Design and synthesis of inhibitors and probes for sulfoquinovosidases and xylanases

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General Introduction

The intricate world of plant glycans stands as a cornerstone in sustaining life on Earth. These complex biopolymers, pervasive in plant cell walls, contribute not only to structural integrity but also to fundamental ecological processes.[1] The significance of plant glycans extends into bioenergy, pharmaceuticals, and various industrial applications,[2] making a comprehensive understanding of enzymatic processes governing plant polysaccharide degradation imperative. Critical to the biotransformation of plant glycans are microbial communities, particularly saprotrophic microorganisms.[3] Armed with an arsenal of enzymes, such as xylanases, α-glucuronidases, and notably recently discovered sulfoquinovosidases, these microorganisms orchestrate the breakdown of cellulose, hemicellulose, sulfoquinovose and other complex carbohydrates into simpler, absorbable components, playing pivotal roles in nature's carbon and sulfur cycling.[4,5] Sulfoquinovosidases are key players in sulfoquinovose degradation, a unique component of plant biomass. Xylanases target the breakdown of xylan, a major hemicellulosic component, while α-glucuronidases facilitate the removal of α-glucuronic acid residues, enhancing the efficiency of biomass degradation.

The methodology of activity-based protein profiling (ABPP), focusing on the study of active enzymes in their native environments, provides a robust framework for such investigations.[6] Building upon pioneering work,[7–9] numerous research groups have developed activity-based probes (ABPs) targeting various enzymes responsible for breaking down major contributors to biomass, such as cellulose and xylan.[10–13] This Thesis describes the synthesis of covalent inhibitors and ABPs for various retaining glycosidases involved in the degradation of sulfoquinovose and xylan, as well as the design and synthesis of ^{13}C-encoded inhibitors and probes for xylanases.

1.1. Plant biomass, hemicellulose and sulfoquinovose

Unraveling the complexity of hemicellulose

To deepen the understanding of biomass degradation, it is vital to have a profound comprehension of the composition of plant biomass, which is predominantly composed of cellulose and hemicellulose.[14] Hemicellulose, a complex polysaccharide composed of various monosaccharides including xylose, arabinose, glucose, and mannose, exhibits structural diversity that poses distinct challenges for its controlled breakdown (Figure 1).[15,16] The intricate arrangement of these sugar units within the hemicellulose matrix necessitates the action of specialized enzymes capable of recognizing and cleaving specific glycosidic linkages. Moreover, unlike cellulose, which consists of repeating glucose units in a linear fashion, the

branched and heterogeneous nature of hemicellulose requires a diverse array of hydrolytic enzymes to efficiently degrade its diverse components.

