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1 An overview of activity-based probes for glycosidases

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11

12 Abstract

13 As the scope of modern genomics technologies increases, so does the need for informative chemical
14 tools to study functional biology. Activity-based probes (ABPs) provide a powerful suite of reagents to
15 probe the biochemistry of living organisms. These probes, featuring a specificity motif, a reactive
16 chemical group and a reporter tag, are opening-up large swathes of protein chemistry to investigation
17 *in vitro*, as well as in cellular extracts, cells and living organisms *in vivo*. Glycoside hydrolases, by virtue
18 of their prominent biological and applied roles, provide a broad canvas on which ABPs may illustrate
19 their functions. Here we provide an overview of glycosidase ABP mechanisms, and review recent ABP
20 work in the glycoside hydrolase field, encompassing their use in medical diagnosis, their application
21 for generating chemical genetic disease models, their fine-tuning through conformational and
22 reactivity insight, their use for high-throughput inhibitor discovery, and their deployment for enzyme
23 discovery and dynamic characterization.

24 Highlights

- 25 • Glycosidases carry out many essential functions across all domains of life
- 26 • Activity-based probes can interrogate complex biological samples for glycosidase activity
- 27 • Glycosidase probes can characterize enzymes of biomedical/biotechnological interest
- 28 • Analysis of conformational itineraries may inform the design of future probes.
- 29 • Activity-based probes assist high-throughput discovery of inhibitors

30

31 1. Introduction and History

32 The development of modern genomic technologies has provided us with the ability to sequence (and
33 even edit) the genetic code of essentially any organism. However, our understanding of how this
34 wealth of genetic information links to biological function *in vivo* is still limited. The majority of the
35 biochemical reactions that underpin life are catalyzed by protein enzymes, whose primary sequences
36 are encoded by genes, but whose functions are often difficult to deconvolute from the genetic code.
37 Enzyme structures and functions are hard to predict from primary sequence alone, and their activities
38 may be further modulated by post-translational modifications, processing, intermolecular interactions
39 and subcellular localization.

1 Activity-based protein profiling (ABPP) has gained prominence as a powerful tool for the functional
2 annotation of enzymes within complex biological milieu. ABPP relies on the availability of suitable
3 activity-based probes (ABPs) that react with broad classes of enzymes, allowing investigation of their
4 identity and functions. ABPs are typically comprised of 3 main components: 1) a specificity endowing
5 motif that directs the ABP towards a particular group of enzymes through key-interactions to the
6 enzyme active-site, 2) a chemical 'warhead' that reacts (often irreversibly) with catalytic residues
7 within the active-site, 3) a reporter group that enables detection and/or capture of the ABP after
8 reaction with the enzyme (**Figure 1a**).

9 ABPs are intimately linked with the concept of mechanism-based inhibitors. The first ABPs were
10 derived from linking mechanism-based inhibitors of particular enzyme classes to reporter moieties
11 that could be exploited for analysis. Early work by Mason *et al.* showed that cathepsin B and L specific
12 ABPs could be developed by simple modification of existing inhibitors to incorporate a ¹²⁵I
13 radioisotope[1]. Tuning the 'peptide' sequence of these ABPs enabled targeting of different classes of
14 proteases, such as the profiling of proteasomal β -subunits by ¹²⁵I-nitrophenyl-(Leu)₃-vinylsulfone as
15 demonstrated by Bogyo, Ploegh and coworkers[2]. This strategy of converting inhibitor to ABP was
16 also used by Cravatt and coworkers in their seminal 1999 report, showing that reactive
17 fluorophosphonates could be derivatized to produce broad spectrum serine hydrolase ABPs, with a
18 biotin reporter group allowing for detection by peroxidase conjugated avidin[3].

19 ABPs have now been developed for many enzyme classes, including methyltransferases[4], kinases[5],
20 phosphatases[6], ubiquitin ligases[7], and glycosidases[8-11] (**Figure 1b**). This review summarizes
21 recent work in the development of glycosidase targeting ABPs, with some comments regarding
22 possible future directions for the field. For a more historical perspective, the reader is invited to
23 consult the comprehensive review of Rempel and Withers[12], and references therein.

24 **2. ABPs for glycoside hydrolases**

25 Carbohydrate containing biomolecules (oligosaccharides, polysaccharides, small and macro-
26 molecular glycoconjugates) play important metabolic, signaling and structural roles across all domains
27 of life. Glycoside hydrolases (GHs; also known as glycosidases) are a major class of enzymes
28 responsible for hydrolytic breakdown of carbohydrates, and are essential for diverse biological
29 processes. ABPP of glycosidases can provide insights into the role these enzymes play in complex
30 biological systems, as well as enabling the identification of previously unknown glycosidases.

31 Mechanistically, glycosidases are classified as inverting or retaining, depending on whether the
32 enzymatic reaction proceeds with net inversion or retention of stereochemistry at the cleaved
33 glycoside anomeric center[13]. Inverting glycosidases typically catalyze hydrolysis *via* a single step
34 nucleophilic displacement at the substrate anomeric center. In contrast, most retaining glycosidases
35 utilize a two-step mechanism mediated by two key catalytic residues: a nucleophile and a general
36 acid/base, and proceed *via* a covalent glycosyl-enzyme intermediate (**Figure 2a**). Trapping this
37 covalent intermediate is the conceptual basis for most mechanism-based inhibitors and ABPs that
38 target retaining glycosidases.

39 *2.1. Glycosidase ABPs that stabilize the covalent intermediate lifetime*

40 A major group of retaining glycosidase mechanism-based inhibitors function by extending the lifetime
41 of the covalent 'glycosyl'-enzyme intermediate, which can be achieved using activated fluorosugar[14-
42 16] (**Figure 2b**) or carbasugar[17] based molecules. In their seminal work, Vocadlo and Bertozzi
43 reported the use of 6-azido-2,6-dideoxy-2-fluoro- β -D-galactosyl fluoride ABPs to label β -
44 galactosidases in crude cell lysates. The modified enzymes were detected by means of Staudinger

1 ligation to a phosphine-FLAG tag, followed by immunoblot[9]. Fluorosugar based ABPs have since
2 been synthesized for the labeling of β -glucosaminidases[18], β -xylanases[19,20] and acid β -
3 glucocerebrosidase (GBA)[21]. Although fluorosugars work well against many glycosidase classes, they
4 are rapidly turned over in some cases (*e.g.* 2-deoxy-2-fluoro- α -D-glucosides by α -glucosidases)[15],
5 precluding the use of these compounds as general probes for all retaining glycosidases.

