ACS Catalysis

pubs.acs.org/acscatalysis
Research Article

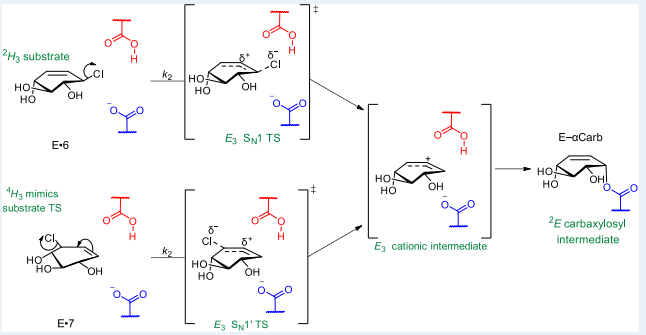
Development of Tunable Mechanism-Based Carbasugar Ligands that Stabilize Glycoside Hydrolases through the Formation of Transient Covalent Intermediates

Sandeep Bhosale, Sachin Kandalkar, Pierre-André Gilormini, Oluwafemi Akintola, Rhianna Rowland, Pal John Pal Adabala, Dustin King, Matthew C. Deen, Xi Chen, Gideon J. Davies, David J. Vocadlo,* and Andrew J. Bennet*

Cite This: *ACS Catal.* 2024, 14, 14769−14779
Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Mutations in many members of the set of human lysosomal glycoside hydrolases cause a wide range of lysosomal storage diseases. As a result, much effort has been directed toward identifying pharmacological chaperones of these lysosomal enzymes. The majority of the candidate chaperones are active site-directed competitive iminosugar inhibitors but these have met with limited success. As a first step toward an alternative class of pharmacological chaperones we explored the potential of small molecule mechanism-based reversible covalent inhibitors to form transient enzyme−inhibitor adducts. By serial synthesis and kinetic analysis of candidate molecules, we show that rational tuning of the chemical reactivity of glucose-configured carbasugars delivers cyclohexenyl-based allylic carbasugar that react with the lysosomal enzyme β-glucocerebrosidase (GCase) to form covalent enzyme-adducts with different half-lives. X-ray structural analysis of these compounds bound noncovalently to GCase, along with the structures of the covalent adducts of compounds that reacted with the catalytic nucleophile of GCase, reveal unexpected reactivities of these compounds. Using differential scanning fluorimetry, we show that formation of a transient covalent intermediate stabilizes the folded enzyme against thermal denaturation. In addition, these covalent adducts break down to liberate the active enzyme and a product that is no longer inhibitory. We further show that the one compound, which reacts through an unprecedented S_N1'-like mechanism, exhibits exceptional reactivity−illustrated by this compound also covalently labeling an α-glucosidase. We anticipate that such carbasugar-based single turnover covalent ligands may serve as pharmacological chaperones for lysosomal glycoside hydrolases and other disease-associated retaining glycosidases. The unusual reactivity of these molecules should also open the door to creation of new chemical biology probes to explore the biology of this important superfamily of glycoside hydrolases.

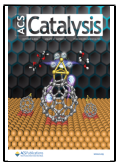


KEYWORDS: *tunable, glycoside hydrolases, covalent inhibition, mechanism, protein stabilization*

INTRODUCTION

As the number of glycoside hydrolases implicated in diseases continues to rise, the pursuit of ligands for these enzymes has gained increasing attention.[1,2] Ligands for this broad and diverse class of enzymes have entered the clinic[3,4] and several have gained clinical approval.[5,6] Many genetic diseases associated with glycoside hydrolases are driven by point mutations causing misfolding and consequent failure of the mutant enzyme to traffic properly to its target cellular compartment. Particular attention has been given to lysosomal glycoside hydrolases because once they exit the endoplasmic reticulum (ER) and reach the acidic environment of the lysosome they exhibit improved stability. Accordingly, one approach to therapeutic exploitation of active site-targeted ligands has been to explore their potential as pharmacological chaperones, which are generally high affinity iminosugars that have a weakly basic amine functionality.[6−8] Such pharmacological chaperones are able to bind to the enzyme within the ER, which leads to stabilization of the enzyme and a reduction in its premature degradation by the quality control mechanisms of the cell. This pharmacological stabilization accordingly enables increased trafficking of the chaperoned enzyme to lysosomes. Once in the lysosome, the pharmacological chaperone gradually diffuses out of this compartment

Received: July 30, 2024
Accepted: August 22, 2024
Published: September 20, 2024



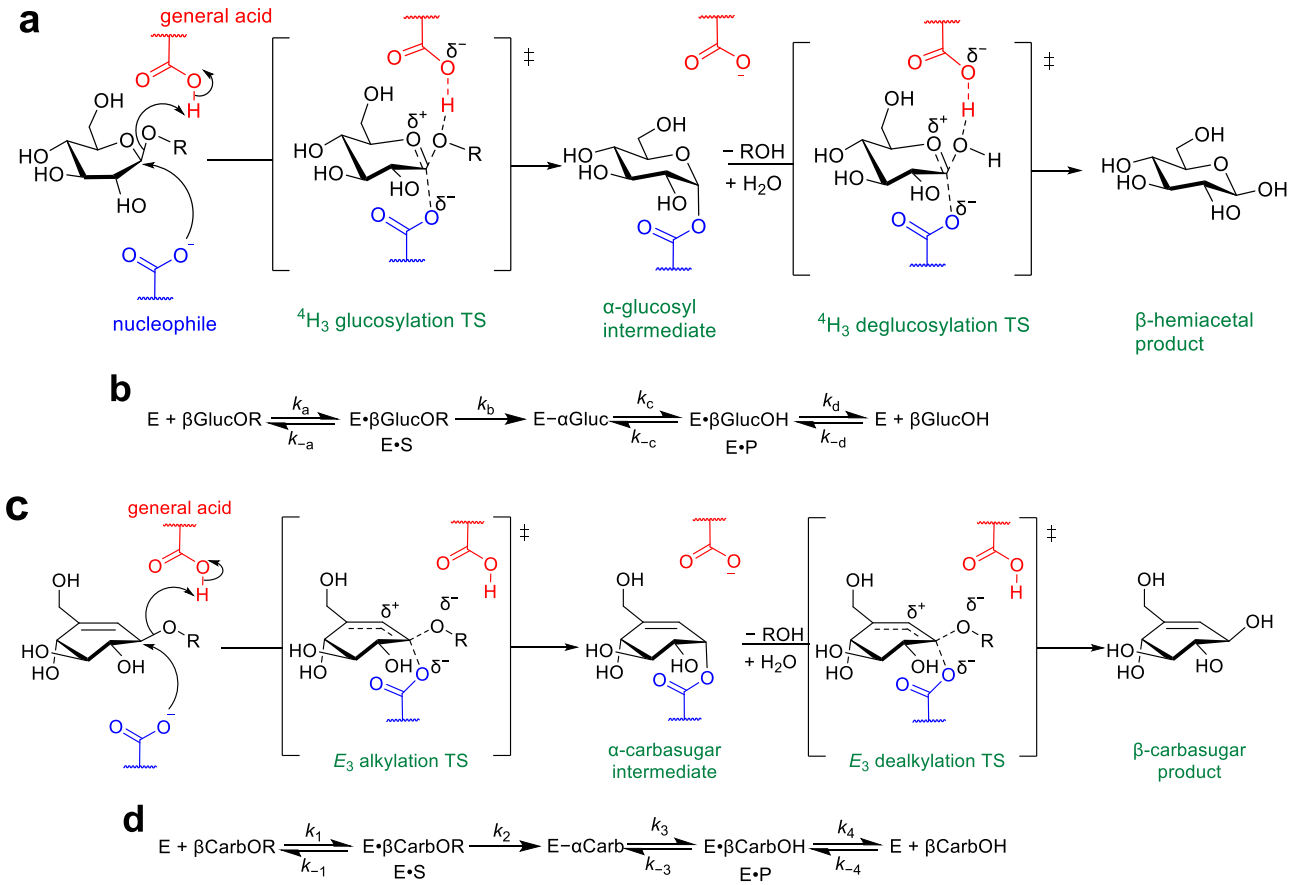


Figure 1. Small molecule covalent inhibitors and the mechanism of action of retaining glycosidases. (a) Mechanism used by glucocerebrosidase for hydrolysis of its natural *β*-glucoside substrate. (b) Kinetic scheme for GCase-catalyzed turnover of *β*-D-glucopyranoside substrates. (c) Mechanism of action for carbasugar covalent inhibitors. (d) Kinetic scheme for covalent carbasugar inhibitors.

allowing the enzyme to execute its essential function of degrading its glycoconjugates substrates.

