Communication

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A New Class of Glycoside Hydrolase Mechanism-Based Covalent Inhibitors: Glycosylation Transition State Conformations

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Supporting Information Placeholder

ABSTRACT: The design of covalent inhibitors in glycoscience research is important for the development of chemical biology probes. Here we report the synthesis of a new carbocyclic mechanism-based covalent inhibitor of an α-glucosidase. The enzyme efficiently catalyzes its alkylation via either an allylic cation or a cationic transition state. We show that this allylic covalent inhibitor has very different catalytic proficiencies for pseudo-glycosylation and deglycosylation. Such inhibitors have the potential to be useful chemical biology tools.

The catalytic transfer of carbohydrate moieties frequently involves anomeric positive charge delocalization onto the endocyclic oxygen atom; we show for the first time that an alkene can perform the same task. Of note, enzymes that either add or remove carbohydrates are often critical to cellular regulation.[1-3] The enzymes that remove sugar residues by hydrolysis of glycosidic bonds are called glycoside hydrolases (GHs).[4] Nature has evolved several strategies for catalysis by GHs, with most enzymes using a pair of active site aspartic and/or glutamic acid (Asp/Glu) residues.[5-7]

Retaining glycoside hydrolases with two catalytic Asp/Glu residues operate via two sequential inversions of configuration. The first results in the formation of a covalent glycosyl-enzyme intermediate (Figure 1) and the second, not shown, involves intermediate hydrolysis. The transition states (TSs) for glycosylation and deglycosylation possess pyranosylium ion-like character, and the six-membered ring adopts one of several allowed conformations.[6,8] In the current example, retaining GH13 α-glucosidases[9] react via pyranosylium ion-like TSs that are traversed during the catalytic cycle. Moreover, the pyranoside conformation at the TSs is postulated to be a $^4H_3$ half-chair.[10] Also, for GH13 enzymes the structure of the enzyme bound intermediate is a $^4C_1$ chair (Figure 1).[11] Kinetic isotope effect data suggests that the productive Michaelis complex is not a ground state chair conformation, but is likely a $^1S_3$ skew boat (Figure 1).[12,13]

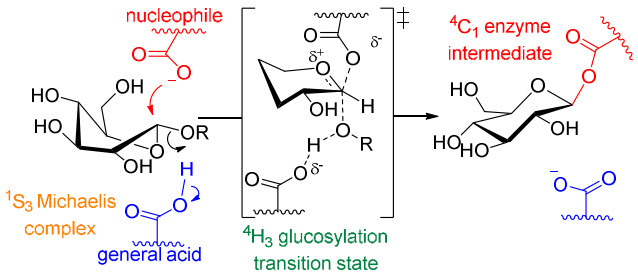


Figure 1. Proposed mechanism of a GH13 retaining α-glucosidase; for clarity, some hydroxyl groups are not shown. Conformations are shown for the Michaelis complex ($^1S_3$), the glucopyranosylium ion-like transition state ($^4H_3$) and the enzyme-bound intermediate ($^4C_1$).

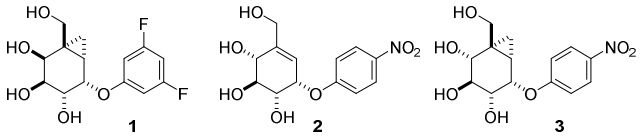


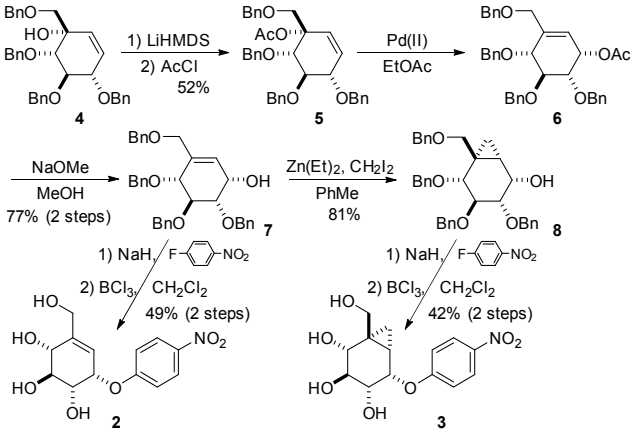
Figure 2. Structures of the mechanism-based covalent inhibitor **1** (GH36 enzyme), and the GH13 inhibitors (**2** and **3**) evaluated as covalent inhibitors in the current study.