6 Cyclopropyl and vinyl carbasugar inhibitors operate in a conceptually similar manner to fluorosugars,
7 by forming a stable 'glycosyl' intermediate that accumulates on enzyme[22]. However, these
8 molecules also suffer from similar drawbacks to fluorosugars, being turned over too rapidly in some
9 cases to be of use as probes. To ameliorate problems of rapid turnover, fluorosugar and carbasugar
10 moieties can be combined. Thus cyclopropyl and vinyl 2-deoxy-2-fluoro-carbagalactose inhibitors have
11 been observed to form longer lived α -galactosidase-conjugates compared to both their 2-deoxy-2-
12 fluoro-galactose and carbagalactose congeners[23,24].

13 2.2. Electrophilic glycosidase ABPs

14 Cyclitol epoxides, such as conduritol B epoxide (CBE; **Figure 2c**), employ an electrophilic trap
15 mechanism to label their target. Pioneering work by Legler in the 1960s provided a possible route into
16 glycosidase ABPs through the use of ^{14}C radiolabeled CBE[25,26]. However, the intrinsic promiscuity
17 of CBE (partially related to its internal symmetry) renders it a poor tool for the study of glycosidase
18 and cellular biology. A marked improvement on CBE was realized with the discovery of the natural
19 product cyclophellitol. First isolated from *Phellinus sp*[27], cyclophellitol resembles β -D-glucose in
20 configuration, but with a highly electrophilic epoxide instead of an aglycone. Engagement of
21 cyclophellitol by retaining β -glucosidases results in attack of the epoxide by the β -glucosidase catalytic
22 nucleophile, leading to irreversible alkylation of this residue[28] (**Figure 2c**).

23 Since its discovery, multiple synthetic routes towards cyclophellitol have been reported[29], however
24 these typically required multi-step synthetic transformations and expensive starting materials. The
25 facile synthesis of cyclophellitol from D-xylose by Madsen and coworkers in 2005 paved the way for
26 exploitation of this scaffold for the development of ABPs[30]. In 2010 Witte *et al.* reported the first
27 cyclophellitol derived ABPs, in which a reporter group was appended to the C6 position of the
28 cyclophellitol 'glucosidic' ring[10]. This produced highly potent (nanomolar K_i) and selective ABPs for
29 lysosomal glucocerebrosidase (GBA), enabling detection of this enzyme in lysates from various
30 biological samples. Although useful for investigating GBA activity, the bulky C6 substituent of first
31 generation cyclophellitol-derived ABPs precluded their activity against other retaining β -glucosidases.
32 This was addressed by the development of *N*-acylated and *N*-alkylated cyclophellitol aziridines, in
33 which the reporter moiety is projected towards the direction normally occupied by the substrate
34 aglycon, leading to broad spectrum ABPs that label all retaining β -glucosidases[31]. Modulation of
35 substitution and stereochemistry around the 'glycosidic' ring of the cyclophellitol scaffold has now
36 provided ABPs for a wide range of different retaining glycosidases[32-35].

37 Haloketone (typically bromoketone) glycosides are another example of electrophilic glycosidase ABP,
38 which inhibit retaining glycosidases *via* alkylation of nucleophilic residues within the enzyme active
39 site (**Figure 2d**). C-bromoketone glycosides and *N*-bromoacetyl-glycosylamines have been developed
40 for the inhibition of β -glucosidases, β -glucanases[36], and endo-xyloglucanases[37]. Although there
41 are relatively few reports of ABPs based upon haloketone type inhibitors, Keresztessy *et al.* reported
42 the use of ^{14}C radiolabeled *N*-bromoacetyl- β -D-glucosylamine to identify the catalytic acid/base
43 residue of cyanogenic β -glucosidase (linamarase) from *Manihot esculenta Crantz* (cassava)[8].
44 Anderson *et al.* also reported the use of an azido *N*-iodoacetyl-glycosylamine probe to profile secreted
45 cellulolytic enzymes of several *Trichoderma reesei* strains[38]. It should be noted that whilst there

1 seems no obvious reason why haloketone glycosides should not also label inverting glycosidases, to
2 our knowledge only inhibition of retaining glycosidases has been observed to date.

3 2.3. Glycosidase ABPs that generate reactive intermediates *in situ*

4 Photoactivatable affinity-based probes (AfBPs) have been explored as potential tools for glycosidase
5 profiling (**Figure 2e**). A 1992 study by Kuhn *et al* showed that thioglycosides functionalized with a
6 photoactive diazirine moiety could label the active site of human lysosomal β -hexosaminidase A[39].
7 Similarly, Orth *et al* demonstrated that benzophenone containing GlcNAc configured AfBPs label the
8 active site of recombinant bacterial *N*-acetylglucosaminidase NagZ[40]. Gandy *et al* utilized a suite of
9 iminosugars functionalized with photoactivatable aromatic azides to profile both recombinant
10 glycosidases and glycosidases in crude *E. coli* lysates[41]. Notably, the authors of this study reported
11 labeling of both retaining and inverting glycosidases by their probes.

12 One general ‘disadvantage’ of photoactive AfBP profiling is that these molecules do not only target
13 enzymes, but can in principle label any carbohydrate binding protein of appropriate specificity, as
14 demonstrated by the labeling of a GM2-activator protein by a ganglioside based diazirine AfBP[42].
15 Furthermore, photoactivation of probes in free solution can contribute to off target labeling of even
16 non-carbohydrate binding proteins. A true ABP based strategy for labeling glycosidases has been to
17 employ probes that only release a reactive group upon enzymatic attack, which can then proceed to
18 label nearby residues around the enzyme active site. The glycosylmethyltriazines pioneered by Sinnott
19 and coworkers are early examples in this class[43], which fragment upon enzyme mediated
20 protonation to form a reactive carbocation that alkylates nearby nucleophilic amino acid sidechains.
21 However, glycosylmethyltriazines are prone to non-enzymatic degradation at acidic pHs, making the
22 study of glycosidases (which typically require acidic pH for optimal activity) using these molecules
23 problematic.