While iminosugars continue to attract much attention as pharmacological chaperones, with one even gaining clinical approval,[5,6] an emerging class of alternative ligands for these enzymes include various elegant mechanism-based covalent inactivators including epoxides,[9] fluorosugars,[10] and aziridines.[11,12] All of these take advantage of the natural catalytic machinery of these enzymes to bind covalently to the catalytic nucleophile within the active site. Indeed, in a manner similar to iminosugars, irreversible binding of covalent inhibitors within the active site has been shown to stabilize the target protein against unfolding.[10,13] Such stabilization against unfolding has been found to correlate with improved trafficking of these lysosomal glycosidases, presumably by decreasing their misfolding. For example, a cyclophellitol-derived inactivator has been shown to increase the lysosomal quantity of the L444P variant *β*-glucocerebrosidase (GCase), a mutant that is prone to unfolding in the ER, but this did not translate into increased lysosomal activity of the enzyme.[14] Indeed, these three classes of covalent inactivators tend to either be irreversible or to result in inactive enzyme-adducts that have very long half-lives. For this reason, none of the currently known covalent ligands can serve as pharmacological chaperones because they cannot modulate enzyme activity in a useful controlled manner.

Nevertheless, we were intrigued by the idea of creating glycoside hydrolase covalent inhibitors that lead to formation of transient covalent glycosyl enzyme intermediates, but which can still stabilize the enzyme through favorable interactions within the active site (Figure 1c).[15] We noted that recently carbasugars, which resemble various bioactive natural products, have been modified to function as covalent inhibitors of various glycoside hydrolases.[16−18] This class of inhibitor makes use of strategic positioning of an alkene or cyclopropyl moiety to stabilize the carbocation that results from departure of a leaving group from the pseudoanomeric center, leading to formation of a covalent inhibitor-enzyme adduct (Figure 1d). However, unlike the aziridine and epoxide inactivators that form a dead-end enzyme−inhibitor complex, the carbasugar-enzyme adduct can be turned over by the enzyme leading to time dependent recovery of its activity. Notably, carbasugar derivatives exhibit differing behaviors in terms of their turnover by different enzymes.[16−18] These observations suggested to us that it may be possible to tune the reactivity of carbasugar analogues to create compounds that rapidly and covalently modify the enzyme within the active site but then turnover during a desirable time scale to liberate the active enzyme (Figure 1).

We decided to pursue this problem by initially focusing on the family 30 glycoside hydrolase (GH30) human *β*-glucocerebrosidase (GCase), which is of high interest. Homozygous loss-of-function mutations in the gene (GBA1) encoding GCase cause Gaucher Disease (GD),[19] a lysosomal storage disease that is characterized by the accumulation of glucosylceramide (GlcCer) within various organs of the body.

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

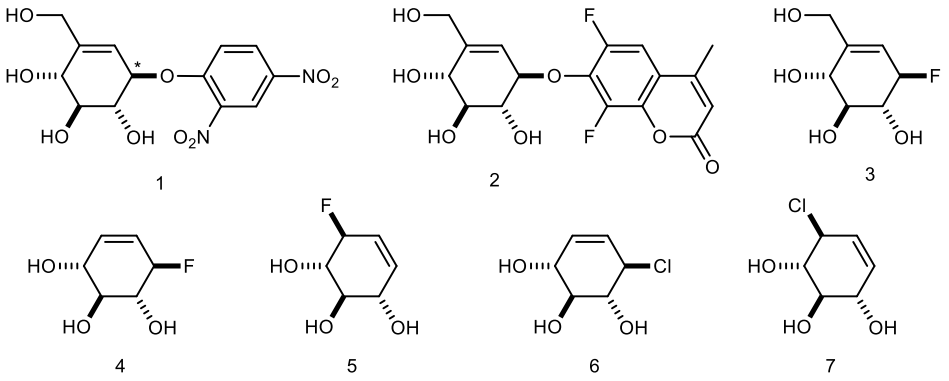


Figure 2. Structures of carbasugars used in this study and their in vitro characterization. Carbasugar covalent inhibitors used in the current study. The pseudoanomeric carbons (shown by the asterisk on 1) are drawn in identical positions for all compounds.

Moreover, heterozygous destabilizing mutations in GBA1 that lead to premature degradation of GCase have been linked to Parkinson Disease (PD),[20] greatly raising interest in this enzyme. Here we describe the design and synthesis of a series of covalent inhibitors of GCase, demonstrate that these compounds can be tuned to have desirable kinetic properties, determine the structure of these ligands bound to GCase, and show that formation of the covalent enzyme intermediate leads to an increase in the thermal stability of this enzyme. We further show the ability of such carbasugar analogues to engage with enzymes having varied substrate requirements by examining a structurally distinct covalent inhibitor lacking a pseudoanomeric center leaving group against both GH30 GCase and the yeast GH13 α-glucosidase.

RESULTS

Most retaining glycosidases, including GCase and yeast α-glucosidase, act on their respective substrates using a two-step catalytic mechanism[15] involving formation of a transient covalent glycosyl enzyme intermediate, which typically has a half-life for hydrolysis in the millisecond time regime[21,22] (Figure 1a). The first step, known as glycosylation (k_b, Figure 1b), involves departure of the aglycon leaving group and formation of the glycosyl enzyme intermediate. The second step, known as deglycosylation (k_c, Figure 1b), involves breakdown of this intermediate by the attack of water at the anomeric center. The processing of substrates by these enzymes can be monitored by incorporating chromogenic or fluorogenic leaving groups.

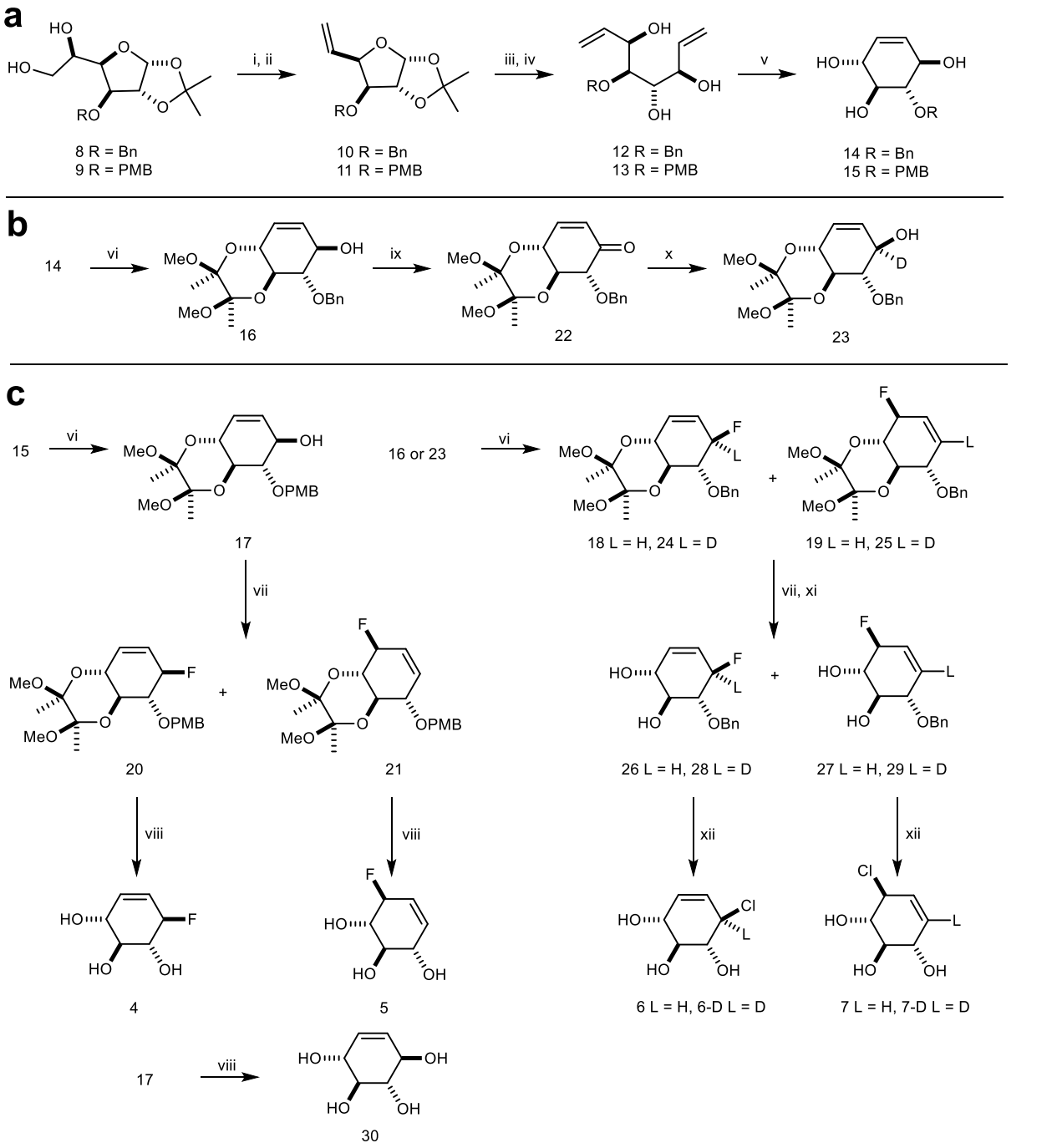
Analogously, allylic carbasugars also form transient covalent adducts that break down to regenerate active enzyme (Figure 1c) at a rate that is governed by k_3 (Figure 1d).[17,18]