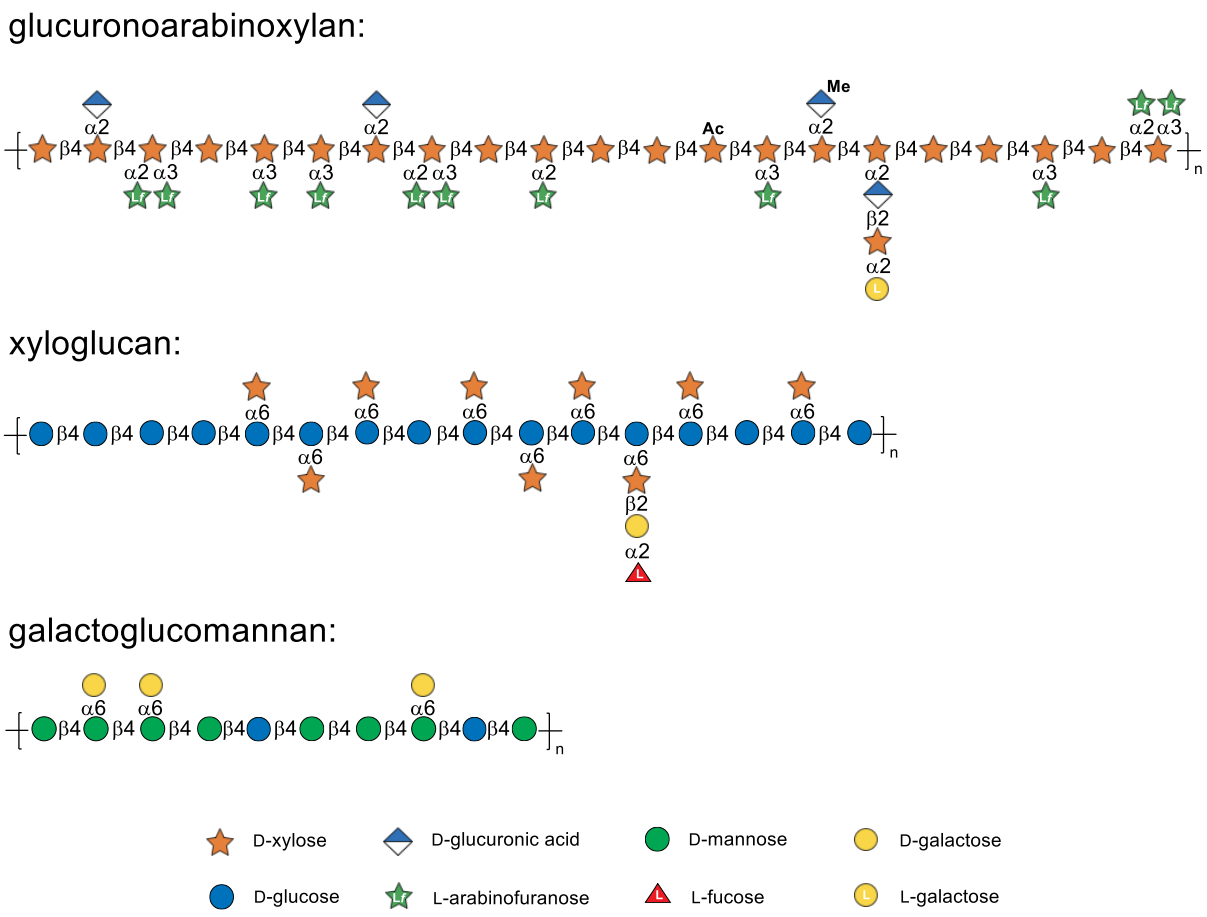

Figure 1 Schematic structures of hemicelluloses. Hemicelluloses can be categorized as xylans, mannans, and xyloglucans, according to variances in their structure, including backbone linkages and side groups. The primary structure of many hemicellulose backbones consists of β-1,4-linked-D-pyranosyl units, including glucose, xylose, and mannose. Xylan may exhibit branching, while in some instances, mannan incorporates glucose into its backbone structure.

The predominant hemicellulosic polysaccharide found in plant cell walls is xylan. It is characterized by a backbone composed of a β-1,4-linked xylopyranose polymer, with the xylose monomers either non-substituted or carrying α-L-arabinofuranoside, α-D-glucuronopyranosode, 4-*O*-methyl-α-D-glucuronopyranoside, or acetate modifications, amongst others (Figure 2). Fungi, yeasts, and bacteria able to degrade hemicellulose employ a suite of enzymes collectively termed hemicellulases, which cooperatively depolymerize hemicellulose in mono- and oligosaccharides. These enzymes include α-D-glucuronidases, α-L-arabinofuranosidases, (acetylxylan-, feruloyl-, and coumaroyl-) esterases, exo-β-xylosidases, and endo-β-xylanases.[17]

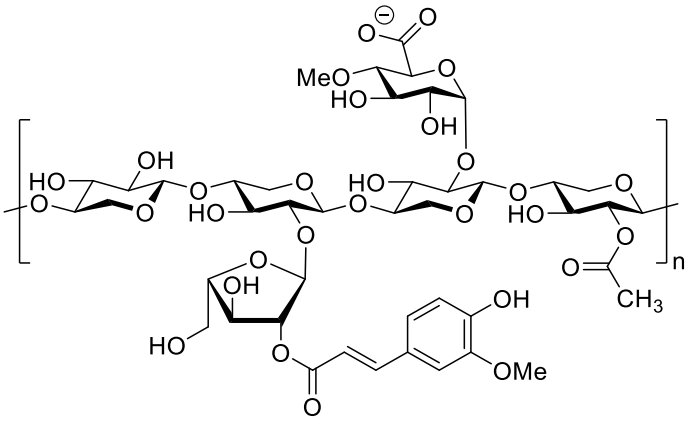

Figure 2 Composition of hemicellulosic xylan. The polysaccharide features a backbone composed of xylose units linked together through β-1,4-glycosidic bonds. The side chains, such as α-L-arabinofuranose, α-D-glucuronopyranose, 4-*O*-methyl-α-D-glucuronopyranose, acetyl groups, and other substituents, are attached to the xylose backbone.

