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McGregor, N.G.S.; Coines, J.; Borlandelli, V.B.; Amaki, S.; Artola Perez de Azanza, M.E.; Nin-Hil, A.; ... ; Davies, G.J.

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Enzyme Mechanisms

Cysteine Nucleophiles in Glycosidase Catalysis: Application of a Covalent β-L-Arabinofuranosidase Inhibitor

Nicholas G. S. McGregor[+], Joan Coines[+], Valentina Borlandelli, Satoko Amaki, Marta Artola, Alba Nin-Hill, Daniël Linzel, Chihaya Yamada, Takatoshi Arakawa, Akihiro Ishiwata, Yukishige Ito, Gijsbert A. van der Marel, Jeroen D. C. Codée, Shinya Fushinobu, Herman S. Overkleeft, Carme Rovira, and Gideon J. Davies**

Abstract: *The recent discovery of zinc-dependent retaining glycoside hydrolases (GHs), with active sites built around a Zn(Cys)$_3$(Glu) coordination complex, has presented unresolved mechanistic questions. In particular, the proposed mechanism, depending on a Zn-coordinated cysteine nucleophile and passing through a thioglycosyl enzyme intermediate, remains controversial. This is primarily due to the expected stability of the intermediate C–S bond. To facilitate the study of this atypical mechanism, we report the synthesis of a cyclophellitol-derived β-L-arabinofuranosidase inhibitor, hypothesised to react with the catalytic nucleophile to form a non-hydrolysable adduct analogous to the mechanistic covalent intermediate. This β-L-arabinofuranosidase inhibitor reacts exclusively with the proposed cysteine thiol catalytic nucleophiles of representatives of GH families 127 and 146. X-ray crystal structures determined for the resulting adducts enable MD and QM/MM simulations, which provide insight into the mechanism of thioglycosyl enzyme intermediate breakdown. Leveraging the unique chemistry of cyclophellitol derivatives, the structures and simulations presented here support the assignment of a zinc-coordinated cysteine as the catalytic nucleophile and illuminate the finely tuned energetics of this remarkable metalloenzyme clan.*

First identified in 2014 and 2018 respectively, glycoside hydrolase families[1] GH127 and GH146 share a common active site structure centred around an unusual Zn(Cys)$_3$-(Glu) coordination complex.[2,3] Hydrolysis of β-L-arabinofuranosides was shown to proceed with retention of configuration,[4] yet the structures of the active site and substrate preclude all known retaining mechanisms, including the classical Koshland double displacement mechanism,[5,6] the NAD^+-dependent mechanism,[7] the neighbouring group participation mechanism,[8,9] and the recently described 1,2-epoxide intermediate mechanism.[10]

The first crystallographic complex of a GH127 enzyme with L-arabinofuranose was solved in 2014.[3] This structure revealed that *Bifidobacterium longum* GH127 (HypBA1) recognizes L-arabinofuranose with the anomeric carbon positioned adjacent to C417, as if hydrolysed from a thioglycosidic linkage. A structure solved for *Bacteroides thetaiotaomicron* GH146 (*Bt*GH146) found similar positioning of L-arabinofuranose adjacent to C416 (which is analagous to C417 in HypBA1).[2]

Site-directed mutagenesis of HypBA1 supported the status of C417 as the catalytic nucleophile, but was confounded by the profound loss of activity resulting from mutagenesis of any residue forming part of the Zn(Cys)$_3$(Glu) coordination complex.[3] Theoretical analysis, using a small active site model, suggested that a previously proposed hydrolytic mechanism passing through a thioglycosyl enzyme intermediate (tGEI) is plausible. However, the tGEI was found to be more stable than the product complex, and experimental evidence for the formation of this intermediate has remained lacking. To determine experimentally if the enzyme possesses a viable catalytic nucleophile, and to