Synthesis and Testing of First-Generation Allylic Carbasugar Covalent Inhibitors. As a first step toward our goal of creating allylic carbasugar covalent inhibitors that have a tunable reactivation rate constant (k_3), we synthesized the known carbaglucose 1 (Figure 2),[18] which bears a chromogenic 2,4-dinitrophenolate (2,4DNP) leaving group, and analogue 2, which contains a fluorogenic 6,8-difluoro-4-methylumbelliferone (6,8DiFMU) (Scheme S1, Supporting Information). Both aglycons have similar leaving group abilities as judged by the pK_a values of the corresponding phenols (2,4DNP pK_a = 3.96, 6,8DiFMU pK_a = 4.81). We found these carbaglucose compounds were good inhibitors with IC_{50} values of 0.17 ± 0.03 and 14.3 ± 1.7 μM for 1 and 2, respectively (Figure S1, Tables S1 and S2, Supporting Information), IC_{50} values that are comparable to many competitive inhibitors of GCase.[23] Nonlinear least-squares fitting of our kinetic data showed these compounds displayed classic signs of mixed inhibition with $K_{i,mixed}$ values for 1 and 2 of 0.16 ± 0.03 and 17 ± 7 μM, respectively (Figure S2, Supporting Information). Such mixed inhibition is consistent with transient covalent labeling of an enzyme,[24] and we therefore monitored GCase-catalyzed turnover of these two carbasugars and determined that the rate constant governing their turnover (k_{cat}) as being quite similar (~0.013−0.018 s^{-1}) (Figure S3, Supporting Information). We next checked for accumulation of a covalent intermediate on the enzyme using a series of classical "jump-dilution" experiments but could not observe any time dependent inhibition with either compound. Given the similarity of the observed k_{cat} values for 1 and 2 and the relatively low measured IC_{50} values, we reasoned that both steps (k_2 and k_3) are likely kinetically significant and that processing of these carbasugars proceeds through a common pseudoglycosyl enzyme intermediate. To try and increase the rate of the pseudoglycosylation step we changed the leaving group to fluoride and synthesized carbasugar 3 (Scheme S1, Supporting Information). We reasoned that as fluoride ion is intrinsically a better leaving group than a phenoxide having a similar basicity[25] and that, within the confines of the active site of GCase, a fluoride leaving group receives H-bonding assistance whereas leaving groups such as 2,4-dinitrophenoxide do not.[26] Unfortunately, carbasugar 3 displayed similar inhibition of GCase (IC_{50} = 41 ± 15 μM) as carbasugars 1 and 2 (Figure S1 and Table S2, Supporting Information), with no observable time-dependent loss of GCase activity. Thus, pseudodeglycosylation is also not rate-limiting for carbasugar 3. We therefore considered approaches to modulate the relative rates of each enzymatic step to obtain a situation where the pseudodeglycosylation step (k_3) becomes markedly slower than the pseudoglycosylation step (k_2), which would lead to an increased lifetime of the GCase covalent intermediate.

Synthesis and Testing of Second-Generation Allylic Carbasugar Covalent Inhibitors. Based on an examination of the literature, we reasoned that removing the hydroxymethyl (C6-CH_2OH) group from the cyclohexene system should decrease both rate constants. In particular, we note that GCase cleaves both xylosylceramide and 4-methylumbelliferyl β-D-xylopyranoside, with k_{cat}/K_m values that are lower than the corresponding glucopyranoside substrates.[27] Given these observations we reasoned that an analogous change to the carbasugar structure, in which the hydroxymethyl group is removed, could lead to molecules having reactivity that would

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

Scheme 1. Synthesis of D- and L-Carbaxylopyranosyl Halides; (a) Synthesis of Intermediates 14 and 15, (b) Synthesis of Intermediates 16 and 23, (c) Synthesis of Target Carbasugars[a]



[a]Reagents: (i) $NaIO_4$, H_2O, rt; (ii) $Ph_3P^+CH_3$, BuLi, 0 °C; (iii) dioxane/water, cat H^+, 90 °C; (iv) $CH_2CHMgBr$, THF, 0 °C; (v) Grubbs' 2nd generation, CH_2Cl_2, heat; (vi) $CH_3COCOCH_3$, $HC(OCH_3)_3$, cat H^+; (vii) DAST, diglyme, 0 °C; (viii) TFA, CH_2Cl_2 (for 4 and 5 from 20 and 21, respectively); (ix) Dess-Martin periodinane, CH_2Cl_2; (x) $NaBD_4$ (MeOD); (xi) TFA, CH_2Cl_2, H_2O; (xii) BCl_3, CH_2Cl_2, −78 °C (for 6, 7, 6-D, and 7-D from 26 to 29, respectively). Abbreviations: DAST, (diethylamino)sulfur trifluoride; TFA, trifluoroacetic acid.

enable accumulation of a covalent intermediate. Accordingly, we set out to make the D-xylo configured carbasugar 4 containing a pseudoanomeric fluoride leaving group. We selected fluorine as a leaving group because it could benefit from H-bonding catalysis, which would accelerate the pseudoglycosylation step and thereby allow rapid formation of the pseudoglycosyl enzyme intermediate. In this way we expected that we might observe a situation where the rate of formation of the pseudoglycosyl enzyme intermediate was much faster than its breakdown.

To prepare carbasugar 4 we developed a synthetic route involving the protected cyclohex-2-en-1-ol 14 (Scheme 1) as a convenient intermediate. Periodate cleavage of the known[28] 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose 8 and subsequent Wittig homologation gave olefin 10. Standard functional group interconversion and ring closing metathesis

