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

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ABSTRACT: The design of covalent inhibitors in glycoscience research is important for the development of chemical biology tools. 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 this allylic covalent inhibitor has different catalytic efficiencies for pseudoglycosylation 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 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 glycosidase 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 pyranosyl ion-like character, and the six-membered ring adopts one of several allowed conformations.8−10 In the current example, retaining

GH13 α-glucosidases9 react via pyranosyl ion-like TSs that are traversed during the catalytic cycle. Moreover, the pyranoside conformation at the TSs is postulated to be a 4H3 half-chair.10 Also, for GH13 enzymes the structure of the enzyme bound intermediate is a 4C1 chair (Figure 1).11 Kinetic isotope effect data suggests the productive Michaelis complex is not a ground state chair conformation, but is likely a 4S1 skew boat (Figure 1).12,13

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) (Figure 2).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 these compounds lead to a single covalent labeling of the enzyme, and importantly 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 3 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 subjected to a Furukawa modified Simmons–Smith cyclopropanation to give 8. Both 7 and 8 underwent facile S8Ar reactions followed by global debenzylation with BCl3 to give 2 and 3, respectively.

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
Scheme 1. Synthesis of Carbocyclic Inhibitors 2 and 3

function of the concentration of the carbosugar analogue. Notably, the allylic inhibitor 2 (red circles) is less active than the bicyclo[4.1.0]heptyl inactivator 3 (blue circles).

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.

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 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 67 275.7, whereas that after reaction with 2 shows the intact enzyme and a monoalkylated species (C₆H₁₂O₄ = 158.1) at 67 433.9, and the mass spectrum for the enzyme modified by 3 displays a single peak at 67 448.8, which corresponds to addition of the carbocyclic skeleton of 3 (C₆H₁₂O₄ = 172.1 + H).

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 and cyclopropylcarbinyl compounds undergo S₉,1 reactions at accelerated rates via allylic and nonclassical bicyclobutonium cationic intermediates, 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 pseudoquartetary aglycone for effective σ-bond participation (Figure 5, Panel A), whereas allylic participation in carbocyclic inhibitor 2 entails a pseudoaxial aglycone (Figure 5, Panel B).

On the basis of current theories that GH13 enzymes stabilize pyranosylum ion-like 2H₁ TSs from a bound 1S₃ Michaelis complex, we propose 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 1,4B boat). That is, the evolved reaction coordinate for α-glucopyranoside hydrolysis, which involves a rate-determining nonchemical step, exhibits a second-order rate constant (k_inact/K_m = 6.7 × 10⁸ M⁻¹ s⁻¹) for 4-nitrophenyl α-glucopyranoside. However, in order to assess the efficiency of covalent labeling it is important to calculate the relative enzymatic proiciencies for formation of the covalent glycosyl–enzyme intermediates (k_cat/K_m × 1/k_inact or k_cat/K_m × 1/k_inact). Because of the extremely slow spontaneous hydrolysis rates of glycosides, we used the kinetic data for the unsubstituted model compounds 9 and 11 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 (pseudodeglycosylation) and for cleavage of the glycosidic bond in the enzyme intermediate (pseudodeglycosylation). Listed in Table 2 are the second-order rate constants ($k_{\text{cat}}/K_m$ and $k_{\text{inact}}/K_i$) for yeast α-glucosidase reacting with 4-nitrophenyl α-D-glucopyranoside and our two covalent inhibitors 2 and 3, the uncatalyzed first-order rate constants for model compound hydrolysis, and the relative catalytic efficiencies (CP), with a value of 1.0 for the 4-nitrophenyl glucoside ($k_{\text{cat}}/K_m \times 1/k_{\text{inact}}$) rel. 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 ($k_{\text{inact}}/K_i$) for enzyme labeling.

Even though the ground state conformations that permit π-bond participation in 2 (2H3 half-chair or a B1,4 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 the enzyme binds 2 in a 2H3 half-chair with a resulting catalyzed formation of an E3 allylic cation or allylic cation-like TS (rose box, Figure 5), a species that is conformationally similar to the glycosylation TS (H3). In the case of covalent inhibitor 3, π-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 covalent labeling by 2, relative to 3, involves a reaction coordinate that more closely matches that of the natural substrates.