[*] Dr. N. G. S. McGregor,[+] Prof. G. J. Davies
York Structural Biology Laboratory, Department of Chemistry,
The University of York
Heslington, York, YO10 5DD (UK)
E-mail: gideon.davies@york.ac.uk
Dr. J. Coines,[+] Dr. A. Nin-Hill, Prof. C. Rovira
Departament de Química Inorgànica i Orgànica (Secció de Química Orgànica) & Institut de Química Teòrica i Computacional (IQTCUB),
Universitat de Barcelona
Martí i Franquès 1, 08028 Barcelona (Spain)
E-mail: c.rovira@ub.edu
V. Borlandelli, Dr. M. Artola, D. Linzel, Prof. G. A. van der Marel, Dr. J. D. C. Codée, Prof. H. S. Overkleeft
Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2300 RA Leiden (The Netherlands)
S. Amaki, Dr. C. Yamada, Dr. T. Arakawa, Prof. S. Fushinobu
Department of Biotechnology, The University of Tokyo
Bunkyo-ku, Tokyo 113-8657 (Japan)
A. Ishiwata, Y. Ito
Synthetic Cellular Chemistry Laboratory,
RIKEN Cluster for Pioneering Research
2-1 Hirosawa, Wako, Saitama 351-0198 (Japan)
Y. Ito
Graduate School of Science, Osaka University
Toyonaka, Osaka 560-0043 (Japan)
Prof. C. Rovira
Institució Catalana de Recerca i Estudis Avançats (ICREA)
Passeig Lluís Companys 23, 08020 Barcelona (Spain)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
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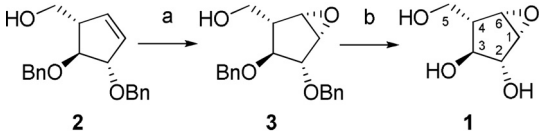
enable more accurate computational study, we prepared a cyclophellitol-derived inhibitor;[11,12] cyclophellitol and its derivatives are glycoside mimics which react with the catalytic nucleophiles of retaining GHs to form non-hydrolysable adducts analogous to the mechanistic covalent intermediate.

The synthesis of β-L-arabinofuranose-configured epoxide **1** relied on the stereoselective epoxidation of key cyclopentene **2**.[13] This was achieved by exploiting the directing effect of the neighbouring primary alcohol through hydrogen bonding with *m*-CPBA at low temperature (Scheme 1).[13] Final β-L-arabinofuranosyl epoxide **1** was obtained by hydrogenation of partially benzylated epoxide **3** with Pearson's catalyst.

To determine whether **1** had the capacity to label the $Zn(Cys)_3(Glu)$ active site, the covalent inactivation of HypBA1 (GH127) by **1** was followed by intact mass spectrometry and residual activity measurement. Samples taken following 0, 10, 60, and 1300 minutes of incubation with 0.1 mM epoxide **1** at 37 °C in 50 mM pH 4.5 NaOAc buffer confirmed time-dependent single labelling, giving a species with a mass difference of 146 Da (Figure 1 A). The ≈55 % labelling measured following 10 minutes of incubation allows estimation of a performance constant (k_i/K_I) of ≈20 $M^{-1}s^{-1}$ (assuming $K_I \gg 0.1$ mM). This was in good agreement with the inhibition performance constant determined via residual activity measurements ($k_i/K_I = 14.5 \pm 0.7\ M^{-1}s^{-1}$, Figure S1), confirming that labelling was concordant with enzyme inhibition. The measured performance constant for the interaction between HypBA1 and compound **1** is comparable to the inhibition by β-D-xylo-configured cyclophellitol epoxide of *Anid*XlnD (GH3) (170 $M^{-1}s^{-1}$) or the inhibition by α-L-arabinofurano-configured cyclophellitol aziridine of *An*AbfA (GH51, 39 $M^{-1}s^{-1}$) or *Ak*AbfB (GH54, 28 $M^{-1}s^{-1}$).[13,14] An activity assay using the synthetic 4-nitrophenyl-β-L-arabinofuranoside substrate also confirmed a loss of activity over time in the crystallised enzyme sample (Figure S2). This result demonstrates that the electrophilic addition typical of appropriately configured cyclophellitol derivatives can occur efficiently within the $Zn(Cys)_3(Glu)$ active site, inhibiting enzymatic activity.



Scheme 1. Synthesis of β-L-arabinofuranosyl-configured epoxide **1**. Reagents and conditions: a) *m*-CPBA, H_2NaPO_4/HNa_2PO_4, CH_2Cl_2, 4 °C, 4 days, 74 %; b) H_2, $Pd(OH)_2$, MeOH, r.t., 19 h, 18 %.