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

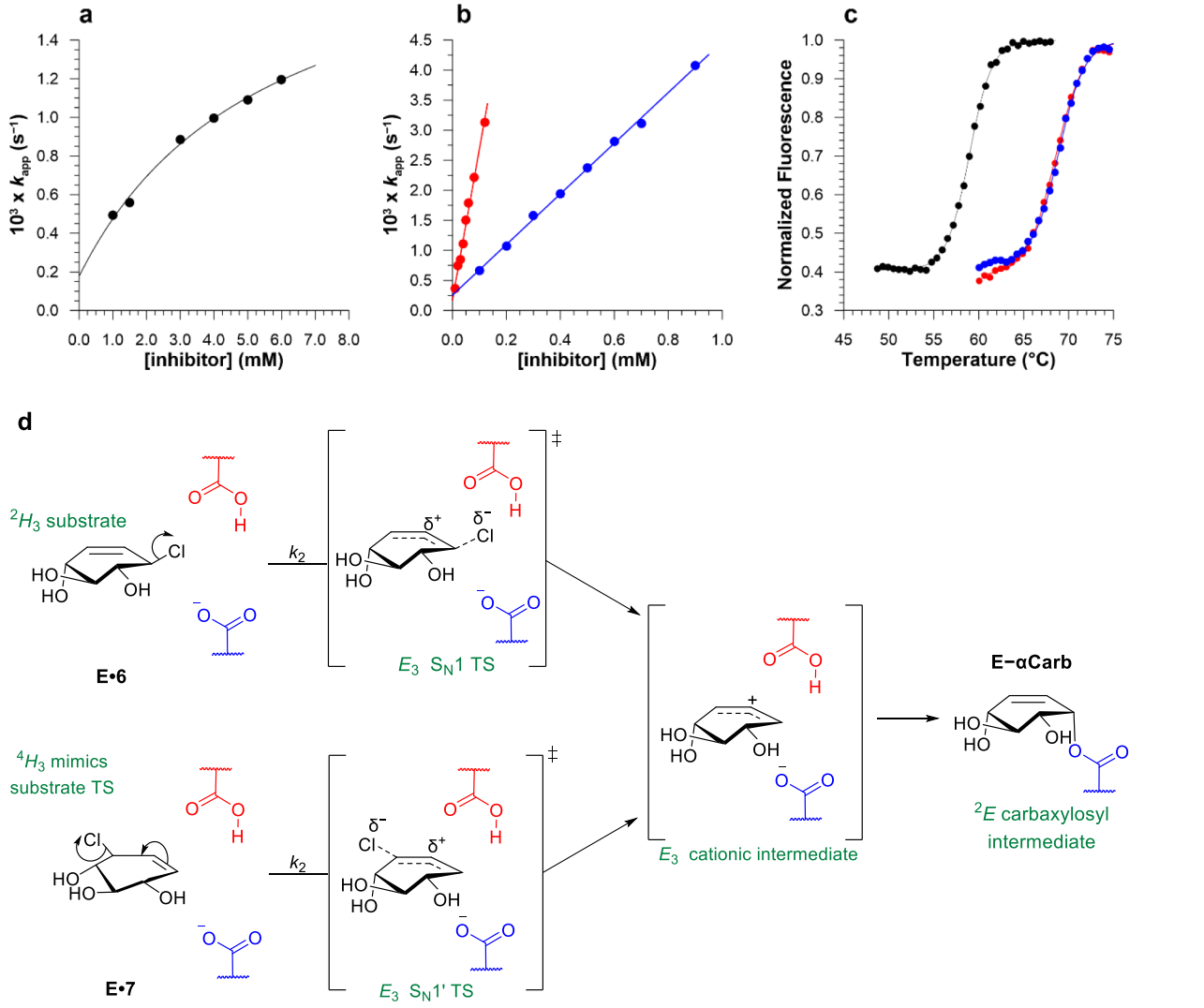


Figure 3. In vitro characterization of carbasugars used in this study. (a) Nonlinear least-squares fit of k_{app} values to a modified Michaelis−Menten expression (eq S3) for the approach to steady-state GCase activity on incubation with D-carbaxylosyl fluoride 4. (b) Linear fit of k_{app} values for the approach to steady state for covalent inhibition of GCase by D-carbaxylosyl chloride 6 (blue) and L-carbaxylosyl chloride 7 (red). (c) Stabilization of folded GCase measured using differential scanning fluorimetry (DSF) in the presence of D-carbaxylosyl chloride 6 (blue), L-carbaxylosyl chloride 7 (red), or vehicle along (black). All lines are the best nonlinear least-squares fit to the appropriate equation. All experiments were carried out at T = 25 °C in McIlvaine buffer pH 5.2 containing 0.1% Triton X-100, 0.24% sodium taurodeoxycholate, and 0.1% BSA. (d) Mechanisms for the GCase pseudoglycosylation step by single turnover chaperones 6 and 7, which after diffusion of chloride out of the active site give identical allylic cation intermediates that are trapped by the enzymatic nucleophile (Glu340) to give the same covalent intermediate (E-αCarb).

of compound 12 provided us with 14, which was converted to allylic alcohol 16 by protecting the *trans*-diol as a butane 2,3-bisacetal (Scheme 1).[29] To install fluorine we used (diethylamino)sulfur trifluoride (DAST) given that it reacts with allylic cyclohexenols through a stereochemically desirable S_Ni-S_Ni' reaction mechanism.[30] Specifically, allylic alcohol 16 reacted with DAST to afford two allylic fluorides (18:19, ratio 3:7). These protected carbasugar fluorides could not be separated, however, we found that removal of the bis-acetal protecting group enabled separation of the partly deprotected allylic fluorides 26 and 27.

Subsequent BCl_3-promoted debenzylation of 26 and 27 led to the expected debenzylation but also an adventitious S_Ni reaction that yielded the corresponding D- and L-carbaxylosyl chlorides 6 and 7 in approximately 90% yields. We therefore changed our protecting group strategy to install a 4-methoxybenzyl (PMB) ether at C3 (9, Scheme 1). Following an identical reaction sequence to that described above, we obtained isomeric carbasugar fluorides (20:21, 1:2 ratio), which we readily separated. Acid-catalyzed deprotection proceeded without incident to give both 4 and 5 in ∼80% yields. With these desired fluorides in hand, we turned our attention to assessing their reactivity with GCase.

In contrast to the reactions of GCase with carbaglucoses 1−3, coincubation of these compounds with the fluorogenic substrate 4-methylumbelliferyl β-D-glucopyranoside (MU-Glc), revealed that D-carbaxylosyl fluoride 4 led to time-dependent inhibition of GCase, with steady state levels of activity being reached after ∼30−60 min. This time-dependent decrease in activity to a steady state level is consistent with transient covalent modification of GCase. To understand the kinetics governing formation of the pseudoxylosyl enzyme intermediate we fit the reaction progress curve[31] of fluorescence arising from turnover of MU-Glc versus time, at each inhibitor concen-

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

tration (Figure S4, Supporting Information), where F_t is the initial fluorescence, ν_i is the initial velocity at $t = 0$, ν_f is the velocity at $t = \infty$, and k_{app} is the apparent rate constant (eq S1, Supporting Information). Next, we fit the resulting calculated k_{app} values as a function of the concentration of covalent inhibitor 4 and, taking into account the concentration of the substrate and its K_m value (eqs S2 and S3, Supporting Information), obtained the inhibition parameters $k_{inact} = (2.0 \pm 0.3) \times 10^{-3}$ s^{-1}, $K_i = 3.4 \pm 1.5$ mM, and $k_{inact}/K_i = 0.59 \pm 0.27$ M^{-1} s^{-1} (Figure 3a and Table S3, Supporting Information).

These inhibition parameters suggests that carbasugar 4 slowly labels GCase but also that the resulting covalent carbaxylosyl GCase intermediate must have a significantly longer half-life ($t_{1/2}$) than the corresponding intermediate formed during the turnover of gluco-configured carbasugars 1, 2, and 3. Yet, the slow approach to steady state in combination with the comparatively similar rate for breakdown of the pseudoxylosyl enzyme intermediate make fluoride 4 ill suited for examining the potential stabilizing effects associated with formation of a transient covalent intermediate on folded β-glucocerebrosidase.

Synthesis and Testing of Third-Generation Allylic Carbasugar Covalent Inhibitors. To obtain a situation where greater steady state levels of the covalent carbaxylosyl intermediate could be obtained, we decided to speed the first step of the reaction by using a carbaxylose inhibitor having a better leaving group. We therefore turned to testing the adventitiously obtained carbaxylosyl chloride 6 (Figure S5, Supporting Information). By using the same kinetic analysis as described above, we observed that the covalent labeling of GCase ($k_{inact}/K_i = 6.9 \pm 0.1$ M^{-1} s^{-1}) occurred ~10−fold faster for chloride 6 as compared to fluoride 4 (Table 1 and Figures S4 and S5, Supporting Information). We also considered another possible strategy we could exploit using these carbaxylose compounds. In particular, we noted that glycoside hydrolases have been found to use catalytic mechanisms that generally involve significant charge delocalization.[15] This observation has led to development of substrates and inactivators that influence the stability of the transition state through installation of electron withdrawing groups at C5.[32] Moreover, we recently showed for an α-galactosidase acting on a carbasugar substrate that the enzyme efficiently stabilized positive charge development in the transition state at both the pseudoanomeric center (C1) as well as at C5.[33,34] Given this observation we decided to test the L-carbaxylosyl chloride 7 (Figure S6, Supporting Information), which owing to its leaving group being situated at C5, would necessarily react through a mechanism involving significant TS positive charge development at this center as the leaving group departs (Figure 3d). Remarkably, using the same series of kinetic analyses, we found that compound 7 covalently labels GCase and, moreover, does so more rapidly than D-carbasugar 6 ($k_{inact}/K_i = 41 \pm 2$ M^{-1} s^{-1}; Figure 3b and Table 1). Notably, the rate constants we measured for breakdown of the carbaxylosyl enzyme intermediates formed by reaction of 6 and 7 with GCase were indistinguishable ($t_{1/2} \approx 50-55$ min, Figure S7 and Table S3, Supporting Information), which suggests that the intermediates formed from labeling GCase with either compound are identical. However, the surprisingly greater rate constant governing formation of this common intermediate on GCase for L-carbaxylosyl chloride 7, as compared to D-carbaxylosyl chloride 6, drew into question our assignments for the structures of 6 and 7. We therefore synthesized the deuterated carbaxylosyl fluorides 28 and 29 (Scheme 1), and performed the BCl_3 reaction. The resulting chloride products that we isolated (6-D and 7-D) exhibited NMR spectra in accord with the chlorination occurring solely via a S_Ni reaction with no evidence for a S_Ni' process (Figure S8, Supporting Information). These data indicate that our original assignments were correct and confirm that GCase−and likely glycoside hydrolases in general−have the surprising ability to react through a novel S_N1' reaction with allylic carbasugar derivatives having a leaving group at C5. Collectively, these data show that the reactivity of carbasugar substrates of GCase can be tuned to obtain situations where the putative covalent intermediate can be rapidly formed and persist for varying lengths of time.

