ conformation,

is a slow and weak inactivator of β-glucosidases because the $^4C_1$ conformation does not match that of a typical β-glucosidase Michaelis complex.[43] These results illustrate that the cyclophellitol-based inhibitors' conformations substantially impact their inhibitory potency. So far, the conformational mimicry strategy based on the cyclophellitol template has been widely applied for other retaining glycosidases, including those that follow similar $^1S_3 \rightarrow {}^4H_3 \rightarrow {}^4C_1$ or $^4C_1 \rightarrow {}^4H_3 \rightarrow {}^1S_3$ reaction itineraries such as GH27 α-galactosidases[44-46], GH79 β-glucuronidases[17] and GH10 β-xylanases[47], and also those following other conformational trajectories.[48-52]

1.3 Competitive glycosidase inhibitors based on transition state mimicry

The design of most competitive glucosidase inhibitors is based on mimicking either the positive charge developed along the bond between the anomeric carbon and the endocyclic oxygen or the canonical half chair conformation of the transition state (Figure 1.5A).[53,54] Iminosugars that feature a nitrogen atom in the carbohydrate-mimetic ring are perhaps the best known of the competitive glycosidase inhibitor class. Nojirimycin (NJ, **8**, Figure 1.5B), the first iminosugar isolated from a *Streptomyces* strain in 1966,[55] is a close glucopyranose analogue that has a nitrogen in place of the endocyclic oxygen. NJ is a potent inhibitor of various α- and β-glucosidases, however, the presence of the hemiaminal moiety in its structure renders this compound unstable in physiological conditions.[56] In 1967, Paulsen and co-workers reported the synthesis of 1-deoxynojirimycin (DNJ, **9**)[57] which was also later found to exist as a natural compound.[58,59] DNJ lacks the hydroxyl group at C1 and is hence much more stable than NJ while possessing similar biological properties. On the basis of DNJ, a lot of configurational analogues[60] and *N*-substituted derivatives[61] have been developed and some of them are employed for therapeutic applications. For example, the α-glucosidase inhibitor miglitol (**10**), which contains a hydroxyethyl group at the nitrogen atom, is used for the treatment of type II diabetes.[62] The synthetic isofagomine (**11**), another type of iminosugar in which the anomeric carbon is replaced by a nitrogen atom, was developed by Bols *et al.* in 1994 and was shown to be a more potent inhibitor of β-glucosidases than α-glucosidases.[63] A previous study on the thermodynamics of binding of **9** or **11** to a β-glucosidase enzyme suggested that the 2-OH of **9** contributed significantly to binding enthalpy.[64] Therefore noeuromycin **12**, an analogue of **11** where the 2-hydroxyl group is present, also synthesized by Bols and co-workers, proved to be a strong inhibitor of both α- and β-glucosidases.[65] The scope of the isofagomine-type 1-*N* iminosugars was further expanded by Ichikawa and co-workers, who showed that configurationally isosteric compounds are potent inhibitors of their corresponding β-

glycosidases.[53] It has been proposed that the protonated form of the iminosugar inhibitors to some extent mimic the positive charge developed at the endocyclic oxygen or the anomeric carbon of the glycosidase transition state.

A different approach for the design of competitive inhibitors is to mimic the conformation of the transition state, which can be achieved by introduction of bicyclic structures. Nojiritetrazole **13**, for example, has a tetrazole moiety fused onto the pseudo-glycoside ring which causes distortion of the ring into a $^4H_3$ conformation. This compound was shown to be a potent retaining β-glucosidase inhibitor.[66] Following this, a series of cyclitol-azole type derivatives, such as glucotriazole (**14**) and glucoimidazole (**15**, **16**), with varying inhibitory potencies for β-glucosidases were developed by different research groups.[53,67] Based on the $^4H_3$ conformation adopted by cyclophellitol **2** (Figure 1.4A), *carba*-cyclophellitol **17**, with the epoxide oxygen replaced by a carbon atom, proved to be a potent reversible inhibitor (K_i = 8.2 nM) of *Tm*GH1 β-glucosidase.[68] Distortion of the pseudo-glycoside ring to emulate the transition state conformation can also be done by incorporation of an unsaturated double bond. Acarbose **18**, for example, is a reversible α-glucosidase inhibitor which has been employed for clinical treatment of type II diabetes.[69] Constrained by the double bond between C5 and C7 (corresponding to the endocyclic O5 of glucopyranose), the valienamine unit in the compound may adopts a half chair conformation, which was also observed in X-ray crystallographic studies of acarbose-glucoamylase complex.[70]