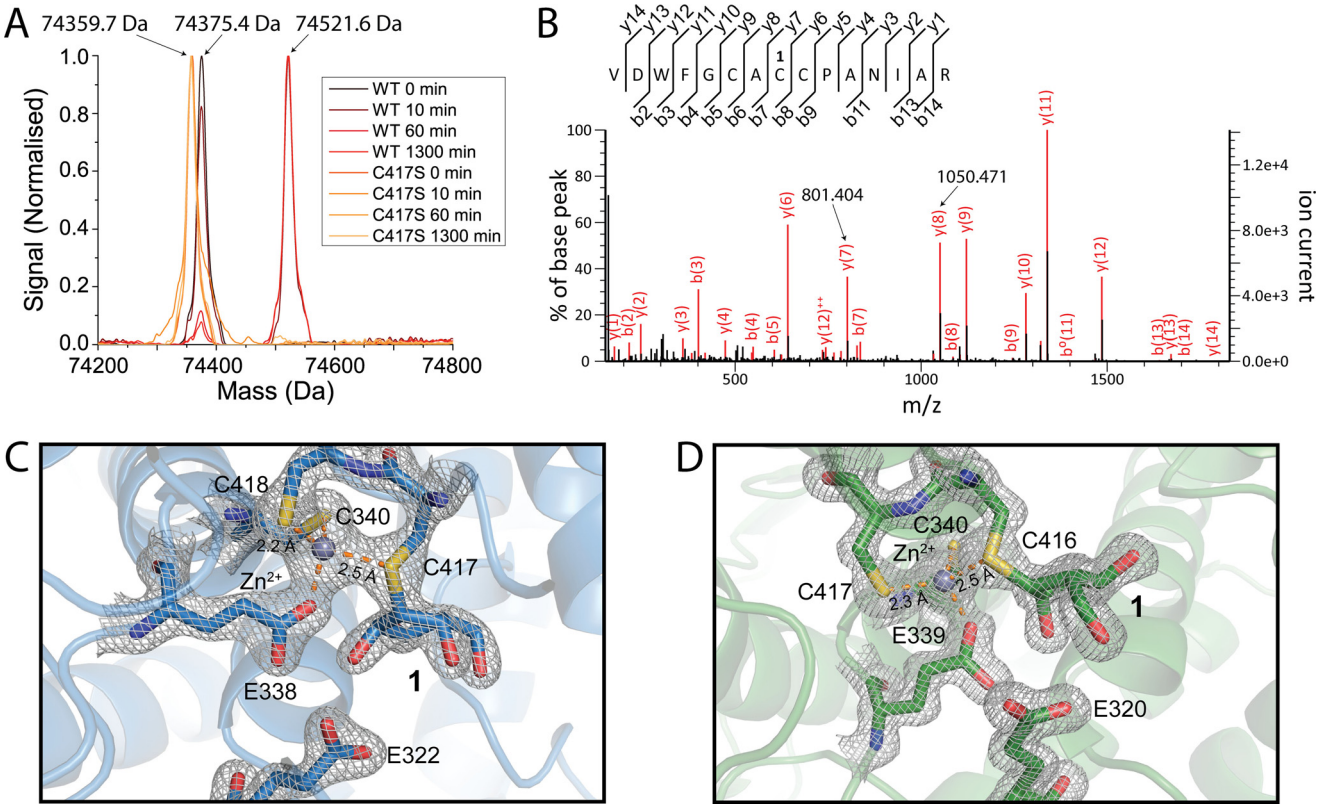


Figure 1. Labelling of β-L-arabinofuranosidases by **1**. A) Overlay of intact MS spectra collected for HypBA1 and HypBA1 C417S following different incubation periods with **1**. Expected mass (native) = 74376.5 Da, with **1** = 74522.6 Da, C417S = 74360.5 Da. B) Active site peptide MS/MS spectra from **1**-labelled HypBA1. *m/z* values for the major diagnostic y_7 and y_8 fragment signals are given (see Table S2 for all y and b fragment *m/z* values). C) Active site structure of HypBA1 bound to **1**. The $2F_o - F_c$ map is shown contoured to 2σ for key residues. The bonds coordinating the zinc ion are shown as orange dashed lines with key bond lengths shown. D) Active site structure of *Bt*GH146 following reaction with **1** with density and coordination shown as in panel C.

To determine whether labelling occurs exclusively at the putative catalytic nucleophile (C417), A search of MS/MS spectra collected from the tryptic digest of HypBA1 labelled with **1** for 1 hour, identified 458 MS/MS spectra which corresponded to peptides derived from HypBA1 (78% coverage, FDR 0.9%). Labelling with **1** was detected exclusively at C417 (Figure 1B). Furthermore, intact MS was run under identical conditions with HypBA1 C417S, giving no detectable labelling after 1300 minutes (Figure 1A).

