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β -L-Arabinofurano-cyclitol Aziridines Are Covalent Broad-Spectrum Inhibitors and Activity-Based Probes for Retaining β -L-Arabinofuranosidases

Valentina Borlandelli, Wendy Offen, Olga Moroz, Alba Nin-Hill, Nicholas McGregor, Lars Binkhorst, Akihiro Ishiwata, Zachary Armstrong, Marta Artola, Carme Rovira, Gideon J. Davies,* and Herman S. Overkleeft*

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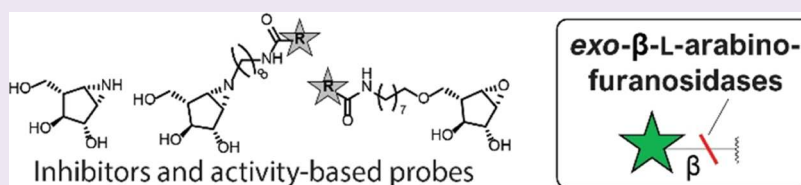
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ABSTRACT: GH127 and GH146 microorganismal retaining β -L-arabinofuranosidases, expressed by human gut microbiomes, feature an atypical catalytic domain and an unusual mechanism of action. We recently reported that both *Bacteroides thetaiotaomicron* BtGH146 and *Bifidobacterium longum* HypBA1 are inhibited by β -L-arabinofuranosyl cyclophellitol epoxide, supporting the action of a zinc-coordinated cysteine as a catalytic nucleophile, where in most retaining GH families, an aspartate or glutamate is employed. This work presents a panel of β -L-arabinofuranosyl cyclophellitol epoxides and aziridines as mechanism-based BtGH146/HypBA1 inhibitors and activity-based probes. The β -L-arabinofuranosyl cyclophellitol aziridines both inhibit and label β -L-arabinofuranosidase efficiently (however with different activities), whereas the epoxide-derived probes favor BtGH146 over HypBA1. These findings are accompanied by X-ray structural analysis of the unmodified β -L-arabinofuranosyl cyclophellitol aziridine in complex with both isozymes, which were shown to react by nucleophilic opening of the aziridine, at the pseudoanomeric carbon, by the active site cysteine nucleophile to form a stable thioether bond. Altogether, our activity-based probes may serve as chemical tools for the detection and identification of low-abundance β -L-arabinofuranosidases in complex biological samples.

β -L-Arabinofuranosidases hydrolyze β -L-arabinofuranosyl units from arabino-oligosaccharides (AOS), complex pectic dietary glycans,¹ and plant-derived glycoproteins such as solanaceous lectins.^{2,3} As is the case for retaining α -L-arabinofuranosidases,^{4–8} known β -L-arabinofuranosidases have been identified in the proteome of prokaryotic micro-organismal species colonizing the human gut¹ including the gut commensal bacteria *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*. Gut microbiomes express several carbohydrate-active enzymes (CAZymes)⁹ for the utilization of specific plant-sourced polysaccharide substrates, which act as nutrients for bacteria and fungi in the gastrointestinal tract.

β -L-Arabinofuranosidases are encoded in AOS utilization genes by several *Bifidobacterium* species,¹⁰ whereas some *Bacteroides* gut microbial species express these enzymes specifically for pectin degradation.¹ Two representative examples of gut microbiome β -L-arabinofuranosidases and the subject of the herein-presented studies are HypBA1 (classified within GH127 and expressed by *B. longum*) and BtGH146 (GH146, expressed by *B. thetaiotaomicron*). We here show that tagged β -L-arabinofuranosyl cyclophellitol aziridines

are versatile reagents for studying HypBA1 and BtGH146 by activity-based protein profiling.