24 Quinone methide based molecules are now predominant amongst the class of reactive group
25 generating ABPs, and have been used to study glycosidases in both biomedical and biotechnological
26 contexts[44-46] (**Figure 2f**). Although useful against both retaining and inverting glycosidases, the use
27 of quinone methide ABPs can also lead to unspecific protein labeling, due to diffusion of activated
28 quinone methide electrophiles out of the enzyme active sites of their initial formation. Such off-target
29 labeling suggests that quinone methide ABPs are better suited for broad labeling of macromolecular
30 complexes or cellular structures[11,44,45], rather than precision labeling of individual glycosidases.

31 3. Glycosidase conformational itineraries and ABP reactivity

32 One of the defining features of glycosidase mediated substrate cleavage is the complex series of
33 conformational distortions undergone by the substrate glycone ring during the catalytic cycle. These
34 distortions are necessary to accommodate the oxocarbenium ion-like transition state of glycoside
35 hydrolysis, in which at least 4 atoms of the glycoside ring (including the endocyclic oxygen) must be
36 coplanar[47]. The sequence of conformational distortions employed by a glycosidase to process its
37 substrates is known as the conformational itinerary. These itineraries are typically conserved between
38 related glycosidase families (**Figure 3a**).

39 Recent years have seen an increasing use of conformational free energy landscape (FEL) analysis as a
40 tool to predict the conformational itineraries employed by different glycosidases[47]. FELs can also be
41 used to calculate conformations favored by glycosidase ABPs and mechanism-based inhibitors, which
42 can shed light on differences in labeling efficiency between different classes of glycosidase ABPs, and
43 inform the design of new probes with improved potency and/or specificity. FEL analysis of
44 cyclophellitol-derived inhibitors/ABPs has shown that these molecules typically adopt a 4H_3 half-chair

1 ground state conformation, due to the stereochemical constraints placed on the cyclitol ring by the
2 strained epoxide/aziridine moiety[48]. Significantly, many families of retaining glycosidases process
3 their substrates via a 4H_3 transition state complex[47], and thus cyclophellitol-derived ABPs act as
4 transition state mimics for these enzymes, typically showing low μM to nM equilibrium constants for
5 initial binding ($K_i = \sim 15 \mu\text{M}$ for cyclophellitol against the *TmGH1* β -glucosidase from *Thermotoga*
6 *maritima*)[28]. Indeed, even neutral non-electrophilic carba-cyclophellitols, containing a cyclopropyl
7 in place of the epoxide, are competent reversible glycosidase inhibitors due to conformational
8 mimicry (**Figure 3b**)[48].

9 The Michaelis complex conformation, adopted by substrates upon initial enzyme binding, can also be
10 exploited for the design of mechanism-based glycosidase inhibitors. Cyclophellitol cyclosulfate
11 inhibitors were designed based on this principle, with the first (glucose configured) inhibitors of this
12 class showing exquisite selectivity for inhibition of α -glucosidases compared to β -glucosidases. The
13 ground state conformation of cyclophellitol cyclosulfates is a 4C_1 chair, which closely resembles the
14 Michaelis complex conformation utilized by α -glucosidases, but not the 1S_3 Michaelis complex
15 conformation utilized by β -glucosidases. Thus α -cyclophellitol cyclosulfates rapidly inactivate α -
16 glucosidases, whilst their β -configured congeners are only marginal β -glucosidase inhibitors[49]
17 (**Figure 3c**).

18 Interestingly, mechanism-based inhibitors/probes that employ non-native conformational itineraries
19 are well tolerated by some glycosidases. The α -D-galacto configured cyclopropane-carbasugars
20 pioneered by Bennet and coworkers adopt an unusual 2H_3 Michaelis complex conformation in the
21 active site of α -galactosidases, owing to conformational restriction across the C5–O5 equivalent bond
22 (the O5 position being occupied by a tertiary carbon in these molecules)[24]. Despite their non-native
23 Michaelis conformation, these molecules react efficiently with GH36 α -galactosidases, although their
24 ability to label GH27 α -galactosidases is more limited[17]. Taken together, it appears that mechanism-
25 based inhibitors employing non-native conformational itineraries can selectively target some classes
26 of glycosidases, hinting at a strategy for the development of family specific inhibitors/probes.

27 **4. Recent developments in the glycosidase ABP field**

28 *4.1. Glycosidase ABPP for biomedical diagnostics*

29 Several pathophysiological states, including mucopolysaccharoidoses and various lysosomal storage
30 diseases (LSDs), arise from insufficient levels of glycosidase activity. Diagnosis of these conditions
31 typically involves the use of activity assays to determine residual glycosidase activities in patient
32 tissues. Such assays can be non-trivial, with specific conditions or inhibitors required to deconvolute
33 activity of the enzyme of interest from overlapping enzymes in the tissue. ABPP is uniquely suited to
34 address this challenge, and ABPs against various classes of glycosidases have demonstrated promise
35 as possible diagnostic tools (**Figure 4a**).

36 GBA is perhaps the most well-studied glycosidase from an ABPP-centric perspective; GBA deficiency
37 is the causal driver of Gaucher's disease, caused by abnormal buildup of the GBA substrate
38 glucosylceramide in cells. Symptoms of Gaucher's disease include thrombocytopenia,
39 hepatosplenomegaly, and osteoporosis, with the type II variant also involving severe neuropathy with
40 patients typically succumbing in infancy. ABPP using cyclophellitol-derived probes allows for rapid
41 evaluation GBA activity in patient tissues, and can identify GBA deficiency in Gaucher's disease
42 samples with sensitivity comparable to immunoblotting (a considerably more laborious
43 technique)[10]. Use of suitably configured ABPs has also demonstrated that lysosomal α -glucosidase,
44 lysosomal β -galactocerebrosidase, and lysosomal α -galactosidase are all amenable to ABPP profiling,

1 opening up avenues for the rapid diagnosis of Pompe[34], Krabbe[50] and Fabry[32] diseases
2 respectively. Interestingly, the irreversible inhibitory nature of many ABPs can also be used to
3 chemically 'knock out' enzyme activity *in vivo*. Zebrafish treated with selective cyclophellitol
4 derivatives accumulate glucosylsphingosine (derived from acid ceramidase mediated conversion of
5 glucosylceramide), indicating 'knock out' of GBA activity and recapitulation of a Gaucher's disease
6 phenotype[51].