Biosulfur and sulfoquinovose

Sulfoquinovose is a sulfonated sugar derivative that represents a significant portion of the organosulfur biomass and has emerged as a vital player in the global carbon and sulfur cycles. Sulfoquinovose (6-deoxy-6-sulfoglucose, Figure 3) is characterized by its unique sulfonated sugar moiety and is predominantly found in the sulfolipid, α-sulfoquinovosyl diacylglycerol (SQDG).[5]

The presence of sulfoquinovose in plant biomass underscores its physiological importance. It is primarily found in thylakoid membranes of chloroplasts, where it serves as a precursor for sulfolipids, crucial components involved in photosynthetic electron transport.[5] The degradation of sulfoquinovose represents a key aspect of carbon and sulfur cycling in natural environments. Microorganisms equipped with sulfoquinovosidases) able to hydrolyse sulfoquinovosides play a central role in releasing sulfoquinovose from its precursor for subsequent assimilation into microbial biomass. This process not only contributes to carbon recycling but also facilitates sulfur metabolism in microbial communities, underscoring the interconnection of biogeochemical cycles.

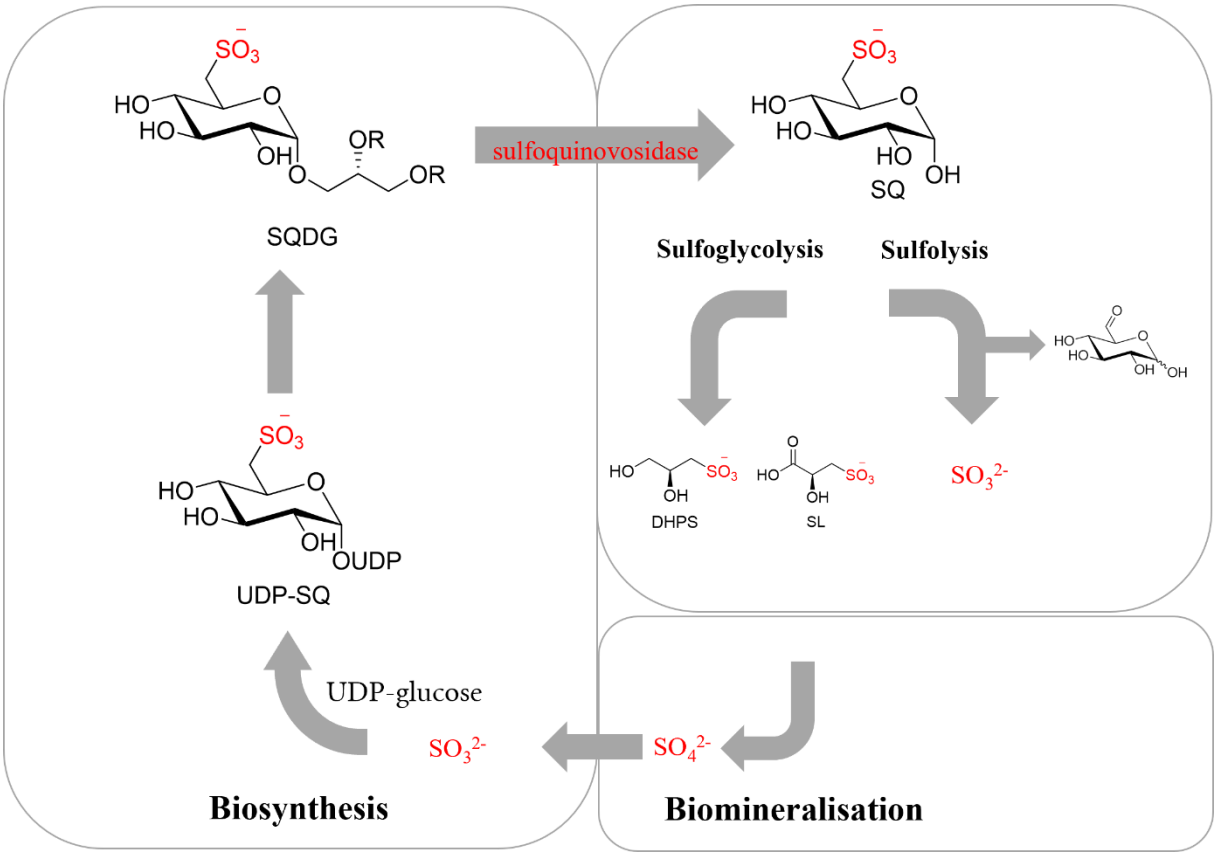

Figure 3 Structure and metabolism of SQ as part of the natural organosulfur cycle.