Similar experiments were run with BtGH146, a recently characterized GH146 β-L-arabinofuranosidase from a distinct phylogenetic clade of retaining glycoside hydrolases, which also possess a $Zn(Cys)_3(Glu)$ active site.[2] Samples taken for intact MS following 0, 60, 240, and 1200 minutes of incubation with 0.1 mM epoxide **1** at 37 °C in 20 mM HEPES pH 7.5 confirmed similar, though slower, labelling (Figure S3A). Digestion of the labelled enzyme followed by LC-MS/MS of the resulting peptides confirmed exclusive labelling of the putative active site peptide at C416 (homologous to C417 in HypBA1, Figure S3B). Thus, the reactivity of **1** exclusively with the putative catalytic cysteine nucleophile appears to be a general phenomenon.

Crystallization of the complex between HypBA1 and **1** (crystallographic data and refinement statistics given in Table S1) yielded a structure which contains clear ligand density bound to the $Zn(Cys)_3(Glu)$ cluster through C1 (Figure 1C). Key hydrogen bonding interactions between O2 and both E338 and H270, between O3 and H194, and between O5 and H142 are preserved between the covalent complex with **1** and the previously determined complex with L-arabinose (PDB ID: 3WKX, Figure S4A). Relative to the L-arabinose complex, C1 has undergone electrophilic migration towards C417, forming a covalent bond with this putative nucleophile and shifting the ring conformation to the somewhat higher energy ^4E (Figure S5).

Crystallization of *Bt*GH146 labelled with compound **1** yielded an unexpected structure, in which the C416 sulphur had attacked C6 of the inhibitor (Figure 1D). Comparison to the L-arabinofuranose complex (PDB ID: 5OPJ) shows that binding occurred with a net rotation of the ring by ≈75° such that O5 formed a hydrogen bond with E320 instead of E217 (Figure S4B), preserving other key interactions. The epoxide oxygen formed an apparent hydrogen bond with E339, which retained its dative bond to zinc.

To understand the origin and mechanistic relevance of our observed complexes, molecular dynamics (MD) simulations were performed to reconstruct the binding of **1** in unreacted Michaelis complexes. The crystallised complexes between each enzyme and **1** were taken as initial structures for MD simulations (Figures S6–S9),[15] after manually breaking the bond between C417/C416 and the anomeric carbon, and rebuilding the epoxide group (see methods). The unreacted epoxide was found to be stable in the active site of HypBA1 with E322 protonated and E338 deprotonated (Figure S6A, S10). The sulphur atom of C417 is closest to C1, being at proper distance and orientation to perform a nucleophilic attack (Figure S6C). This explains the formation of the C1-S bond in the covalent complex. The simulations also show that the protonated acid/base side chain is very mobile (Figure S7). Due to the positioning of the epoxide oxygen away from the normal glycosidic oxygen position, the acid/base does not interact with it directly. Instead, it is likely that epoxide protonation by E322 is mediated by a water molecule.

Analysis of the Michaelis complex for *Bt*GH146 reveals a different scenario. The reconstructed Michaelis complex for *Bt*GH146 interacting with **1** showed that this complex is generally unstable unless both E320 and E339 were protonated (Figure S11). While it is unlikely that protonation of both of these groups would occur at pH 7, this type of binding, in an unstrained E_3 conformation,[16,17] shows how this unexpected binding mode favours reactivity with C6 over C1; the sulphur atom of C416 is closer to C6 than to C1 throughout the simulation (Figure S6B,C). Thus, we believe that the observed covalent *Bt*GH146:**1** complex is derived from an atypical binding mode, in which the epoxide binds across the C416-Zn-E339 axis priming C6 for attack by the cysteine nucleophile. We speculate that this is made possible by the lack of steric bulk in the inhibitor structure since any extension from the glycosidic or epoxide oxygen would clash with H264 and E320 in the observed binding position, and thus the epoxide reacts "erroneously".