Table 1. Kinetic Parameters for the Covalent Inhibition and Reactivation of Human GCase by the Carbasugars 4−7[c]

inhibitor	k_{inact}/K_i (M^{-1} s^{-1})	k_{react} (s^{-1})	$t_{1/2react}$ (min)
4	0.59 ± 0.27[a]		
5	N.I.[b]		
6	6.9 ± 0.1	$(2.2 \pm 0.2) \times 10^{-4}$	52.5 ± 4.8
7	41 ± 2	$(2.1 \pm 0.1) \times 10^{-4}$	55.0 ± 2.6

[a] $k_{inact} = (2.0 \pm 0.3) \times 10^{-3}$ s^{-1}, $K_i = 3.4 \pm 1.5$ mM. [b] N.I. no time-dependent inhibition observed at 6 mM. [c] Conditions: $T = 25$ °C in McIlvaine buffer pH 5.2 containing 0.1% Triton X-100, 0.24% sodium taurodeoxycholate, and 0.1% BSA.

X-ray Structural Analysis of Michaelis Complexes and Covalent Intermediates of GCase. To better understand how these carbasugars interact with GCase and to clarify whether carbasugars 6 and 7 indeed reacted to give a common covalent enzyme adduct as our kinetic analyses suggested, we set out to obtain cocrystal structure of human GCase[35] in complex with these compounds. We therefore soaked crystals of GCase, produced recombinantly from insect cells, with carbasugar 6 with a view to capturing and visualizing the transient carbaxylosyl enzyme adduct. Following a 30 min soak, we obtained crystals that diffracted to 1.58 Å. Analysis of the diffraction data revealed unambiguous electron density showing 6 had reacted with the catalytic nucleophile (Glu340) to form a covalent bond to the pseudoanomeric carbon (Figure 4a). Analysis of the structure of the resulting enzyme−inhibitor complex showed this ester C−O bond has a length of 1.38 Å, which we modeled at 75% occupancy. This lower occupancy likely reflects hydrolytic turnover of the covalent complex ($t_{1/2} \approx 55$ min in solution—Table S3, Supporting Information) during crystallization. The carbasugar ring of 6 in this complex adopts an envelope conformation (^{2}E), presumably with the planarity enforced by the endocyclic double bond and the formation of hydrogen bonding interactions to active site residues Asp127, Trp179, Asn234, Glu340, and Trp381. Similar experiments using the L-carbasugar 7, led to a structure in which GCase has reacted to form the same adduct having the same ring conformation, hydroxyl stereochemistry, and hydrogen bonding network (Figure 4b). The observed enzyme−inhibitor complex, with a C−O covalent bond length of 1.40 Å, was modeled at 70% occupancy. These data supported our proposal that 7, being the enantiomer of 6, reacted through an alternative and unprecedented S_N1' mechanism (Figure 3d) to generate the same observed covalent adduct as seen when incubating 6 with GCase, which is consistent with the reactivation rate constant

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

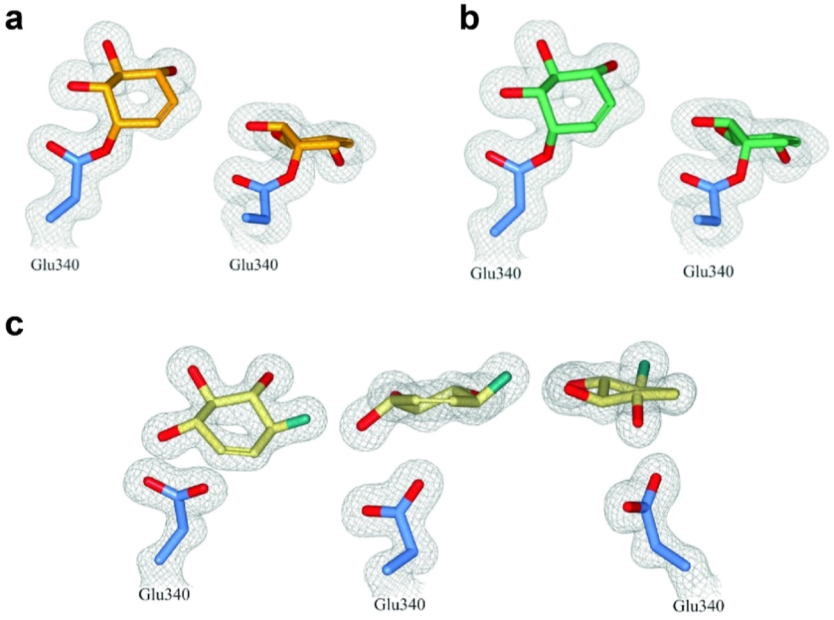


Figure 4. Structural characterization of covalent intermediates and Michaelis complex between GCase and carbasugars. (a) Crystal structure of the GCase-6 complex, showing two orientations of the covalent complex in which 6 adopts an envelope (^{2}E) conformation. The maximum-likelihood σ_A-weighted $2F_o - F_c$ electron density map is contoured to 1 σ (0.37 e/Å^3). (b) Crystal structure of the GCase-7 complex, showing two orientations of the covalent complex in which, the carbasugar adopts an envelope (^{2}E) conformation, numbering of the ring starts at the covalent attachment to the enzyme. The maximum-likelihood σ_A-weighted $2F_o - F_c$ electron density map is contoured to 1 σ (0.36 e/Å^3). Carbasugar average *b*-factor = 20 Å^2. (c) Crystal structure of the GCase-5 complex, showing three orientations of L-carbasugar fluoride bound noncovalently in the active site in a half-chair (^{4}H$_3$) conformation. Green atom indicates fluorine. The maximum-likelihood σ_A-weighted $2F_o - F_c$ electron density map is contoured to 1 σ (0.37 e/Å^3).

governing the breakdown of this intermediate being the same for both 6 and 7. We also tested whether the C_2-symmetric tetraol 30, which lacks a good leaving group, affected GCase activity at 0.25 mM (Figure S9, Supporting Information) and found this compound showed neither competitive inhibition nor time dependent inhibition.

To obtain a "Michaelis" complex with these carbasugars noncovalently bound by GCase, we performed structural studies using the less reactive L-carbaxylosyl fluoride 5. These experiments led to a cocrystal structure in which electron density for a single molecule of 5, bound noncovalently within the enzyme active site, was observed (Figure 4c). Although F and OH were indistinguishable within the electron density map, the absolute L-stereochemistry of the carbasugar bound within the active site shows unambiguously that 5 had not reacted. This "Michaelis" complex provides insight into how inhibitor 5 and, by extension, 7 bind prior to reacting within the active site of GCase. The electron density unambiguously shows that 5 was bound in a half-chair (^{4}H$_3$) conformation, which is enforced by the endocyclic double bond, that mimics the expected partial double bond character seen in the oxocarbenium ion-like transition states stabilized by this enzyme (Figures 1a and 4c). This carbasugar engages in hydrogen bonding interactions with active site residues Asp127, Trp179, Asn234, Glu340, and Trp396, showing an overall similar pattern of hydrogen bonds as seen for the covalent adduct of 7 linked to Glu340.