Previously, we utilized the requirement for transition state charge delocalization, a fundamental factor in catalysis by most GHs, in our design of a cyclopropyl-containing mechanism-based covalent inhibitor (**1**) of a retaining α-galactosidase (GH36).[14,15] Here we describe the synthesis of two carbocyclic analogues of D-glucose (**2** and **3**) that are covalent inhibitors of a GH13 retaining α-glucosidase. We show that these compounds lead to a single covalent labeling of the enzyme, and importantly that the rate constants for 'pseudo'-glycosylation and deglycosylation for these inhibitors are distinct and provide insight into the conformational itinerary for this GH family.

We synthesized **2** and **3** (Scheme 1) from **4**, which we made in four steps (42% yield) from 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose.[16] First, we acetylated the tertiary alcohol in **4** to give **5**, which underwent a palladium-catalyzed [3,3]-sigmatropic rearrangement to give the pseudo anomeric acetate **6**.[17] Deacetylation gave allylic alcohol **7**, which was sub-

jected to a Furukawa modified Simmons-Smith cyclopropanation to give **8**. Both **7** and **8** underwent facile S_NAr reactions followed by global debenzylation with BCl_3 to give **2** and **3**, respectively.

Scheme 1. Synthesis of carbocyclic inhibitors 2 and 3.



We tested carbasugar analogues **2** and **3** for their activity against a yeast α-glucosidase. Shown in Figure 3 are the measured pseudo-first-order rate constants for the loss of enzyme activity, each data point is calculated from remaining enzyme activity as a function of preincubation time, as a function of the concentration of the carbasugar analogue. Notably, the allylic inhibitor **2** (red circles) is less active than the bicyclo[4.1.0]heptyl inactivator **3** (blue circles).

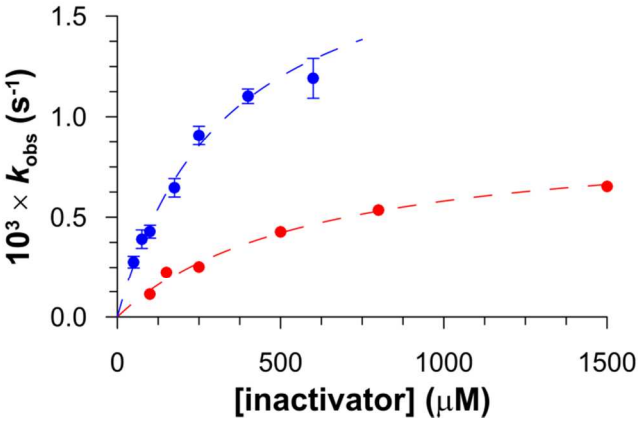


Figure 3. Kinetics for covalent inhibition of yeast α-glucosidase. Shown in red circles are the data for allylic inhibitor **2**, while the blue circles represent labeling by bicyclic inhibitor **3**. Error bars that are not visible are encompassed within the symbol. The dashed lines are the best non-linear fits to a standard Michaelis–Menten equation. Conditions were T=25 °C, sodium phosphate buffer (50 mM, pH 6.84, [BSA] = 1 mg/ml).

We then measured the reactivation rate constants for the covalently-modified enzyme (Figure 4). Remarkably, the recovery of enzyme activity following inhibition by allylic inhibitor **2** (red circles) is more rapid than that for bicyclo[4.1.0]heptyl inactivated enzyme (blue circles). The kinetic data were fit to the standard kinetic scheme for covalent inhibition (Scheme 2) and the derived rate and equilibrium constants are tabulated in Table 1.