1.2 Glycoside hydrolases

Catalytic diversity within GH families

The glycoside hydrolase (GH) superfamily contains a large number of enzymes with different substrate specificities and catalytic mechanisms.[18,19] Enzymes within GH families exhibit specificity towards particular glycosidic bonds or substrates, determined by active site architecture. They employ different catalytic mechanisms, retaining or inverting the anomeric configuration of substrates. Regioselectivity contributes to the diversity of hydrolysis products during biomass degradation, while some enzymes also possess transglycosylation activity, forming glycosidic bonds between molecules. Structural and sequence diversity in GH families reflect evolutionary adaptations to ecological niches and substrate sources, enabling enzymes to accommodate diverse substrates and reactions. This diversity underscores the complexity of carbohydrate metabolism and biomass degradation across organisms.

Classification of Glycoside Hydrolases

Glycoside hydrolases can be classified based on amino acid sequence similarities, structural homologies, and catalytic mechanisms.[19,20] The Carbohydrate-Active enZymes (CAZy)

database (http://www.cazy.org)[18] provides a comprehensive classification system of the glycoside hydrolase (GH) families based on amino acid sequence similarities.

Glycosidases can be categorized as two classes based on their catalytic mechanisms: inverting glycosidases and retaining glycosidases. As first described by Daniel Koshland in 1953,[21] inverting glycosidases catalyze the hydrolysis of glycosidic bonds by following a single-displacement mechanism. During this process, the anomeric configuration of the substrate undergoes inversion, resulting in the opposite configuration in the product (Figure 4a), whereas retaining glycosidases employ a double-displacement mechanism during the hydrolysis of glycosidic bonds, which allows for the retention of the anomeric configuration of the substrate in the reaction product (Figure 4b).