7 Glycosidase overexpression can also be linked to pathophysiologies. Heparanase is an endo- β -
8 glucuronidase principally responsible for heparan sulfate (HS) breakdown in the body. Aberrant HPSE
9 overexpression results in excessive HS degradation within the extracellular matrix surrounding cells,
10 and releases of growth factors stored by extracellular HS networks. These factors increase cell motility
11 and growth, and thus heparanase drives metastasis in a wide range of cancers. β -D-glucuronide
12 configured cyclophellitol-derived ABPs detect heparanase in human tissue lysates, and may be useful
13 diagnostic tools to track aberrant heparanase upregulation during cancer development[35].

14 4.2. Glycosidase ABPP for imaging applications

15 Many glycosidase ABPs are cell permeable, allowing for their use in cells, tissues, and even whole
16 organisms, enabling a variety of imaging applications (**Figure 4a**). van Smeden *et al* have shown that
17 epidermal GBA in intact skin sections can be visualized by confocal microscopy following application
18 of fluorescent cyclophellitol-derived ABPs[52]. Similar GBA imaging in human fibroblasts was
19 demonstrated by van Meel *et al*, who also applied correlated light and electron microscopy (CLEM) to
20 confirm specific staining of the lysosomal compartment[53].

21 *In situ* imaging to visualize cellular uptake and trafficking may be a useful technique to assess the
22 efficacy of therapeutic enzyme uptake following enzyme replacement therapy regimens. Phenix *et al*.
23 demonstrated that ^{18}F -fluorosugar pre-labeled recombinant GBA could be tracked in mice by PET
24 imaging, allowing for the uptake of GBA into organs following enzyme administration to be
25 quantitated[21]. Higher resolution imaging of enzyme trafficking into cellular organelles has also been
26 reported, albeit in cell culture systems. van Meel *et al*. used CLEM to visualize lysosomal accumulation
27 of recombinant ABP pre-labeled GBA by human fibroblasts following its addition into culture media.
28 The authors found that lysosomal accumulation of recombinant GBA occurred even in the absence of
29 LIMP-II (the protein responsible for lysosomal trafficking of endogenous GBA) suggesting that
30 lysosomal trafficking of recombinant GBA is a LIMP-II independent process[53]. Similarly, Artola *et al*.
31 tracked ABP pre-labeled α -L-iduronidase (IDUA) uptake by mucopolysaccharidosis (MPD)I and
32 mucopolysaccharidosis II (MD)II fibroblasts. In this case, application of mannose-6-phosphate abrogated uptake
33 of recombinant IDUA, confirming that IDUA uptake was dependant on the mannose-6-phosphate
34 pathway[54].

35 4.3. Glycosidase ABPP for inhibitor discovery

36 Fluorescent glycosidase ABPs can be exploited to screen for inhibitors in compound libraries through
37 the use of the high-throughput fluorescence polarization (FluoPol) assay format. When excited with
38 plane polarized light, enzyme bound fluorescent ABPs will retain a greater degree of polarization in
39 their emitted light compared to free ABPs in solution. Thus, incubation of enzyme-ABP mixtures with
40 compounds that reduce ABP binding will lead to reduced fluorescence polarization (**Figure 4b**). Lahav
41 *et al*. used cyclophellitol aziridine equipped with a TAMRA-fluorophore in a FluoPol assay to identify
42 inhibitors of non-lysosomal glucocerebrosidase (GBA2), which displayed 100,000 fold selectivity for
43 GBA2 over GBA[55]. GBA2 is involved in the degradation of glucosylceramide and is a potential
44 therapeutic target in Niemann-Pick type C disease[56]. FluoPol-ABPP may provide a general strategy

1 to identify inhibitors of retaining GHs, without the requirement for screening assays based on
2 fluorogenic or chromogenic substrates.

3 4.4. Glycosidase ABPP for biotechnology

4 Although many early glycosidase ABPP studies were biomedical in focus, recent years have seen an
5 increasing drive to employ ABPP techniques towards biotechnological applications (**Figure 4c**). One
6 early study in this arena, by Hekmat and coworkers, showed the promise of ABPP for the profiling of
7 bacterial secretomes for biomass degrading enzymes[19]. The authors used 2-deoxy-2-fluoro-
8 disaccharides to profile the secretome of *Cellulomonas fimi* for cellobiohydrolase and
9 xylobiohydrolase activity. Coupling isotopically tagged and biotinylated versions of these probes to
10 mass spectrometry, the authors quantified active enzymes under variable growth conditions and also
11 identified a previously unknown xylanase[57]. Schröder *et al.* recently carried a similar analysis of β -
12 xylosidases and β -xylanases in *Aspergillus niger* secretomes using cyclophellitol-derived ABPs. The
13 authors demonstrated by both fluorescent labeling and proteomic analyses that *A. niger* secreted
14 distinct catabolic enzymes depending on the carbon source utilized during its growth[58].

15 A comprehensive ABPP study of biomass degradation carried out by Chauvigné-Hines *et al.* utilized a
16 suite of glucose and cellobiose configured ABPs to study the *Clostridium thermocellum* secretome[44].
17 A wide range of ABP chemistries were used in this study, encompassing fluorosugar, bromoketone
18 and quinone methide based probes. Impressively, the authors were able to identify a large subset of
19 the *C. thermocellum* glycosidases involved in biomass breakdown, including many proteins of the
20 macromolecular cellulosome complex. The use of such 'suites' of ABPs may represent a useful strategy
21 to overcome limitations inherent to each class of glycosidase ABP (see section 3).

22 ABPP has also been used as a tool to study intestinal microbiota. Whidbey and coworkers recently
23 used quinone methide generating β -glucuronidase ABPs for labeling of whole cells derived from
24 mouse gut microbiomes[59]. The authors sorted labeled cells using fluorescence activated cell sorting
25 (FACS) to produce both labeled and unlabeled subpopulations. The identities of the β -glucuronidase
26 positive community members were subsequently revealed by 16S rRNA sequencing of
27 subpopulations. β -glucuronidase active taxa were compared between hosts and under antibiotic
28 treatment conditions, revealing distinct taxa active in different hosts. This is a powerful technique as
29 it clearly links function to taxa without the need for genomic context.