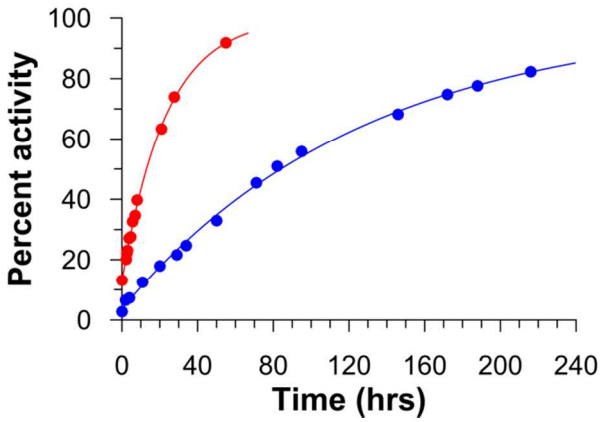
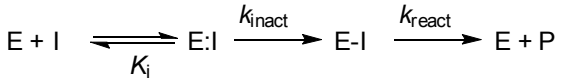


Figure 4. Reactivation kinetics for covalently inhibited α-glucosidase. Shown in red circles are the data for the dealkylation of enzyme labeled by inhibitor **2**, while the blue circles represent reactivation of enzyme treated with **3**. The solid lines represent the best non-linear fits to a standard first-order rate equation. Conditions were T=25 °C, sodium phosphate buffer (50 mM, pH 6.84, [BSA] = 1 mg/ml).

Scheme 2. Kinetic scheme for the covalent inhibition of GH13 yeast α-glucosidase by carbasugar analogues 2 and 3.



We tried to identify the sites of labeling by incubating the enzyme with excess inhibitor, followed by ESI tandem mass spectrometry (MS/MS) of the tryptic (and peptic) peptides obtained by digestion of both the inactivated and the untreated enzymes. Unfortunately, we were unable to obtain satisfactory peptide fragmentation that remained covalently modified after tryptic digestion. However, we showed that yeast α-glucosidase is singly labeled by the expected mass addition of the carbon skeleton portions of **2** and **3** to the molecular weight of the enzyme (Figure S1 Supporting Information). That is, the mass spectrum of enzyme shows a single peak for the native enzyme at 67275.7, while that after reaction with **2** shows the intact enzyme and a monoalkylated species ($C_7H_{10}O_4$ = 158.1) at 67433.9, and the mass spectrum for the enzyme modified by **3** displays a single peak at 67448.8, which corresponds to addition of the carbocyclic skeleton of **3** ($C_8H_{12}O_4$ = 172.1 + H).

Table 1. Kinetic parameters for the covalent inhibition and reactivation of yeast α-glucosidase by the inhibitors 2 and 3. Conditions were T=25 °C, sodium phosphate buffer (50 mM, pH 6.84, [BSA] = 1 mg/ml).

Inactivator	K_i (μM)	k_{inact} (s^{-1})	$t_{½}$ (mins)	k_{inact}/K_I (M^{-1} s^{-1})	k_{react} (s^{-1})	$t_{½}$ (hrs)
2	570 ± 90	(9.05 ± 0.63) × 10^{-4}	12.8	1.59 ± 0.27	(1.19 ± 0.07) × 10^{-5}	16.3
3	285 ± 45	(1.82 ± 0.14) × 10^{-3}	6.3	6.4 ± 1.1	(2.17 ± 0.14) × 10^{-6}	88.7

The data in Table 1 shows two remarkable features: (i) both the first- (k_{inact}) and second-order (k_{inact}/K_i) rate constants for inactivation of yeast α-glucosidase are larger for the bicyclic inhibitor; and (ii) the reactivation of labeled enzyme (k_{react}) is faster for the allylic covalent adduct.

Both allylic[18] and cyclopropylcarbinyl compounds[19-21] undergo S_N1 reactions at accelerated rates via allylic and non-classical bicyclobutonium cationic intermediates,[22,23] respectively. Of note, distinct conformations are required for formation of delocalized carbocations from inhibitors **2** and **3**. Specifically, enzyme-catalyzed labeling within the active site requires a conformation in which a π-type molecular orbital can assist glycosidic C–O bond cleavage, a process that occurs from an oxygen n-type lone pair for natural substrates. In the current study, the cyclopropyl-containing inhibitor **3** requires a pseudo-equatorial aglycone for effective σ-bond participation (Fig. 5, Panel A),[24-26] while allylic participation in carbocyclic inhibitor **2** entails a pseudo-axial aglycone (Fig. 5, Panel B).