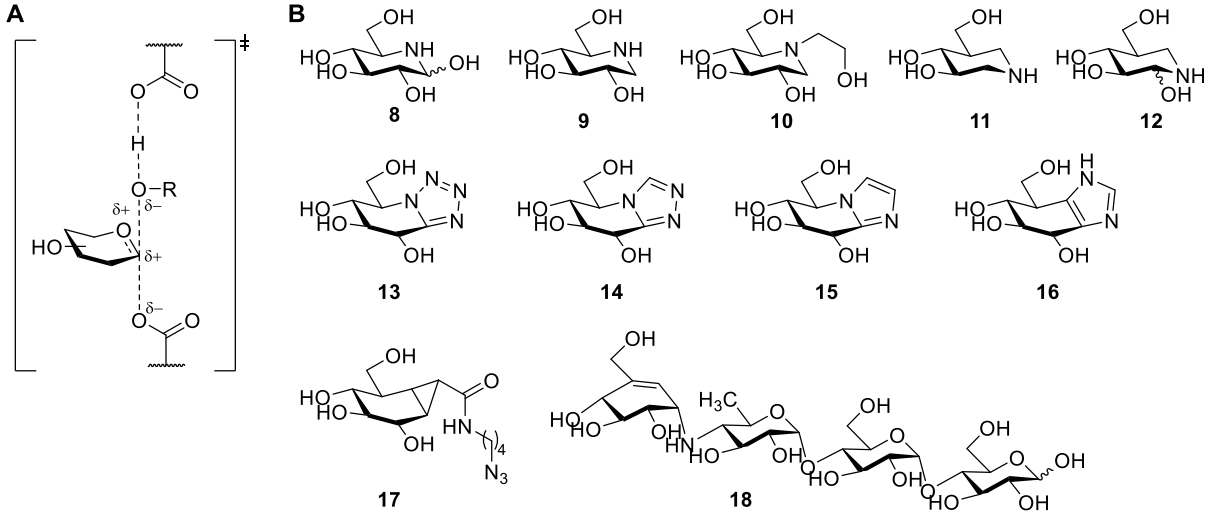

Figure 1.5. A) Transition state for the glycosylation step of a retaining glycosidase; B) Structure of nojirimycin (**8**), 1-deoxynojirimycin (**9**), miglitol (**10**), isofagomine (**11**), noeuromycin (**12**), nojiritetrazole (**13**), glucotriazole (**14**), glucoimidazoles (**15** and **16**), *carba*-cyclophellitol (**17**), and acarbose (**18**).

1.4 Activity-based protein profiling

Activity-based protein profiling (ABPP), first described by Benjamin F. Cravatt in 1999,[71] has become a powerful tool to study the functional state of enzymes in complex biological systems.[72] This technique relies on the development of suitable activity-based probes (ABPs) that irreversibly inhibit an enzyme or a class of enzymes through covalent attachment, allowing direct detection and/or identification of target enzymes. An ABP generally includes three major structural elements: a reactive moiety (or warhead) sometimes embedded in a recognition motif, a reporter group, and a linker (Figure 1.6A). Reactive moieties can be derived from known mechanism-based inhibitors that react covalently and irreversibly with the catalytic amino acids in the active site of the enzyme (class) of interest. Reporter groups are usually fluorescent dyes (for instance a BODIPY, cyanine or rhodamine fluorophore) or enrichment/capture agents (such as biotin or a bio-orthogonal group) that enable ABP-labeled proteins to be visualized and enriched for subsequent studies. A linker can be a hydrophilic or lipophilic chain to provide enough space between the reactive moiety and the reporter group. The covalent and irreversible cyclophellitol-based glycosidase inhibitors discussed in the above section have proven to be suitable warheads and introduction of a reporter entity on (generally) the aziridine nitrogen generates ABPs targeting different classes of retaining glycosidases.

Since the methods for performing ABPP experiments are diverse, this technology has been applied in various formats. For example, fluorescent probes can be used for the detection of enzyme activities. After incubation of a proteome with a fluorescent probe, the protein mixture is denatured and resolved by SDS-PAGE, and the ABP-modified proteins are then visualized by in-gel fluorescence scanning (Figure 1.6B). Since precursor enzymes, inactive or malfunctioning enzymes are generally not labeled, the band intensity can be correlated with the expression level of active enzymes. In this way, ABPs can be used as diagnostic tools to detect different enzyme activities between patients and healthy individuals.[42,73] Alternatively, when the proteome is incubated with a biotin-tagged ABP, the labeled proteins can be enriched by affinity purification using streptavidin magnetic beads (Figure 1.6C). Enriched proteins are then digested on beads by a proteolytic enzyme (usually the serine protease, trypsin) and the resulting peptide mixture analyzed by LC-MS/MS. The obtained protein sequences are matched against protein sequence databases to identify the target protein.[74] Another major application of ABPP is in the discovery of enzyme inhibitors, using methodologies termed competitive ABPP.[75,76] Compared to conventional inhibitor screening methods, competitive ABPP allows enzymes to be tested in native proteomes, without the need for purification and can be done

even if the identity of an active enzyme is unknown. In this experiment, a biological sample is pre-incubated with an inhibitor followed by labelling with a fluorescent ABP (Figure 1.6D). After denaturation and resolving by SDS-PAGE, the inhibitor potency can be read out by quantification of the reduction in probe labeling intensity. When the ABP used targets multiple enzymes in the same sample, a panel of inhibitors can be tested in parallel and the selectivity of inhibitors can be assessed.