30 5. Future challenges for glycosidase ABPP

31 Several decades after the development of the first mechanism-based glycosidase inhibitors, the field
32 of activity-based glycosidase profiling is now reaching maturity, at least with respect to retaining
33 glycosidases. The field has seen the development of several different warhead chemistries[10,14,36],
34 as well probes tailored to react with specific retaining glycosidase families[10,17,49]. Our experience
35 suggests that, in principle, any retaining glycosidase that employs the Koshland double displacement
36 mechanism is amenable to ABP profiling. Whilst 'monosaccharide' ABPs that target retaining *exo*-
37 glycosidases have been relatively well explored, profiling of retaining *endo*-glycosidases will require
38 more complex ABPs structures to mimic the length and/or branching of their natural substrates. Given
39 past and recent successes in the glycosylation of fluoroglycoside[20,60], cyclophellitol-derived[58,61],
40 and bromoketone[37] warheads, the general profiling of retaining *endo*-glycosidases appears to be
41 within reach.

42 In contrast to fluorophosphonate-type ABPs, which react with large numbers of serine hydrolases, the
43 development of truly broad-spectrum retaining glycosidase ABPs has remained elusive. Removal of
44 hydroxyls from an ABP scaffold, as was recently done for cyclophellitol-derived ABPs[62], yields

1 broader spectrum probes at a cost of markedly reduced potency. The use of multiple ABPs in
2 multiplexed labeling experiments provides a possible strategy to circumvent this problem, which also
3 offers an attractive bonus: the relative specificity rendered by the configuration of the probes
4 translates to the specificity of the glycosidases that are labeled. Interrogation of *e.g.* a microbial
5 secretome derived from biomass growth using a set of differently fluorescent glycosidase ABPs will
6 yield a 'multicolored' fingerprint of the possible glycosidases present. First forays in activity-based
7 secretome profiling indicate the huge potential of glycosidase ABPs in the field of biotechnology, in
8 addition to their more developed applications in biomedicine (inhibitor discovery, target engagement,
9 diagnostics).

10 The enzymatic mechanism of retaining glycosidases proceeds through a covalent enzyme-substrate
11 intermediate, which has provided us with a fortuitous route towards the design of ABPs for this
12 enzyme class. The extensive choice of probe chemistries now on offer for retaining glycosidases may
13 present a daunting choice for researchers when selecting suitable ABPs for their studies. In our
14 experience, cyclophellitol-derived ABPs are excellent first choice molecules for a wide range of *in vitro*,
15 *in cellulo* and even *in vivo* applications. Cyclophellitol-derived ABPs typically adopt a ground state 4H_3
16 conformation that mimics the substrate transition state conformation for many glycosidases[48,58],
17 meaning these probes often label with high potency, producing stable enzyme-cyclitol linkages
18 suitable for various downstream analyses. Conversely, glycosidases that do not employ a 4H_3 transition
19 state conformation can be less reactive towards cyclophellitol-derived ABPs, as has recently been
20 observed with GH11 β -xylanases (proposed to utilize a ${}^{2,5}B$ transition state conformation[58]). In such
21 cases, fluorosugars may be more suitable, as they more closely resemble natural substrate
22 glycones[63]. For β -glucosidase, β -galactosidase, and related glycosidase classes, fluorosugar
23 inhibitors typically form stable, long-lived intermediates well suited for ABP derivation. However, in
24 some cases (notably with 2-deoxy-2-fluoro- α -glycosides and α -glycosidases), fluorosugar inhibitors
25 are too rapidly turned over to be of use as probes. In such cases, combining fluoro- and carbasugar
26 chemistries may help to stabilize the covalent enzyme-probe intermediate[23,24]. Alternatively,
27 controlled turnover of fluorosugar inhibitors can be useful in time-dependent labeling applications,
28 where reactivation of the enzyme is desired.

29 In contrast to the wide range of ABPs available for typical retaining glycosidases, ABPs that efficiently
30 and specifically label other glycoprocessing enzymes (atypical retaining glycosidases utilizing oxidative
31 or substrate assisted mechanisms, inverting glycosidases, glycosyl transferases, polysaccharide lyases,
32 oxygenases) remain elusive. Opening up these enzyme classes to ABPP has huge potential in both the
33 biomedical and biotechnological arenas. Some progress towards ABPP of inverting glycosidases has
34 been made with photo-affinity and quinone methide based probes, which are indiscriminate with
35 respect to catalytic mechanism[41]. However, these labeling chemistries also suffer from a relative
36 lack of efficiency and selectivity. We postulate that widespread adoption of ABPP for other
37 glycoprocessing enzyme classes will only take place after effective irreversible enzyme active site
38 binders are identified. The development of such molecules will require the efforts of high-level
39 synthetic chemistry, coupled to computation and structural analysis of the reaction mechanisms and
40 conformational itineraries utilized by these diverse enzyme classes.