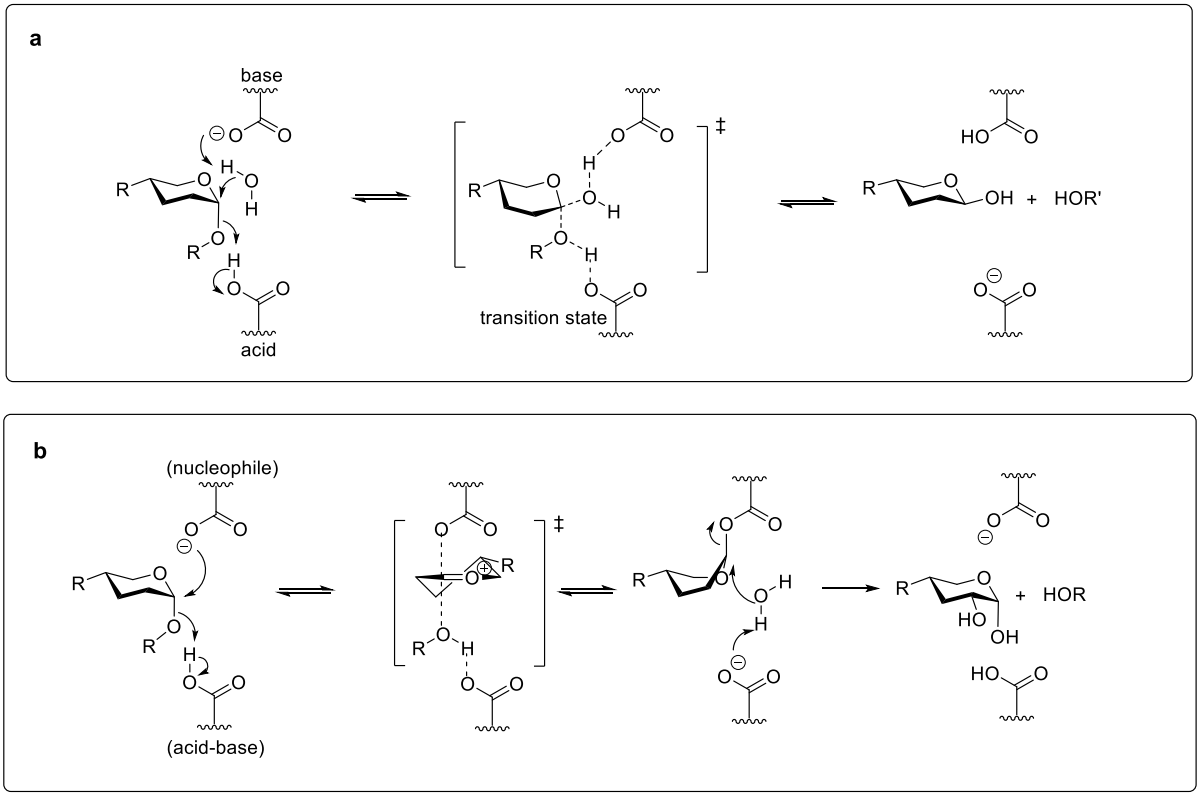

Figure 4 Typical catalytic mechanisms of a) inverting α-glycosidases, and b) retaining α-glycosidases.

Glycosidases can also be classified according to their substrate cleavage sites, again giving two subclasses: exo- and endo-glycosidases. Exo-glycosidases have active sites shaped like pockets and cleave polysaccharides at the non-reducing terminal end, producing a monomer and a shortened polymer chain. In contrast, endo-glycosidases have active sites shaped like tunnels or clefts and cleave a polymer chain internally (Figure 5).

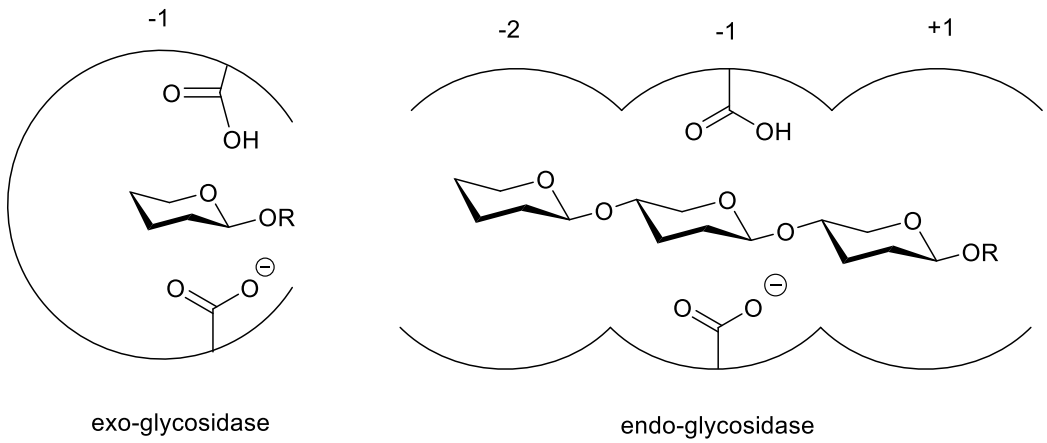

Figure 5 Schematic view of the *exo*- and *endo*-glycosidase active sites.

The synergy of enzymes in biomass degradation

The concerted action of various glycoside hydrolases is essential for the efficient degradation of complex carbohydrates present in biomass. Exo-acting glycosidases, such as β-glucosidases, β-xylosidases, and α-L-arabinofuranosidases, primarily hydrolyze oligosaccharides by cleaving off monosaccharides from the non-reducing ends, though they may also contribute to the breakdown of longer polysaccharides under certain conditions. Endo-acting glycosidases, such as endo-β-xylanases, endo-β-glucanases, and endo-β-mannanases, cleave internal glycosidic bonds within polysaccharides, often acting directly on both branched and linear substrates. In some cases, exo-acting glycosidases cooperate with endo-acting enzymes by further processing the oligosaccharides generated from the cleavage of internal bonds. This synergy facilitates the production of absorbable sugars, which are crucial for their utilization by microbial communities and higher organisms.

1.3 Activity-based protein profiling: illuminating enzyme function

Exploring the intricate enzyme network using ABPP

Activity-Based Protein Profiling (ABPP) represents a technology, first employed about 25 years ago, that allows the study of enzyme activities in the context of complex (not native) biological systems.[22] It employs activity-based probes (ABPs), compounds designed to selectively interact with specific enzymes or enzyme groups. These ABPs are equipped with a reporter molecule, such as a fluorophore, biotin, or bioorthogonal group, facilitating the detection and identification of captured enzymes (Figure 6). Typically, an ABP consists of three main components: a reactive moiety (or 'warhead'), a spacer linking the warhead to the reporter group, and the reporter group itself. The reactive moiety forms a covalent bond with the target enzyme while remaining inert in cell extracts or living cells when not engaged with the enzyme.