Biochemical and structural studies on HypBA1 established the retaining activity of this GH,¹¹ which was found to be capable of hydrolyzing terminal β -L-arabinofuranosides with retention of the configuration at the anomeric center. Crystallographic evidence for the distance (4.9 Å)¹² between two carboxylates (E332 and E338) initially supported a classical two-carboxylate mechanism of action. However, the presence of a coordination system made of three cysteines and one glutamate centered around a zinc cation within the catalytic domain of HypBA1^{12,14} suggested alternative mechanisms to be in play. Site-directed mutagenesis¹¹ and crystallographic^{12,14} experiments revealed the presence of a

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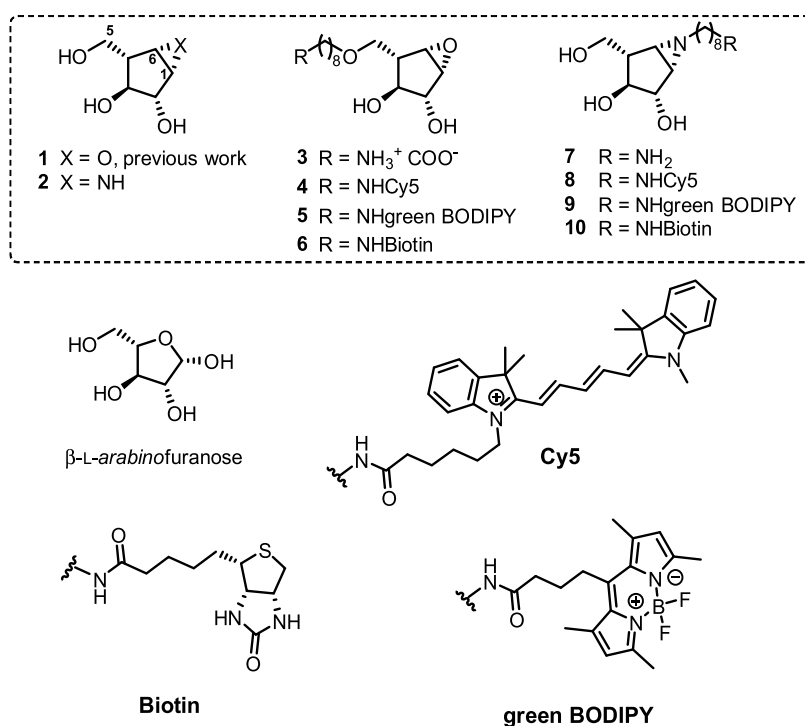


Figure 1. Chemical structures of β -L-arabinofuranose, β -L-arabinofuranosyl cyclophellitol epoxides (3–6) and aziridines (2 and 7–10) presented in this work and structure of unmodified β -L-arabinofuranosyl cyclophellitol epoxide **1**¹⁵ as reported previously.

cysteine nucleophile, rather than a glutamate or aspartate as in canonical glycosidases.

By making use of the mechanism-based inhibitor, β -L-arabinofuranosyl cyclophellitol epoxide¹⁵ (**1**, Figure 1), we recently obtained structural and biochemical evidence for the nature of the nucleophilic residue in HypBA1 and BtGH146, which in both cases turned out to be a cysteine (C417 in HypBA1, C416 in BtGH146) as the nucleophile. The crystal structure of **1** in complex with BtGH146 revealed an unexpected regiochemical reactivity: the catalytic nucleophile C416 appeared to have formed a covalent bond with the pseudoanomeric carbon (that is, C6 in **1**), instead of the expected carbon^{16–20} corresponding to the anomeric center of the parent substrate (C1 in **1**), while the HypBA1 catalytic nucleophilic cysteine reacted at the carbon mimicking the anomeric center (C1). This difference in the regiochemical outcome for the enzymatic reaction with **1** by the two enzymes was attributed to the slow inactivation rate of the epoxide scaffold and to structural differences between the active sites of the two enzymes.

To study the reactivity of HypBA1 and BtGH146 toward cyclophellitol aziridines and with the aim to generate activity-based probes with which gut microbiome β -L-arabinofuranosidase activity can be profiled, we decided to prepare β -L-arabinofuranose-configured cyclophellitol aziridine (**2**) and equip this with various reporters to give a series of potential β -L-arabinofuranosidase ABPs (**8–10**, Figure 1). These ABPs are accompanied by the synthesis of O5-modified epoxide probes **4–6**. Our studies, which include *in silico* conformational behavior of the epoxide and aziridine scaffolds, structural studies of both enzymes reacted with the nontagged mechanism-based inhibitors, enzyme inhibition kinetic studies, and *in vitro* ABPP experiments, reveal that both enzymes react in the expected fashion with the aziridines (aziridine opening

at C1) and that the aziridines are valid ABPs that label both GH families with about equal efficiency.