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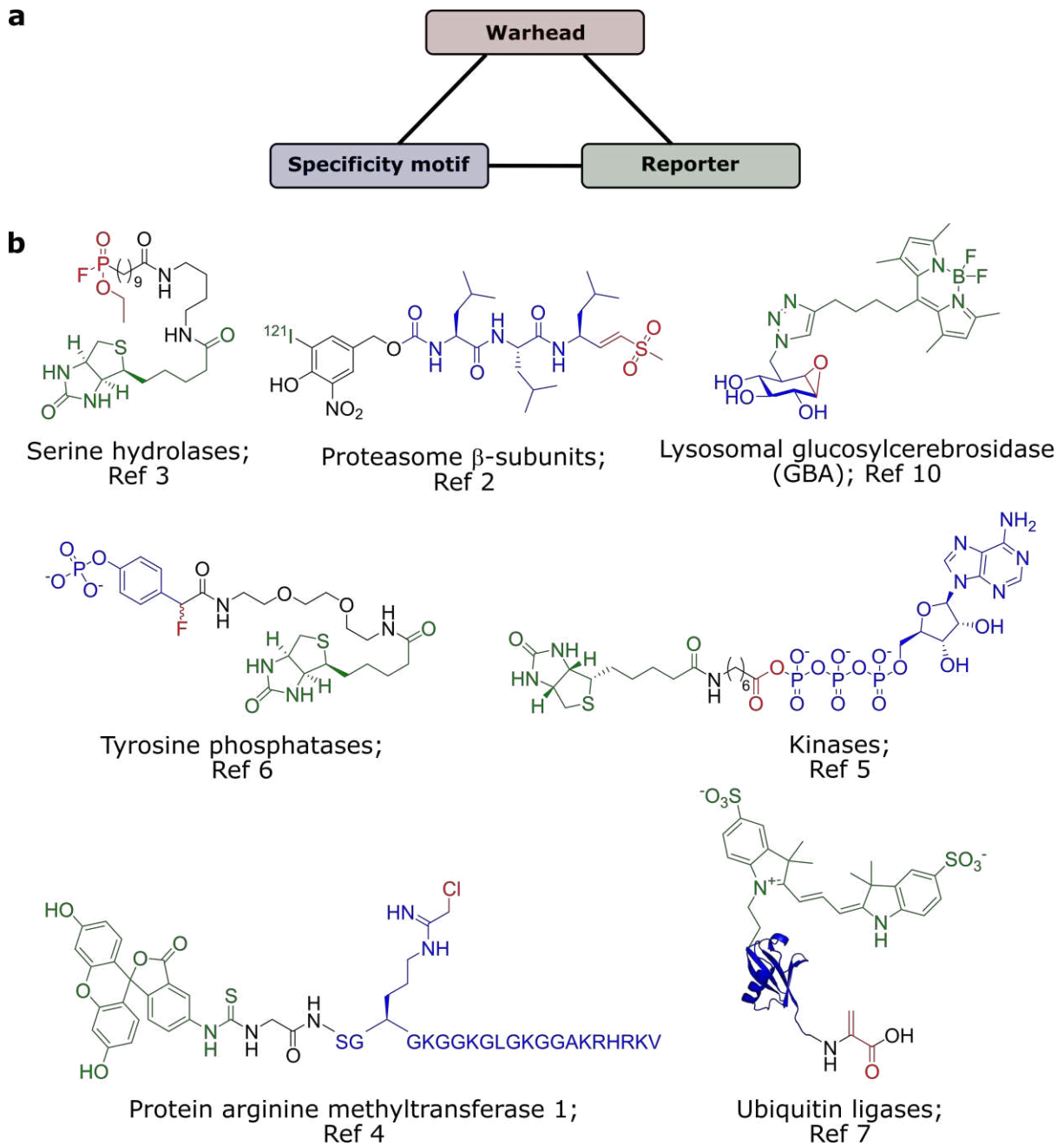
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43 'Endoglycosidase probes' to H.S.O.), the European Research Council (ERC-2011-AdG-290836
44 'Chembiosphing' to H.S.O., and ERC-2012-AdG-32294 'Glycopoise' to G.J.D.), and the Biotechnology

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3 **Declaration of Interest**

4 The authors declare no conflict of interest.

5

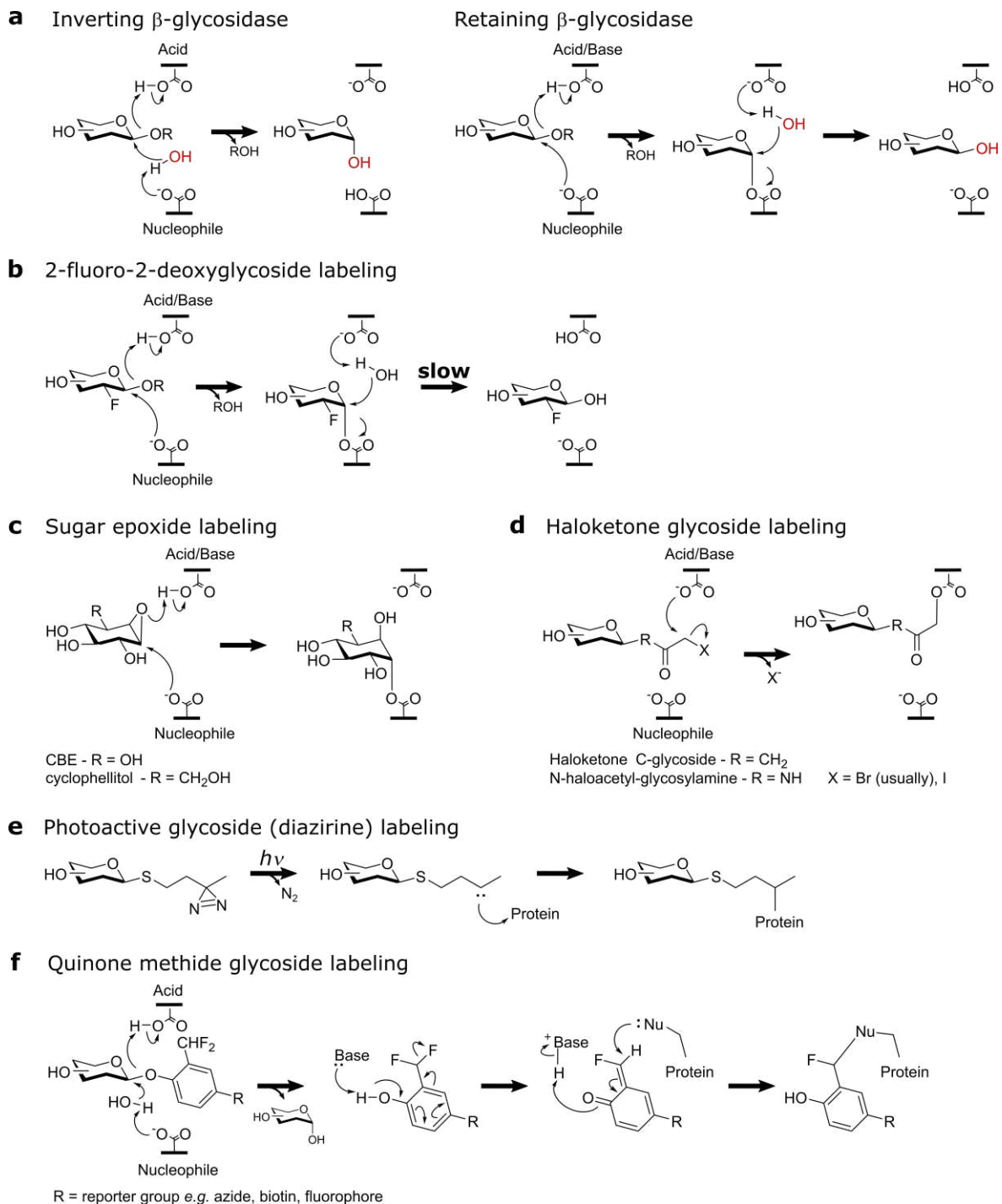


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2 **Figure 1** Conceptual basis for ABP design. **a** The three fundamental components of an ABP. Note -
 3 some probes may lack a specificity motif, endowing them with broad spectrum activity against a range
 4 of enzymes e.g. serine hydrolase probes in Ref [3]. **b** Examples of ABPs designed to target various
 5 enzymes and enzyme classes. Colors correspond to ABP components as outlined in **a**.