The reporter group aids visualization in protein gels or living cells, often utilizing fluorescent groups like BODIPY, rhodamine, or cyanine, or enabling affinity enrichment and subsequent detection via mass spectrometry, commonly using a biotin group.

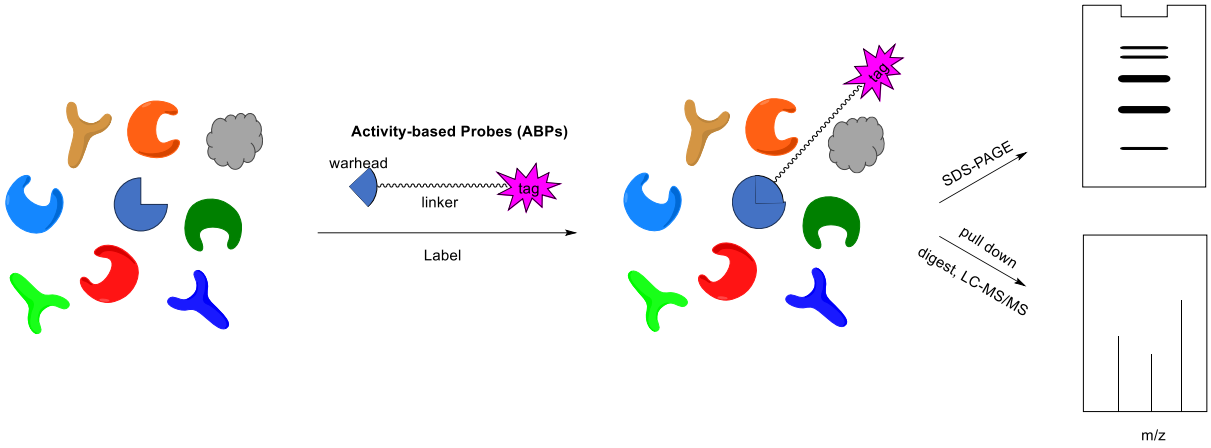

Figure 6 In a standard ABPP procedure, the proteome undergoes treatment with the ABP. Essential to the effectiveness of the probe is its covalent binding to the target enzyme(s). Subsequently, depending on the tag (reporter) group, the labeled enzyme(s) can be directly observed via SDS–PAGE through fluorescent scanning or isolated through streptavidin pull-down for purification. After purification, the enzymes can be digested with trypsin and the resulting peptides sequenced using mass spectrometry.

The role of cyclophellitol derivatives: mechanism-based covalent inhibitors and probes

Central to ABPP are mechanism-based covalent inhibitors and probes.[23] These small molecules are designed to mimic natural substrates and form covalent bonds with the active sites of target enzymes, thus irreversibly modifying the enzyme active sites. Compared to reversible inhibitors, mechanism-based inhibitors form covalent, stable bonds with the target enzyme, allowing for prolonged labeling and detection. Two types of mechanism-based probes (MBPs) have been reported for glycosidases. The first type releases an aglycone upon enzymatic hydrolysis, which can react covalently with target residues near the active site, while the second type directly forms a stable, covalent bond with the catalytic nucleophile of the glycosidase. An example of the first type of MBP is presented by probe **1** (Figure 7), developed for labeling α-fucosidases.[24] This probe integrates an α-L-fucose recognition unit with a latent trapping moiety containing a (di)fluoromethyl group linked to a BODIPY fluorophore. Following enzymatic cleavage, a reactive quinone methide fragment is generated, which is designed to react within the α-fucosidase active site with any available nucleophile. This probe has been effectively utilized for labeling *Thermotoga maritima* α-fucosidase in protein extracts.