In the study of the mechanism of $Zn(Cys)_3(Glu)$-dependent glycoside hydrolases, the absence of a covalent intermediate mimic had previously precluded informed computational studies on the controversial deglycosylation step of the reaction. Enabled by the observed complex between **1** and HypBA1, we simulated the deglycosylation step of the enzyme reaction mechanism. We reconstructed the natural tGEI by replacing the CHOH group at C6 of the covalently bound inhibitor (Figure 1C) with an oxygen atom and then equilibrated the complex with MD and QM/MM MD simulations. A large QM region, including the Zn^{2+} cation and its coordination shell was described with density functional theory (DFT), using the Becke–Lee–Yang–Parr (BLYP) functional,[18,19] previously used in the study of Zn^{2+}-containing GHs,[20] whereas the MM region was described with the Amber force field[21] (further details in Supporting Information). The simulations show that the complex is stable and, most interestingly, the active site accommodates a water molecule bridging the anomeric carbon and the side chain of the (deprotonated) acid/base residue, in a suitable orientation for deglycosylating nucleophilic attack (tGEI in Figure 2A). This is an indication that the cyclophellitol-labelled complex is a *bona fide* mimic of the covalent intermediate of the chemical reaction.

The deglycosylation reaction was modelled using the metadynamics approach, starting from a snap-shot of the equilibration QM/MM MD simulation. The reaction was driven from the tGEI to the product of the reaction (β-L-arabinofuranose) using one collective variable (CV), which combines the main bonds formed and cleaved during the reaction. The CV was defined as the difference of $C1\text{-}S_{Cys}$ and $C1\text{-}O_w$ distances (O_w is the oxygen atom of the nucleophilic water). Analysis of the free energy profile of the simulated reaction provides an atomistic picture of the HypBA1 catalytic mechanism. The nucleophilic attack of the water molecule takes place via a dissociative transition state, in

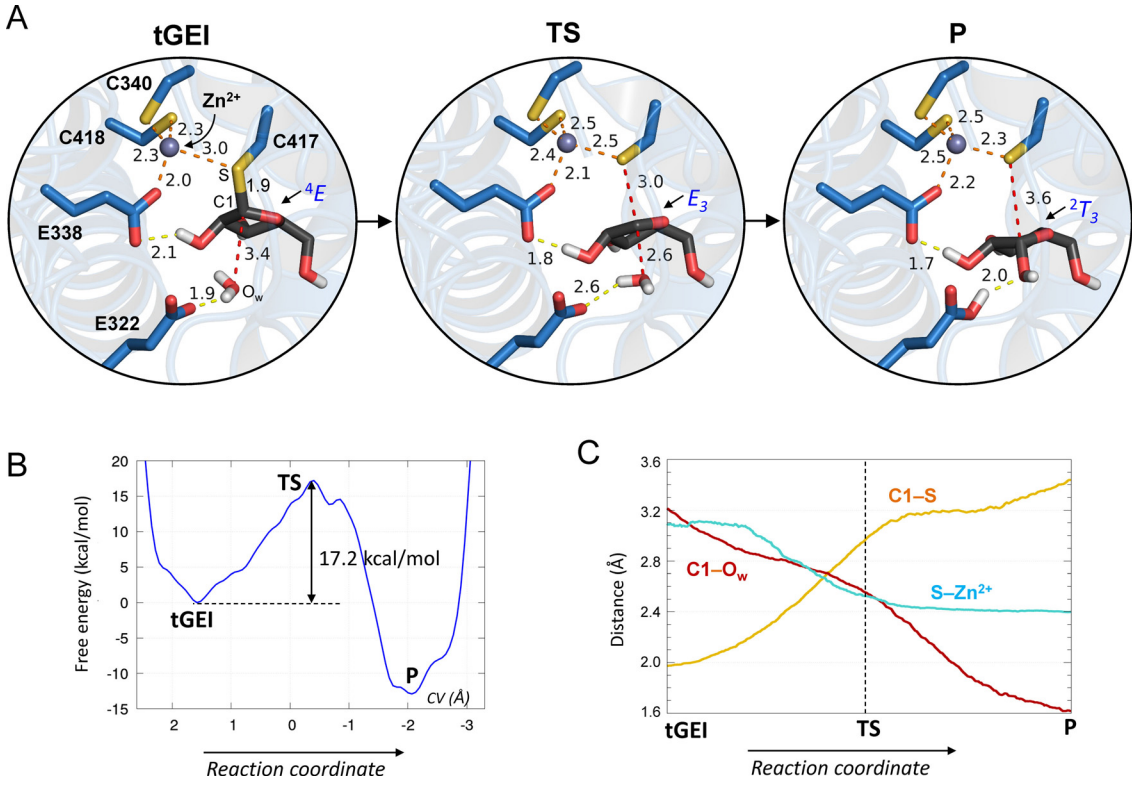


Figure 2. Deglycosylation reaction in HypBA1. A) Active site configuration of the thioglycosyl enzyme intermediate (tGEI), the reaction transition state (TS) and the reaction products (P) obtained from QM/MM metadynamics simulations informed by the X-ray structures obtained in this work. B) Reaction free energy profile. C) Evolution of relevant distances along the reaction coordinate.