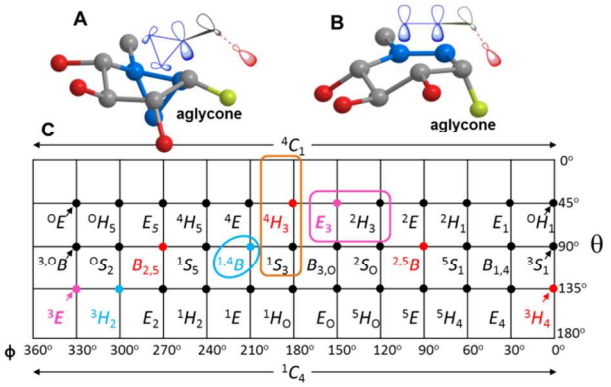
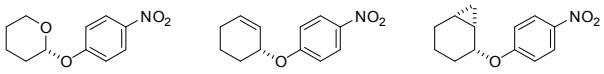


Figure 5. Conformations for π-type orbital participation (blue atoms at back) into the σ* of the glycosidic C–O bond; the C6 hydroxyl group is omitted for clarity. **Panel A**) α-cyclopropyl inhibitor **3**; **B**) α-allylic compound **2**; **C**) Mercator projection of six-membered ring conformations. The currently accepted reaction coordinate for a GH13 enzyme is indicated by the orange box. Possible conformations for a pyranosylium ion-like TS are shown in red. Bisected conformations for cyclopropyl assisted ionization are shown in teal with the closest to the enzymatic reaction coordinate being circled. The two lowest energy conformations for an allylic cation (between C5–C6–C1) are labeled in rose (bold font) with the proposed reaction coordinate for **2** circle by the rose box.

Based on current theories[5,6] that GH13 enzymes stabilize pyranosylium ion-like $^4H_3$ TSs from a bound $^1S_3$ Michaelis complex,[5,6] we propose that our bicyclo[4.1.0]carbasugar **3** reacts from the bisected geometry, required for bicyclobutonium ion formation, that is closest to that for the catalyzed-hydrolysis reactions of GH13 enzymes (Figure 5, panel A, a ^{1,4}B boat). That is, the evolved reaction coordinate for α-glucopyranoside hydrolysis, which involves a rate-determining non-chemical step,[12] exhibits a second-order rate constant (k_{cat}/K_m = 6.7 × 10^4 M^{-1} s^{-1}) for 4-nitrophenyl α-glucopyranoside.[12] However, in order to assess the efficiency of covalent labeling it is important to calculate the relative enzymatic proficiencies for formation of the covalent glycosyl-enzyme intermediates (k_{cat}/K_m × 1/k_{uncat} or k_{inact}/K_i × 1/k_{uncat}).[14,27,28]



9 **10** **11**

Figure 6. Structures of the model compounds (**9–11**) used to estimate relative catalytic proficiencies.

Due to the extremely slow spontaneous hydrolysis rates of glycosides,[29] we used the kinetic data for the unsubstituted model compounds **9** and **11**[14] and we made **10** (Figure 6), by standard procedures (Supporting Information). To evaluate the spontaneous rate constant for the pH-independent hydrolysis of **10** at 25 °C we extrapolated kinetic data acquired at higher temperatures (Supporting Information, Table S1). We then calculated the relative rate constants for covalent labeling of the enzyme (pseudo-glycosylation) and for cleavage of the glycosidic bond in the enzyme intermediate (pseudo-deglycosylation). Listed in Table 2 are the second-order rate constants (k_{cat}/K_m and k_{inact}/K_i) for yeast α-glucosidase reacting with 4-nitrophenyl α-D-glucopyranoside[12] and our two covalent inhibitors **2** and **3**, the uncatalyzed first-order rate constants for model compound hydrolysis, and the relative catalytic proficiencies (CP), with a value of 1.0 for the 4-nitrophenyl glucoside ($k_{cat}/K_m \times 1/k_{uncat})_{rel}$.

Table 2. Relative enzymatic proficiencies for glycoside hydrolysis and covalent-labeling by 2 and 3.

core structure	k_{cat}/K_m *or* k_{inact}/K_i (M^{-1} s^{-1})	k_{uncat} (s^{-1})[a]	CP_{rel}
pyranosyl	67,000[b]	4.61 × 10^{-5}	1.0
cyclohexenyl	1.59	4.20 × 10^{-8}	0.028
bicyclo[4.1.0]	6.4	1.49 × 10^{-6}	0.0033

[a]Rate constant extrapolated to 25 °C, Supporting Information (Table S2). [b]Data taken from reference [12].