An additional coincidental, yet noteworthy, observation we made during structural studies using carbasugar 7 was that an additional molecule was also bound noncovalently to GCase at a site at the surface of the immunoglobulin-like domain of GCase. In contrast to the envelope conformation observed for the covalent adduct of 7 within the active site, this unreacted molecule of 7 molecule adopted a half-chair (^{3}H$_4$) conformation (Figure S10, Supporting Information), forming hydrogen bonding interactions with Arg47, Glu41, and a nearby water molecule. A potential aliphatic–π stacking interaction with the side chain of Arg39 was also seen, with a separation distance of 3.3 Å (Figure S10, Supporting Information). Supporting these observations, we also found L-carbaxylosyl fluoride 5 also bound noncovalently to this same remote binding site (Figure S11, Supporting Information). Carbasugar 5 at this site was also unreacted, based on its clear L-xylo configuration, and we readily modeled it in the same orientation as we had observed for 7. The electron density unambiguously showed that 5 at this site adopted the same half-chair conformation as 7 and formed the same hydrogen bonding network. Interestingly, we were never able to observe enantiomer 6 binding to this site, indicating the stereochemistry of the hydroxyl groups likely influences noncovalent binding to this site. The stereochemical requirements for binding to this site, coupled with reproducibly observing ligands in this location, suggests that this remote binding site may have a physiological function in binding some as yet unidentified ligand.

To probe whether transient formation of these covalent intermediates within the active site of GCase affects enzyme stability we performed differential scanning fluorimetry (DSF) in the presence and absence of both D,L-carbaxylosyl chlorides (6 and 7; Figure 2d). Incubation of GCase in the presence of either covalent inhibitor resulted in an indistinguishable 10 °C increase in melting temperature (6; 68.9 ± 0.1; 7 68.5 ± 0.1; control 59.0 ± 0.1 °C). These data again show that the enantiomeric pair of carbaxylosyl chlorides react with GCase to give an identical covalent intermediate, which confers a stabilizing effect on the enzyme.

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

Reactivity of Carbaxylosyl Chlorides with Other Glycoside Hydrolases. Given the remarkable observation that β-glucocerebrosidase is covalently labeled by L-carbaxylosyl chloride 7, which possesses a sp^2 center at the pseudoanomeric carbon atom, we reasoned that in the absence of an anomeric substituent this compound may also be able to covalently modify mechanistically distinct α-glucosidases. To investigate this idea, we used yeast GH13 α-glucosidase as a test case. Gratifyingly, we note that 7 labeled yeast α-glucosidase in a time-dependent manner (Table 2 and Figures S12 and S13, Supporting Information). Given that GH13 yeast α-glucosidase is a strikingly stable enzyme we reasoned that we could reliably examine the pH-dependence of the inactivation process leading to the covalent intermediate and, in this manner, examine the active protonation states of this retaining α-glycoside hydrolase (Figure 1c).[15,36] We therefore measured the second-order rate constants for the covalent inhibition of yeast α-glucosidase by 7 over a range of pH values (Figures 5 and S14, S15) and found a bell-shaped pH-rate profile, albeit with the best fit being for three ionization events, which is similar to profiles observed for other glycoside hydrolase-catalyzed reactions.[37] These data show that these cyclohexene-derived carbasugars lacking an anomeric substituent can covalent label anomerically distinct glycoside hydrolases in a mechanism-based manner that depends on the enzyme having a protonation state that is also required for turnover of natural substrates.

Table 2. Kinetic Parameters for the Covalent Inhibition and Reactivation of Yeast α-Glucosidase by the Carbasugar 7[a]

inhibitor	k_{inact}/K_i (M^{-1} s^{-1})	k_{react} (s^{-1})	$t_{1/2react}$ (h)
7	7.70 ± 0.07	(2.35 ± 0.17) × 10^{-6}	81.8 ± 5.9

[a]Conditions: experiments were carried out at T = 37 °C in 50 mM sodium phosphate buffer, pH 7.0 containing 0.1% BSA.

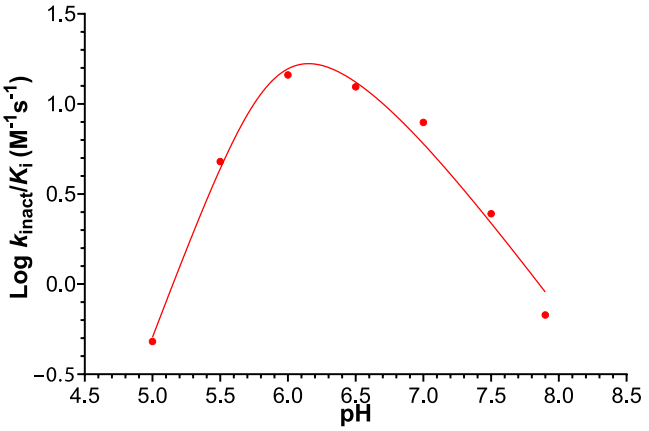


Figure 5. pH-rate profile for the logarithm of the inactivation second-order rate constants (k_{inact}/K_i) for yeast α-glucosidase by 7. All experiments were performed at T = 37 °C in 50 mM buffer (acetic acid-sodium acetate buffer for pH 5.0−5.5, sodium phosphate buffer for pH 6.0−7.5, TAPS buffer for pH 7.9) containing 0.1% BSA. The line through the points is the best fit to a model where two protonation events (low pH) lead to an inactive enzyme, while a single deprotonation at higher pH values gives inactive enzyme.

DISCUSSION

By rationally tuning the reactivity of a glucose-configured carbasugar we have created a new class of reversible covalent glycoside hydrolase inhibitors. These inhibitors derive their ability to transiently inactivate glycoside hydrolases by retaining features of the natural substrate essential for binding, which are suitably oriented hydroxyl groups positioned on a six-membered ring. However, the mechanisms by which these cyclohexenyl inhibitors act are distinct, involving stabilization of the cationic transition states, not by an endocyclic ring oxygen as seen for natural substrates, but rather by a suitably positioned alkene moiety that enables cation stabilization through charge delocalization. Indeed, we suggest that GCase-catalyzed covalent labeling occurs via S_N1 reactions for fluoride 4, chloride 6, and, most unusually, an atypical mechanism that we describe here as S_N1' for chloride 7 (Figure 3d). Specifically, we propose that GCase-catalyzed pseudoglycosylation with D-carbaxylosyl fluoride 4, chloride 6, and the more reactive L-carbaxylosyl chloride 7, occurs via such dissociative processes, which give rise to a common enzyme bound allylic cation intermediate (Figure 3d), based on the following reasoning:

(i) Both experimental[38−40] and theoretical[41] studies indicate that an S_N2 displacement has a significantly lower TS free energy than the corresponding S_N2' counterpart. As a result, if covalent labeling involved S_N2 and S_N2' mechanisms inhibitor 6 should covalently label GCase more rapidly than does 7, a situation that is contrary to our observations

(ii) Contrary to the commonly held view, fluoride is a viable leaving group in bona fide S_N1 reactions performed in polar H-bonding environments. For instance, the difference in S_N1 reactivity of 1-adamantyl chloride (1-AdCl)[42] and 1-adamantyl fluoride (1-AdF)[43] in trifluoroethanol is approximately 760-fold at 25 °C. Moreover, this ratio is almost independent of temperature as the major difference in the corresponding activation parameters is a larger negative entropy ($\Delta S^{\ddagger}$) for the solvolysis of 1-AdF, likely caused by the obligatory strong H-bonding that is needed to assist fluoride ion departure for formation of the 1-adamantyl carbocation[44,45]

(iii) The ratio of the second order rate constants (k_{inact}/K_i) governing the GCase-catalyzed reactions of 6 and 4 is 12 ± 5, a ratio that is consistent with S_N1 reactions in which the presence of an optimally positioned H-bonding donor only assists departure of fluoride to give enzyme-bound allylic cations,[34] and

(iv) L-Carbaxylosyl chloride 7 undergoes pseudoglycosylation faster than does the D-enantiomer 6 because 7 binds in the conformation of the transition state for GCase-catalyzed cleavage of natural substrate glucosidic bonds ($^4H_3$), a conformation that enables the most efficient stabilization of positive charge development at the allylic cation-like transition state (Figure 3d).

These collective points, along with detailed QM/MM computational studies on a retaining galactosidase,[34] support such allylic covalent inhibitors reacting with GCase by S_N1 (4 and 6) and S_N1' (7) mechanisms for these reactions. We expect that this mechanistic knowledge can be exploited in the generation of such carbasugar labeling agents for GCase as well as other enzymes of interest. Further, the ability of GCase to

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

catalyze an S_N1' mechanism for 7 is intriguing and suggests the possibility of designing other substrates or inhibitors that exploit this mechanistic promiscuity for other members of the large class of glycoside hydrolases. Indeed, 7 bears a chloride leaving group that requires little to no catalytic assistance for departure from C5 but, once bound into an active site that has evolved to stabilize the development of positive charge, undergoes rapid pseudoglycosylation and ultimately hydrolytic turnover of this intermediate. As a result, such allylic carbasugar inhibitors lacking a pseudoanomeric leaving group possess the exceptional reactivity needed to inhibit both α- and β-glucosidases.