Interestingly, the relative efficiencies for pseudodeglycosylation are markedly different (Table 3) than those for the initial labeling event (Table 2). That is, the natural β-D-glucopyranosyl enzyme intermediate is hydrolyzed much more efficiently relative to the allylic and bicyclic covalent intermediates. We reason the enzymatic motions that promote distortion of the C1 glycosyl unit in the intermediate so that it undergoes hydrolysis to form α-D-glucopyranose in a 1S 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 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 that irreversibly label glycosidase hydrolyses, 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 (pseudodeglycosylation and deglyco-

![Table 1. Kinetic Parameters for the Covalent Inhibition and Reactivation of Yeast α-Glucosidase by the Inhibitors 2 and 3](Image)

<table>
<thead>
<tr>
<th>inactivator</th>
<th>$K_i$ (μM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
<th>$k_{\text{cat}}/K_i$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{\text{inact}}$ (s$^{-1}$)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>570 ± 90</td>
<td>(9.05 ± 0.63) × 10$^{-4}$</td>
<td>12.8</td>
<td>1.59 ± 0.27</td>
<td>(1.19 ± 0.07) × 10$^{-5}$</td>
<td>16.3</td>
</tr>
<tr>
<td>3</td>
<td>285 ± 45</td>
<td>(1.82 ± 0.14) × 10$^{-3}$</td>
<td>6.3</td>
<td>6.4 ± 1.1</td>
<td>(2.17 ± 0.14) × 10$^{-6}$</td>
<td>88.7</td>
</tr>
</tbody>
</table>

*Conditions were $T = 25$ °C, sodium phosphate buffer (50 mM, pH 6.84, [BSA] = 1 mg/mL).

![Table 3. Relative Proiciencies for Deallylation of the Yeast α-Glucosidase Covalent Intermediates](Image)

<table>
<thead>
<tr>
<th>core structure</th>
<th>$k_{\text{light}}$ or $k_{\text{inact}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)$^a$</th>
<th>CPrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyranosyl</td>
<td>&gt;29 s$^{-1}$</td>
<td>4.61 × 10$^{-5}$</td>
<td>1.0</td>
</tr>
<tr>
<td>cyclohexenyl</td>
<td>1.19 × 10$^{-5}$</td>
<td>4.20 × 10$^{-5}$</td>
<td>&lt;.6 × 10$^{-4}$</td>
</tr>
<tr>
<td>bicyclo[4.1.0]</td>
<td>2.17 × 10$^{-6}$</td>
<td>1.49 × 10$^{-6}$</td>
<td>&lt;.24 × 10$^{-4}$</td>
</tr>
</tbody>
</table>

*Supporting Information (Table S2). $^a$Data for most reactive pyridinium glycoside for which $k_{\text{cat}}$ reports on the glycosylation step.

![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) Enzyme 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 pyranosyl 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 and C6–C1) are labeled in rose (bold font) with the proposed reaction coordinate for 2 circled by the rose box.](Image)

![Figure 6. Structures of the model compounds (9–11) used to estimate relative catalytic efficiencies.](Image)
sylation) both depend on the pyranosylamion-like TS ($^{3}$H$_{2}$
$^{3}$H$_{2}$ B$_{2}$, or $^{5}$P$^{5}$,6,10 and a confirmation 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

The Supporting Information is available free of charge on the
ACS Publications website at DOI: 10.1021/jacs.7b05065.

Full experimental procedures, spectroscopic data, and
rate constants for hydrolysis of 10 (PDF)

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**

(1) Witte, M. D.; van der Marel, G. A.; Aerts, J. M.; Overkleeft, H. S.
(4) Lombard, V.; Ramulu, H. G.; Drula, E.; Coutinho, P. M.;
(7) Mhlongo, N. N.; Skelton, A. A.; Kruger, G.; Soliman, M. E. S.;
(9) Sinnott, M. Carbohydrate Chemistry and Biochemistry: Structure and
(10) Speciale, G.; Thompson, A. J.; Davies, G. J.; Williams, S. J. Curr.
(11) Uitdehaag, J. C. M.; Mou, R.; Kalk, K. H.; van der Veen, B. A.;
Ed. 2016, 55, 14978–14982.
(18) Lowry, T. H.; Richardson, K. S. Mechanism and theory in organic
2520.
(22) Harris, J. M.; Moffatt, J. R.; Case, M. G.; Clarke, F. W.; Polley, J. S.;
1998, 120, 1405–1409.
130, 9168–9172.
4624.
(31) Willems, L. I.; Beenakker, T. J. M.; Murray, B.; Scheij, S.;
Ferraz, M. J.; van Rijssel, E. R.; Florea, B. I.; Codee, J. D. C.;
van der Marel, G. A.; Aerts, J. M. F. G.; Overkleeft, H. S. J. Am.
(33) Kallemeijn, W. W.; Li, K. Y.; Witte, M. D.; Marques, A. R.; Aten,
J.; Scheij, S.; Jiang, J.; Willems, L. I.; Voorn-Brouwer, T. M.;
van Roomen, C. P.; Ottenhoff, R.; Boot, R. G.; van den Elst, H.;
Walvoort, M. T.; Florea, B. I.; Codee, J. D.; van der Marel, G. A.;
Aerts, J. M.;