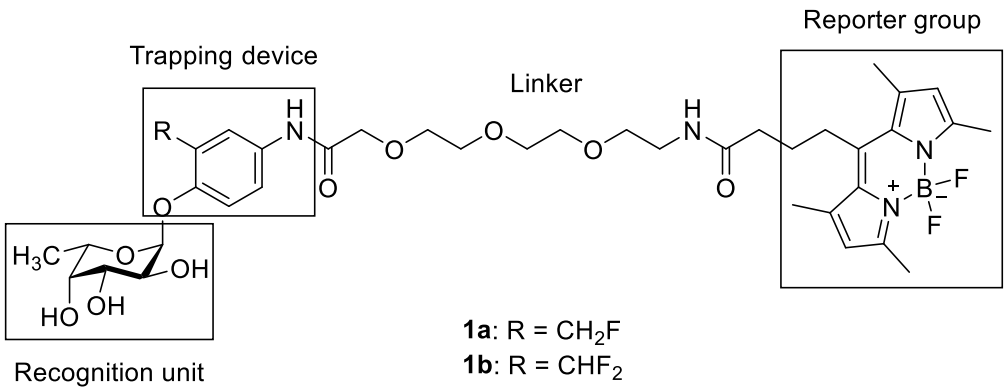

Figure 7 Mechanism-based probes (MBPs) for fucosidases. The trapping device is activated upon release of the aglycone by the target glycosidase.

The second type of MBP exploits the double-displacement mechanism of retaining glycosidases by forming a covalent bond with the catalytic nucleophile. Activated fluorosugars (**Figure 8**, compounds **2**-**5**),[25] represent a prominent example of this class of MBPs and feature an activated anomeric leaving group combined with an electron-withdrawing fluorine substituent[26–28] enabling the formation of a long-lived covalent glycosyl-enzyme intermediate. Epoxide-containing cyclitols like conduritol B-epoxide (CBE, compound **6**)[29] and cyclophellitol (compound **7**)[30] react with the catalytic nucleophile of retaining glycosidases to form a covalent and stable intermediate. Here, the epoxide, upon protonation, acts as the leaving group and in general cyclitol epoxides are more reactive GH inactivators that, once reacted, form more stable covalent enzyme-inhibitor bonds.

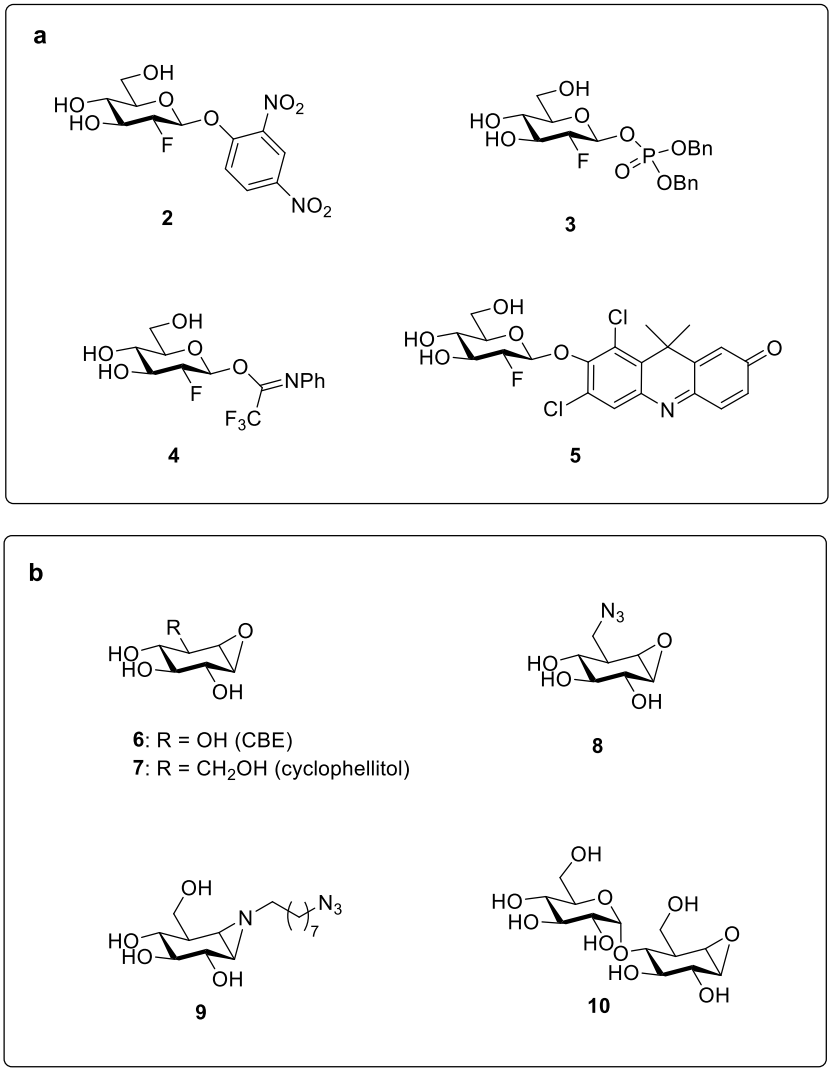

Figure 8 Covalent inhibitors and MBPs for retaining glycosidases based on a) fluorinated glycosides and b) cyclitols containing an epoxide or aziridine motif.