6

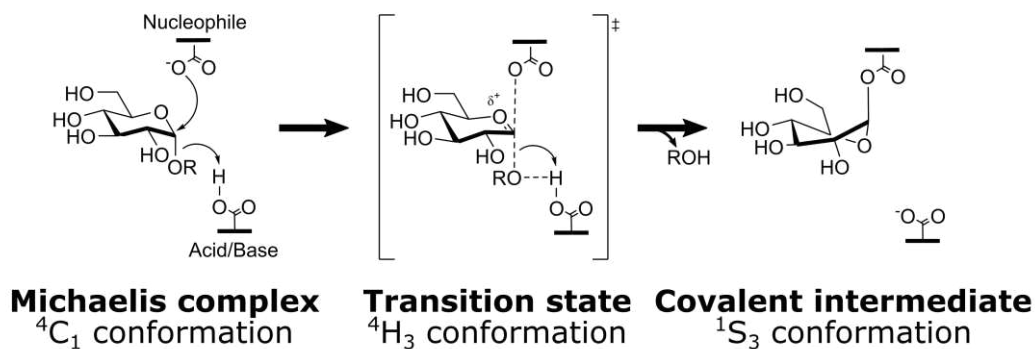
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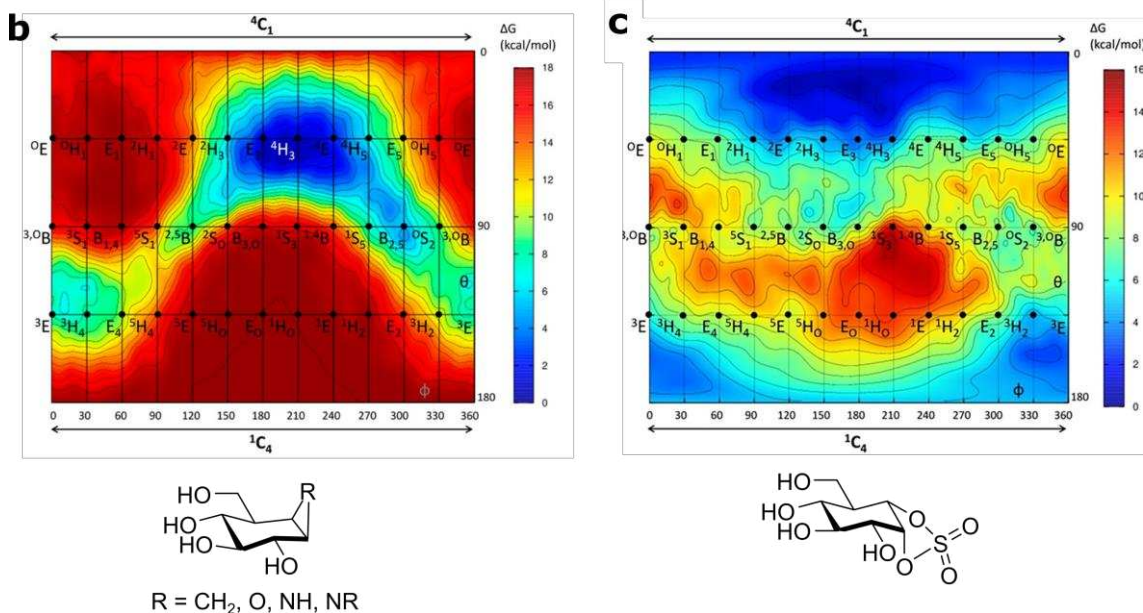
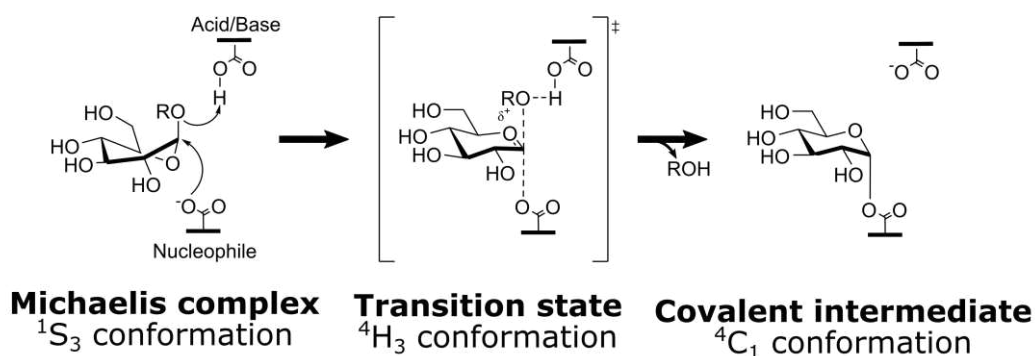
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2 **Figure 2** Mechanisms of glycoside hydrolases and inhibitor action. **a** Mechanism of glycoside cleavage
 3 by inverting (one step) and retaining (two step) glycosidases **b** Fluorosugar inhibitors (2-deoxy-2-
 4 fluoro-glycoside shown here) are processed by retaining glycosidases, but the slow deglycosylation
 5 step results in inactivated enzyme. **c** Sugar epoxides (e.g. CBE or cyclophellitol) are attacked at their
 6 pseudo-anomeric center by glycosidase catalytic nucleophiles, leading to irreversible alkylation of this
 7 residue. **d** Haloketone glycosides employ an electrophilic trap, which can irreversibly alkylate residues
 8 within an enzyme active site (alkylation of the catalytic acid/base residue is shown here). **e** Photoactive
 9 glycoside AfBPs form a reactive species upon photoexcitation (carbene formation from a diazirine
 10 shown here), which reacts with neighboring protein residues to form irreversible linkages. **f** Quinone
 11 methide glycosides release a reactive species when cleaved, which can irreversibly alkylate a nearby
 12 nucleophilic protein residue.