Further support for these carbasugars functioning as mechanism-based labeling agents is seen in the standard bell-shaped pH-rate profile for the inactivation processes (k_{inact}/K_i) that is similar to that seen for the hydrolysis of natural substrates (k_{cat}/K_m).[37] This bell-shaped profile arises from ionization of two key carboxylic acid residues that are needed in the appropriate protonation state[46] to effect the first chemical step involving pseudoglycosylation.[37] This observation may initially appear inconsistent with our conclusion that neither general-acid nor nucleophilic catalysis is required for departure of the chloride leaving group. However, though yeast α-glucosidase did not evolve to provide catalytic assistance for leaving group departure at C5 of 7, the correct protonation state of the enzyme is still needed to provide the correct negatively charged environment that stabilizes positive charge development within the allyl cation-like transition states at C5, C5a, and C1 for pseudoglycosylation.[33]

In summary, we have demonstrated the ability to tailor the reactivity of carbasugar inhibitors, and we anticipate that such principles can be applied, in principle, to refine the properties of such molecules for any glycoside hydrolase that operates via a double displacement mechanism. Such variations can be tailored for the enzyme of interest to generate useful chemical biology tools. As well as being exploited in the development of next generation small molecule pharmacological chaperones that stabilize both wild-type and misfolding variant lysosomal glycoside hydrolases (Figure 3c, covalent adduct), yet yield active enzyme following dealkylation (Figures 1c,d and 3d, liberated enzyme). More broadly, the design of such tunable reversible covalent inhibitors should be generally applicable to the larger set of active-site directed reversible covalent ligands for other enzymes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.4c04549.

General methods and reagents, eqs S1−S3, Tables S1−S3, Scheme S1, Figures S1−S78 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

David J. Vocadlo − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada; Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0001-6897-5558; Email: dvocadlo@sfu.ca

Andrew J. Bennet − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0002-8378-6752; Email: bennet@sfu.ca

Authors

Sandeep Bhosale − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0002-1850-1271

Sachin Kandalkar − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada*

Pierre-André Gilormini − *Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0003-1491-1174

Oluwafemi Akintola − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0001-9790-7132

Rhianna Rowland − *Department of Chemistry, University of York, York YO10 5DD, U.K.;* orcid.org/0000-0002-8717-346X

Pal John Pal Adabala − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada*

Dustin King − *Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada;* orcid.org/0000-0003-1775-2885

Matthew C. Deen − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada*

Xi Chen − *Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada*

Gideon J. Davies − *Department of Chemistry, University of York, York YO10 5DD, U.K.;* orcid.org/0000-0002-7343-776X

Complete contact information is available at: https://pubs.acs.org/10.1021/acscatal.4c04549

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful for support from the Canadian Institutes of Health Research (A.J.B.: grant-173228) and the Natural Sciences and Engineering Council of Canada (A.J.B.: RGPIN-04910 and CRDPJ-480335, and D.J.V.: RGPIN-05426). We thank Diamond Light Source for access to beamline I03 and I04 (proposal numbers mx18598 and mx24948). G.J.D. thanks the Royal Society for the Ken Murray Research Professorship and RJR thanks the BBSRC for White Rose Doctoral Training Partnership funding (BB/M011151/1). D.J.V. thanks the Canada Research Chairs program for support as a Tier I Canada Research Chair in Chemical Biology.

■ REFERENCES

(1) Platt, F. M.; d'Azzo, A.; Davidson, B. L.; Neufeld, E. F.; Tifft, C. J. Lysosomal storage diseases. *Nat. Rev. Dis. Primers* **2018**, *4*, 27.

(2) Varki, A. *Essentials of Glycobiology*, 4th ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor: Cold Spring Harbor, NY, 2022.

(3) Selnick, H. G.; Hess, J. F.; Tang, C. Y.; Liu, K.; Schachter, J. B.; Ballard, J. E.; Marcus, J.; Klein, D. J.; Wang, X. H.; Pearson, M.; Savage, M. J.; Kaul, R.; Li, T. S.; Vocadlo, D. J.; Zhou, Y. X.; Zhu, Y.