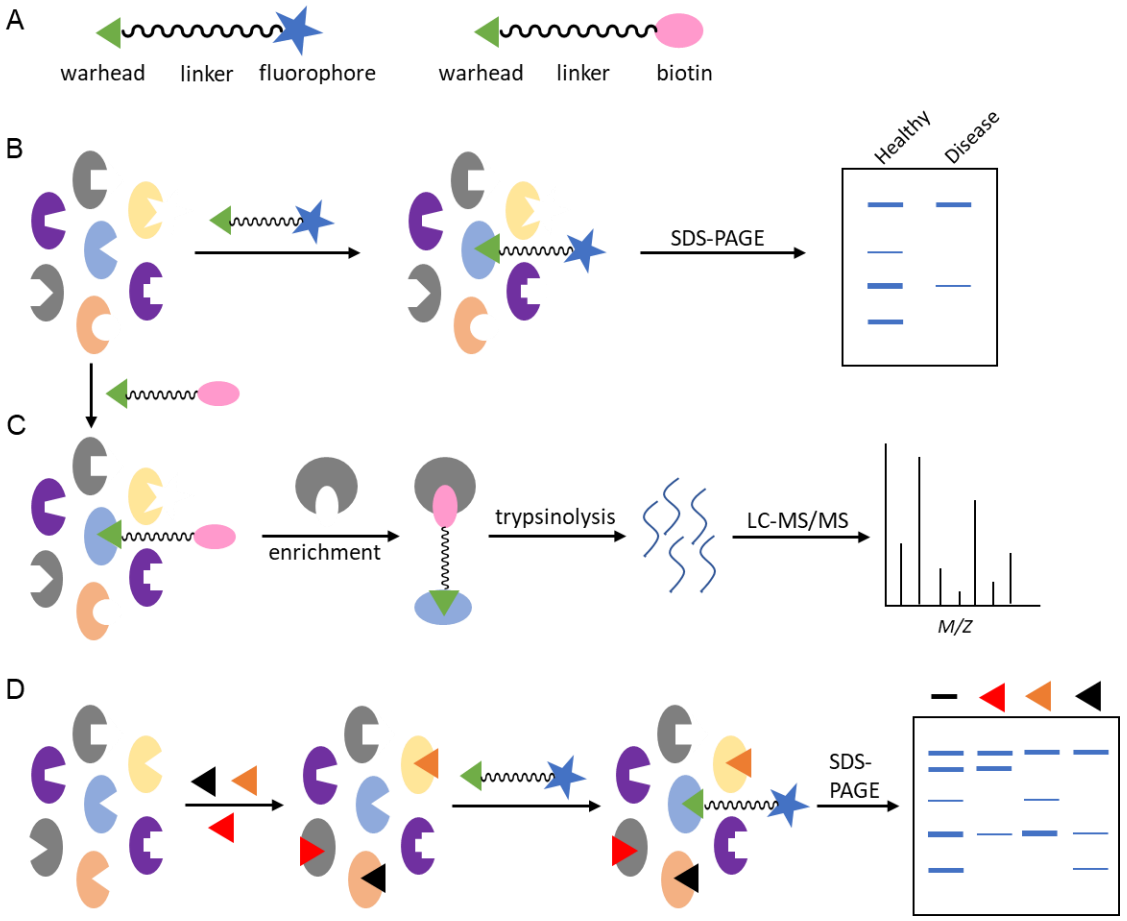

Figure 1.6. Activity-based protein profiling (ABPP). A) General structure of an activity-based probe (ABP); B) Fluorescent ABPs can be used to detect and analyze enzyme activities by SDS-PAGE in a comparative ABPP experiment; C) Identification of the target enzymes by proteomics; D) Inhibitor discovery by competitive ABPP.

1.5 Outline of this thesis

To date, cyclitol-epoxide/azirine/cyclosulfate based inhibitors and ABPs have been applied to the study of various retaining *exo*- and *endo*-acting glycosidases, and the work described in this Thesis was in part focused on expanding this to capture – with ABPP – additional endoglycosidases of biomedical and biotechnological importance. The first two chapters of this Thesis focus on the development of covalent inhibitors and ABPs targeting starch-degrading

enzymes. **Chapter 2** describes the synthesis of a set of maltobiose *epi*-cyclophellitol derived retaining α-amylase inhibitors and probes, and their labeling efficiency is biochemically evaluated on complex biological samples. **Chapter 3** reports on the synthesis of *epi*-cyclophellitol-based pseudotrisaccharides equipped with a suite of reporter entities. The construction of the branched pseudotrisaccharide probes involves selective α-1,4- and α-1,6-glycosylation of proper glucose donors on cyclohexene acceptors followed by further chemical elaboration, giving access to the desired epoxides. **Chapter 4** describes the synthesis of a set of bifunctional cyclophellitol aziridines which are functionalized at both C6 and aziridine nitrogen, as well as the biochemical evaluation of their activity and selectivity towards human lysosomal β-glucocerebrosidase. **Chapter 5** reports on the synthesis of a panel of *gluco*- and *galacto*-configured uronic acid-type 1-*N*-iminosugars as competitive heparanase inhibitors, and how the structural basis for enzyme inhibition by the synthetic iminosugars is studied by X-ray crystallography. **Chapter 6** gives a summary of the Thesis and provides some directions for future work based on the here presented results.

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