Cyclophellitol-based MBPs have undergone structural modifications to enhance their utility, including the introduction of chemical handles for further functionalization (compounds **8** and **9**),[31,32] replacement of the epoxide with an aziridine (compound **9**), and prolonging the sugar chain (compound **10**).[33] These modified derivatives have been applied in various studies, including the identification of acid/base residues in human retaining β-glucosidases,[34] irreversible inhibition of endoglycoceramidase II from *Rhodococcus sp.*,[35] and structural analysis of human pancreatic α-amylase.[33] In recent years, cyclophellitol-based MBPs have also been utilized in ABPP for enzymes involved in biomass degradation. Building upon previous research involving monosaccharidic cyclophellitol epoxide **7** and aziridine **9**, the creation and utilization of ABPs **11**, **12**, **13**, and **14** to target xylan-degrading enzymes,[12] as well as ABPs **15** and **16** for cellulases,[13] have been developed in recent times.

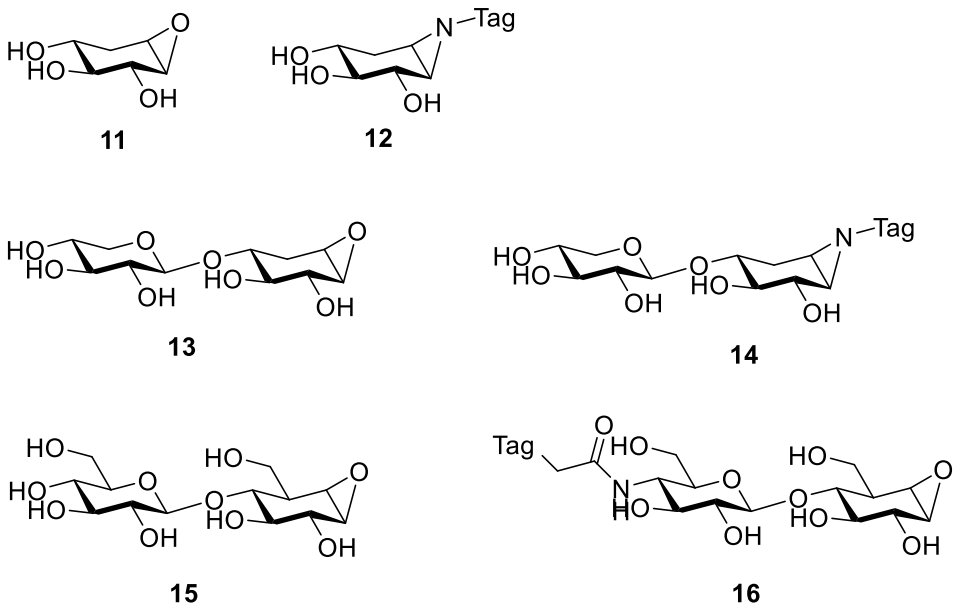

Figure 9 Recently developed cyclophellitol-based MBPs for enzymes involved in biomass degradation.

1.4 Outline of this Thesis

The experimental chapters describe studies on the design and synthesis of a series of new retaining glycosidase inhibitors and ABPs, based on the general cyclophellitol design. **Chapter 2** describes the design and synthesis of putative SQase inhibitors and ABPs using a 1,7-epicyclophellitol aziridine scaffold. **Chapter 3** reports on the design and synthesis of a series of putative ABPs aimed to expand the scope of enzyme detection by incorporating branched oligosaccharide substrates, targeting a range of xylanase activities involved in the breakdown of plant polysaccharides, particularly those enzymes that recognize branched structures. **Chapter 4** describes the synthesis of α-MeGlcA-configured cyclophellitol aziridine that mimics the assumed transition state of glucuronic acid hydrolysis, which may serve as starting point for the synthesis of activity-based probes to identify retaining α-glucuronidases. **Chapter 5** describes the synthesis of two ^{13}C-labeled xylanase ligands (one substrate and one inhibitor), for the purpose of exploring the catalytic mechanisms and structural dynamics of xylanases, with a specific focus on the retaining glycosidase BCX from *B. circulans*. **Chapter 6** gives a summary of the Thesis and provides some recommendations for future work based on the projects described in the thesis.

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