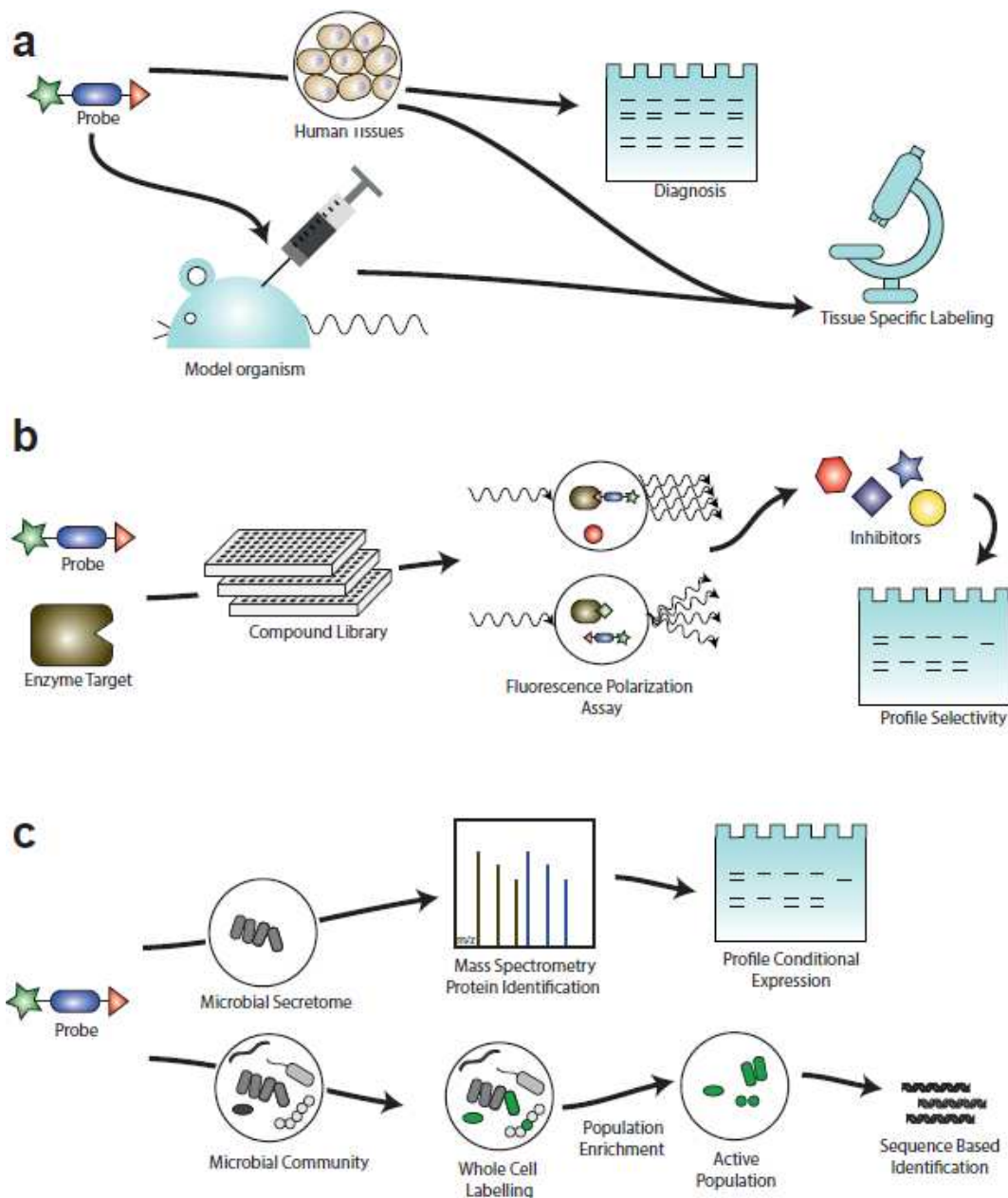
a Retaining α -glucosidase conformational itinerary



Retaining β -glucosidase conformational itinerary



1
2 **Figure 3** Conformational aspects of glycosidase ABP reactivity. **a** Conformational itineraries for half-
3 reactions (initial substrate binding to covalent intermediate formation) catalyzed by typical retaining
4 α - and β -glucosidases. Both classes of enzymes proceed *via* a 4H_3 transition state. **b** Conformational
5 FEL for a cyclophellitol derived molecule (carba-cyclophellitol, R=CH₂), showing a ground state
6 conformation centered around 4H_3 . Cyclophellitol-derived ABPs are transition state mimics and potent
7 inactivators of many retaining classes of retaining glycosidases. **c** Conformational FEL for α -
8 cyclophellitol cyclosulfate, showing a ground state conformation centered around 4C_1 . This
9 conformation matches the typical α -glucosidase Michaelis complex conformation, rendering α -
10 cyclophellitol cyclosulfates potent selective inactivators of α -glucosidases.



1

2 **Figure 4** Recent applications of Glycosidase ABPs for protein profiling. **a** Glycosidase specific probes
 3 can detect the absence of enzyme expression in genetic diseases. ABPs have also been used to detect
 4 the presence of glycosidase activity in tissue samples, as well as in whole organisms *in vivo*. **b** FluoPol
 5 assays for glycosidases involves the use of a fluorescent ABP, the enzyme of interest and compound
 6 libraries. Glycosidase labeling by the ABP results in a maintenance of fluorescence polarization, whilst
 7 competition by inhibitors results in a loss of polarization. The specificity of identified hits can then be
 8 interrogated through SDS-PAGE and other downstream assays. **c** Glycosidase ABPs can be used to
 9 profile biomass degrading enzymes and microbiota community members. Active enzymes present in
 10 microbial secretomes can be profiled by SDS-PAGE and active proteins identified using pull downs and
 11 mass spectrometry. Whole cells can be labeled and sorted with the use of FACS. Communities of either
 12 active or inactive populations can then be investigated through sequence-based identification of
 13 microbial taxa.

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