B.; Mu, C. W.; Wang, Y. D.; Wei, Z. Y.; Bai, C.; Duffy, J. L.; McEachern, E. J. Discovery of MK-8719, a potent O-GlcNAcase inhibitor as a potential treatment for tauopathies. *J. Med. Chem.* **2019**, *62*, 10062−10097.
(4) den Heijer, J. M.; Kruithof, A. C.; van Amerongen, G.; de Kam, M. L.; Thijssen, E.; Grievink, H. W.; Moerland, M.; Walker, M.; Been, K.; Skerlj, R.; Justman, C.; Dudgeon, L.; Lansbury, P.; Cullen, V. C.; Hilt, D. C.; Groeneveld, G. J. A randomized single and multiple ascending dose study in healthy volunteers of LTI-291, a centrally penetrant glucocerebrosidase activator. *Br. J. Clin. Pharmacol.* **2021**, *87*, 3561−3573.
(5) Patterson, M. C.; Vecchio, D.; Prady, H.; Abel, L.; Wraith, J. E. Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study. *Lancet Neurol.* **2007**, *6*, 765−772.
(6) Hughes, D. A.; Nicholls, K.; Shankar, S. P.; Sunder-Plassmann, G.; Koeller, D.; Nedd, K.; Vockley, G.; Hamazaki, T.; Lachmann, R.; Ohashi, T.; et al. Oral pharmacological chaperone migalastat compared with enzyme replacement therapy in Fabry disease: 18-month results from the randomised phase III ATTRACT study. *J. Med. Gen.* **2017**, *54*, 288−296.
(7) Fan, J. Q.; Ishii, S.; Asano, N.; Suzuki, Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat. Med.* **1999**, *5*, 112−115.
(8) Parenti, G.; Andria, G.; Valenzano, K. J. Pharmacological chaperone therapy: Preclinical development, clinical translation, and prospects for the treatment of lysosomal storage disorders. *Mol. Ther.* **2015**, *23*, 1138−1148.
(9) Witte, M. D.; Kallemeijn, W. W.; Aten, J.; Li, K. Y.; Strijland, A.; Donker-Koopman, W. E.; van den Nieuwendijk, A.; Bleijlevens, B.; Kramer, G.; Florea, B. I.; Hooibrink, B.; Hollak, C. E. M.; Ottenhoff, R.; Boot, R. G.; van der Marel, G. A.; Overkleeft, H. S.; Aerts, J. Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.* **2010**, *6*, 907−913.
(10) Scherer, M.; Santana, A. G.; Robinson, K.; Zhou, S.; Overkleeft, H. S.; Clarke, L.; Withers, S. G. Lipid-mimicking phosphorus-based glycosidase inactivators as pharmacological chaperones for the treatment of Gaucher's disease. *Chem. Sci.* **2021**, *12*, 13909−13913.
(11) Adams, B. T.; Niccoli, S.; Chowdhury, M. A.; Esarik, A. N. K.; Lees, S. J.; Rempel, B. P.; Phenix, C. P. N-Alkylated aziridines are easily-prepared, potent, specific and cell-permeable covalent inhibitors of human β-glucocerebrosidase. *Chem. Commun.* **2015**, *51*, 11390−11393.
(12) 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. A. A.; 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.; Overkleeft, H. S. Novel Activity-Based Probes for Broad-Spectrum Profiling of Retaining β-Exoglucosidases In Situ and In Vivo. *Angew. Chem. Int. Ed., Engl.* **2012**, *51*, 12529−12533.
(13) Do, J.; McKinney, C.; Sharma, P.; Sidransky, E. Glucocerebrosidase and its relevance to Parkinson disease. *Mol. Neurodegener.* **2019**, *14*, 36.
(14) Ben Bdira, F.; Kallemeijn, W. W.; Oussoren, S. V.; Scheij, S.; Bleijlevens, B.; Florea, B. I.; van Roomen, C.; Ottenhoff, R.; van Kooten, M.; Walvoort, M. T. C.; Witte, M. D.; Boot, R. G.; Ubbink, M.; Overkleeft, H. S.; Aerts, J. Stabilization of Glucocerebrosidase by Active Site Occupancy. *ACS Chem. Biol.* **2017**, *12*, 1830−1841.
(15) Vocadlo, D. J.; Davies, G. J. Mechanistic insights into glycosidase chemistry. *Curr. Opin. Chem. Biol.* **2008**, *12*, 539−555.
(16) Adamson, C.; Pengelly, R.; Shamsi Kazem Abadi, S.; Chakladar, S.; Draper, J.; Britton, R.; Gloster, T.; Bennet, A. J. Structural snapshots for mechanism-based inactivation of a glycoside hydrolase by cyclopropyl-carbasugars. *Angew. Chem., Int. Ed.* **2016**, *55*, 14978−14982.
(17) Shamsi Kazem Abadi, S.; Tran, M.; Yadav, A. K.; Adabala, P. J. P.; Chakladar, S.; Bennet, A. J. New class of glycoside hydrolase mechanism-based covalent inhibitors: Glycosylation transition state conformations. *J. Am. Chem. Soc.* **2017**, *139*, 10625−10628.
(18) Danby, P. M.; Withers, S. G. Glycosyl cations versus allylic cations in spontaneous and enzymatic hydrolysis. *J. Am. Chem. Soc.* **2017**, *139*, 10629−10632.
(19) Parenti, G. Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO Mol. Med.* **2009**, *1*, 268−279.
(20) Sidransky, E.; Lopez, G. The link between the GBA gene and parkinsonism. *Lancet Neurol.* **2012**, *11*, 986−998.
(21) Sinnott, M. L.; Souchard, I. J. The mechanism of action of β-galactosidase. Effect of aglycone nature and α-deuterium substitution on the hydrolysis of aryl galactosides. *Biochem. J.* **1973**, *133*, 89−98.
(22) Kempton, J. B.; Withers, S. G. Mechanism of Agrobacterium .beta.-glucosidase: kinetic studies. *Biochemistry* **1992**, *31*, 9961−9969.
(23) Breen, I. Z.; Artola, M.; Wu, L.; Beenakker, T. J. M.; Offen, W. A.; Aerts, J. M. F. G.; Davies, G. J.; Overkleeft, H. S. Competitive and covalent inhibitors of human lysosomal retaining exoglucosidases. *eLS*; Wiley & Sons Ltd.: Chichester, 2018.
(24) Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*; Wiley: New York, 1975.
(25) Jones, C. C.; Sinnott, M. L. Leaving ability and basicity of leaving groups attached by first-row elements. *J. Chem. Soc., Chem. Commun.* **1977**, 767−768.
(26) Farren-Dai, M.; Sannikova, N.; Świderek, K.; Moliner, V.; Bennet, A. J. Fundamental insight into glycoside hydrolase-catalyzed hydrolysis of the universal Koshland substrates−glycopyranosyl fluorides. *ACS Catal.* **2021**, *11*, 10383−10393.
(27) Boer, D. E.; Mirzaian, M.; Ferraz, M. J.; Zwiers, K. C.; Baks, M. V.; Hazeu, M. D.; Ottenhoff, R.; Marques, A. R. A.; Meijer, R.; Roos, J. C. P.; Cox, T. M.; Boot, R. G.; Pannu, N.; Overkleeft, H. S.; Artola, M.; Aerts, J. M. Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions. *J. Lipid Res.* **2021**, *62*, 100018.
(28) Beauhaire, J.; Ducrot, P. H. An epoxide derived from D-glucose as the key intermediate for penaresidine and sphingolipids synthesis. *Synth. Commun.* **1998**, *28*, 2443−2456.
(29) Ley, S. V.; Baeschlin, D. K.; Dixon, D. J.; Foster, A. C.; Ince, S. J.; Priepke, W. M.; Reynolds, D. J. 1,2-Diacetals: A new opportunity for organic synthesis. *Chem. Rev.* **2001**, *101*, 53−80.
(30) Shih, T. L.; Liao, W. Y.; Yen, W. C. Regioselective fluorination in synthesis of deoxyfluoro quercitols from D-(−)-quinic acid. *Tetrahedron* **2014**, *70*, 9621−9627.
(31) Baici, A.; Schenker, P.; Wachter, M.; Ruedi, P. 3-Fluoro-2,4-dioxa-3-phosphadecalins as inhibitors of acetylcholinesterase. A reappraisal of kinetic mechanisms and diagnostic methods. *Chem. Biodivers.* **2009**, *6*, 261−282.
(32) McCarter, J. D.; Withers, S. G. 5-Fluoro Glycosides: A New Class of Mechanism-Based Inhibitors of Both α- and β-Glucosidases. *J. Am. Chem. Soc.* **1996**, *118*, 241−242.
(33) Ren, W.; Farren-Dai, M.; Sannikova, N.; Świderek, K.; Wang, Y.; Akintola, O.; Britton, R.; Moliner, V.; Bennet, A. J. Glycoside hydrolase stabilization of transition state charge: New directions for inhibitor design. *Chem. Sci.* **2020**, *11*, 10488−10495.
(34) Akintola, O.; Farren-Dai, M.; Ren, W.; Bhosale, S.; Britton, R.; Świderek, K.; Moliner, V.; Bennet, A. J. Glycoside hydrolase catalysis: Do substrates and mechanism-based covalent inhibitors react via matching transition states? *ACS Catal.* **2022**, *12*, 14667−14678.
(35) Rowland, R. J.; Wu, L.; Liu, F.; Davies, G. J. A baculoviral system for the production of human β-glucocerebrosidase enables atomic resolution analysis. *Acta Crystallogr. Sect. D-Struct. Biol.* **2020**, *76*, 565−580.
(36) Speciale, G.; Thompson, A. J.; Davies, G. J.; Williams, S. J. Dissecting conformational contributions to glycosidase catalysis and inhibition. *Curr. Opin. Struct. Biol.* **2014**, *28*, 1−13.
(37) Matsusaka, K.; Chiba, S.; Shimomura, T. Purification and substrate specificity of brewer's yeast .ALPHA.-glucosidase. *Agric. Biol. Chem.* **1977**, *41*, 1917−1923.
(38) Bordwell, F. G. Are nucleophilic bimolecular concerted reactions involving four or more bonds a myth? *Acc. Chem. Res.* **1970**, *3*, 281−290.

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779

(39) Bordwell, F. G.; Mecca, T. G. Nucleophilic substitutions in allylic systems. Further evidence against existence of concerted S_N2' mechanism. *J. Am. Chem. Soc.* **1972**, *94*, 5829−5837.
(40) Kantner, S. S.; Humski, K.; Goering, H. L. On the solvolysis of 2-cyclohexenyl 3,5-dinitrobenzoate and p-nitrobenzoate in aqueous acetone. Introduction of acyl-oxygen cleavage by basic buffer systems. *J. Am. Chem. Soc.* **1982**, *104*, 1693−1697.
(41) Streitwieser, A.; Jayasree, E. G.; Hasanayn, F.; Leung, S. S. H. A theoretical study of S_N2' reactions of allylic halides: Role of ion pairs. *J. Org. Chem.* **2008**, *73*, 9426−9434.
(42) Bentley, T. W.; Carter, G. E. The S_N2-S_N1 spectrum. 4. The S_N2 (intermediate) mechanism for aolvolysis of tert-butyl chloride: A revised Y scale of solvent ionizing power based on solvolysis of 1-adamantyl chloride. *J. Am. Chem. Soc.* **1982**, *104*, 5741−5747.
(43) Ohga, Y.; Munakata, M.; Kitagawa, T.; Kinoshita, T.; Takeuchi, K.; Oishi, Y.; Fujimoto, H. Solvolyses of bicyclo[2.2.2]oct-1-yl and 1-adamantyl systems containing an ethylidene substituent on the 2-position: typical examples of rate enhancements ascribed to relief of F-strain. *J. Org. Chem.* **1994**, *59*, 4056−4067.
(44) Chan, J.; Tang, A.; Bennet, A. J. A stepwise solvent-promoted S_Ni reaction of α-D-glucopyranosyl fluoride: Mechanistic implications for retaining glycosyltransferases. *J. Am. Chem. Soc.* **2012**, *134*, 1212−1220.
(45) Sinnott, M. L.; Jencks, W. P. Solvolysis of D-glucopyranosyl derivatives in mixtures of ethanol and 2,2,2-trifluoroethanol. *J. Am. Chem. Soc.* **1980**, *102*, 2026−2032.
(46) Wan, Q.; Parks, J. M.; Hanson, B. L.; Fisher, S. Z.; Ostermann, A.; Schrader, T. E.; Graham, D. E.; Coates, L.; Langan, P.; Kovalevsky, A. Direct determination of protonation states and visualization of hydrogen bonding in a glycoside hydrolase with neutron crystallography. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 12384−12389.

https://doi.org/10.1021/acscatal.4c04549
ACS Catal. 2024, 14, 14769−14779