which the C1-S bond is significantly elongated (changing from 1.91 Å at the tGEI to 2.96 Å at the TS; Figure 2A and C) relative to the nucleophilic attack distance (the C1-O_w distance shortening from 3.40 Å at the tGEI to 2.56 Å at the TS). The TS shows a clear oxocarbenium ion-like character: the C1-O5 distance of the β-L-arabinofuranose shrinks from 1.44 Å at the tGEI to 1.31 Å at the TS, and the anomeric charge increases by 0.78 e^-. The β-L-arabinofuranose ring changes conformation during the reaction, following a $^4E \rightarrow [E_3]^{\ddagger} \rightarrow {}^2T_3$ itinerary, in excellent agreement with the conformations observed in the product complex of HypBA1 (PDB 3WKX, see also Figure S5). The computed reaction free-energy profile (Figure 2B) exhibits a single transition state, indicative of a concerted mechanism, with a free energy barrier (17.2 kcal mol^{-1}) consistent with that estimated from the experimental rate constant (≈16.4 kcal mol^{-1}, estimated using the Eyring-Polanyi equation[22] computed with the k_{cat} measured for the reaction with *p*-nitrophenyl-β-Araf,[23] under the assumption that deglycosylation is the rate-limiting step in this reaction). This barrier is also comparable to the 15.5 kcal mol^{-1} barrier calculated for the hydrolysis of the canonical glycosyl-enzyme intermediate of *E. coli* LacZ β-galactosidase using QM/MM methods.[24] Sensibly, the computed reaction is exergonic, that is, the products of the reaction are more stable than the tGEI.

Analysis of the evolution of the C1-S and Zn^{2+}-S distances along the reaction coordinate (Figure 2C) reveals an inverse relationship, reflecting the assistance of the metal cation during the cleavage of the C1-S bond. At the tGEI, C417 is weakly coordinated to the metal cation (Zn^{2+}-S = 3.03 Å). However, the Zn^{2+}-S distance shortens as soon as the C1-S distance starts to elongate until C417 recovers its lowest energy coordination distance (Figure S12). The Zn^{2+}-S distance in the simulated product complex (2.4 Å) is in excellent agreement with the one observed in the X-ray structure of the product complex (2.4 Å, PDB 3WKX). Our results suggest that the presence of the metal ion significantly decreases the nucleophilicity of C417, simultaneously ensuring that the C1-S bond can be efficiently hydrolysed. Altogether, the simulations support the role of C417 as the nucleophile of the reaction catalyzed by HypBA1 and demonstrate that participation of the sulphur nucleophile in the $Zn(Cys)_3(Glu)$ coordination complex is essential for glycoside hydrolysis. They furthermore demonstrate the value of covalent species obtained from cyclophellitol-labeling experiments as starting points to model the reactivity of glycosyl enzyme intermediates.

In summary, here we have presented the first known inhibitor of a β-L-arabinofuranosidase and shown that it covalently modifies GH127 and GH146 active sites to form a tGEI mimic. Structural analysis of prepared complexes revealed an unexpected flexibility in binding mode giving rise to two different reacted structures, which both support the proposed identity of C417 (HypBA1) or C416 (*Bt*GH146), part of the $Zn(Cys)_3(Glu)$ coordination complex, as the catalytic nucleophile. Reconstruction of the tGEI from the coordinates of the complex between HypBA1 and its cyclophellitol-derived inhibitor facilitated analysis of the cleavage

mechanism of the C–S bond within the tGEI. QM/MM metadynamics simulations illuminate the finely tuned energetics of this active site, revealing that the Zn^{2+}-S interaction significantly destabilises the C–S bond, facilitating the attack of water onto C1 and driving the reaction towards a lower energy product state.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: arabinofuranosidase · cyclophellitol · glycoside hydrolase · metalloenzyme · zinc

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