Notably, the catalytic proficiency for covalent labeling by **2** is higher than the corresponding value for reaction with **3**, despite the cyclopropyl inhibitor exhibiting a larger second-order rate constant (k_{inact}/K_i) for enzyme labeling.

Even though the ground state conformations that permit π-bond participation in **2** ($^2H_3$ half-chair or a $B_{1,4}$ boat) are removed from the GH13 α-glucosidase reaction coordinate

(orange box, Figure 5, panel C) it is clear that the enzyme stabilizes formation of an allylic cation-like TS, which should have five coplanar carbon atoms. Thus, we reason that the enzyme binds **2** in a $^{2}H_{3}$ half-chair with a resulting catalyzed formation of an E_{3} allylic cation or allylic cation-like TS (rose box, Figure 5), a species that is conformationally similar to the glycosylation TS ($^{4}H_{3}$). In the case of covalent inhibitor **3**, σ-bond participation requires a bisected geometry; however, the resultant cation likely remains in the original bisected geometry due to the high rotational barrier in bicyclobutonium ions.[30] We conclude that covalent labeling by **2**, relative to **3**, involves a reaction coordinate that more closely matches that of the natural substrates.

Table 3. Relative proficiencies for dealkylation of the yeast α-glucosidase covalent intermediates.

core structure	k_{deglyc} or k_{react} (s^{-1})	k_{uncat} (s^{-1})[a]	CP_{rel}
pyranosyl	>29 s^{-1} [b]	4.61×10^{-5}	1.0
cyclohexenyl	1.19×10^{-5}	4.20×10^{-8}	$<4.6 \times 10^{-4}$
bicyclo[4.1.0]	2.17×10^{-6}	1.49×10^{-6}	$<2.4 \times 10^{-6}$

[a]Supporting Information (Table S2) [b]Data for most reactive pyridinium glycoside for which k_{cat} reports on the glycosylation step.[12]

Interestingly, the relative proficiencies for pseudo-deglycosylation are markedly different (Table 3) than those for the initial labeling event (Table 2). That is, the natural β-glucopyranosyl enzyme intermediate is hydrolyzed much more efficiently relative to the allylic and bicyclic covalent intermediates. We reason that the enzymatic motions that promote distortion of the $^{4}C_{1}$ glycosyl unit in the intermediate so that it undergoes hydrolysis to form α-glucopyranose in a $^{1}S_{3}$ skew boat are much less effective at promoting cleavage of the covalent enzyme-intermediates by formation of allylic and bicyclobutonium ion-like TSs, which based on the principle of microscopic reversibility must involve TS conformations similar to those for intermediate formation.

Finally, we envision that these two families of covalent inhibitors will be useful research tools for biological studies. Our covalent inhibitors, unlike other inactivators such as cyclophellitol and analogues[31-33] that irreversibly label glycoside hydrolases, show a time dependent loss and return of enzymatic activity. Moreover, we should be able to customize the rates of covalent-labeling (by changing the leaving group) and reactivation (by choosing either the cyclohexenyl or the bicyclo[4.1.0]heptyl skeleton). That is, our two classes of reversible covalent inhibitors could be used to monitor cellular responses to time-dependent changes in GH activity. Also, if the rates for each process (pseudo-glycosylation and deglycosylation) both depend on the pyranosylium ion-like TS ($^{4}H_{3}$, $^{3}H_{4}$, $B_{2,5}$, or $^{2,5}B$)[5,6,10] and a conformation for orbital participation we suggest that a simple analysis using Figure 5 will allow researchers to target the optimal carbasugar analogue for their particular GH.

ASSOCIATED CONTENT

Supporting Information

Full experimental procedures, spectroscopic data, and rate constants for hydrolysis of **10**. Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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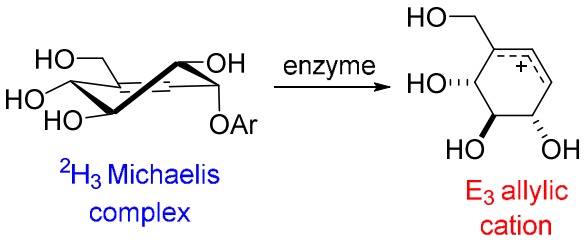
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