RESULTS AND DISCUSSION

Conformational Analysis. In the first instance, the conformational free energy profile of the β -L-arabinofuranosyl cyclitol aziridine (**2**) was computed with *ab initio* metadynamics (Figure 2B) and compared to the ones previously reported for β -L-arabinofuranosyl cyclophellitol epoxide **1** and for the natural substrate β -L-arabinofuranose. As illustrated in Figure 2B, the conformational behavior of aziridine **2** is similar to the epoxide (**1**). Both molecules adopt preferably a conformation closely resembling *E*₃. More energy is needed to twist the conformation of **1** and **2** (free energy = 4.7 and 5.7 kcal/mol, for epoxide and aziridine, respectively) compared to the natural β -L-arabinofuranose (free energy = ca. 2.6 kcal/mol).

Synthesis. The synthesis of both epoxide-based and aziridine-based β -L-arabinofuranosyl cyclitols **2–10** involved the installation of the electrophilic trap prior to incorporation of the linker (Scheme 1) and diverged for the two types of warheads at the stage of L-arabinofuranosyl cyclopentene intermediate **11**. Starting from commercially available α -D-galactopyranoside, bisbenzylated L-arabinofuranosyl cyclopentene **11** was obtained in nine steps following the synthetic strategy recently reported.²¹ L-Arabinofuranosyl cyclopentene **11** was transformed into the aziridine following a set of stereospecific reactions previously applied to six-membered ring cyclitol structures.^{22–24} Specifically, compound **11** was subjected to trichloroimidation of the unprotected primary hydroxyl functionality followed by stereospecific NIS-induced iodocyclization. The resulting iodotrichloroimidate **12** was hydrolyzed under mild acidic conditions to generate *in situ* the unprotected iodoammonium salt, which under mild basic conditions and micromolar concentrations underwent one-pot

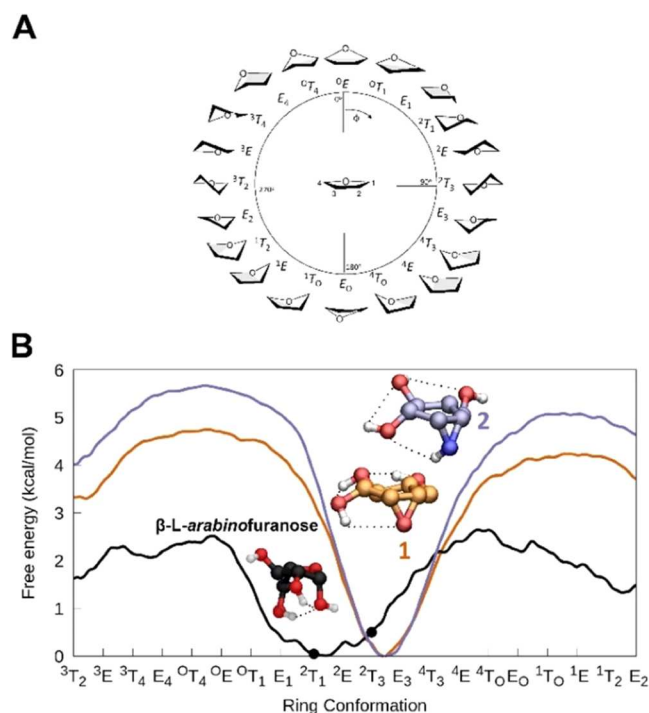


Figure 2. (A) Graphical representation of the conformations of a 5-membered ring according to the Cremer–Pople angle Φ . (B) Conformational free energy profile of isolated β -L-arabinofuranose (black line), compound 1 (orange line) as previously published,¹⁵ and compound 2 (blue line). Conformations observed in product complexes of β -L-arabinofuranosidases are represented with a black circle (PDB entry 3WKX for HypBA1; PDB entry SOPJ for BtGH146).

intramolecular nucleophilic displacement by the primary amine of the adjacent iodine leaving group, affording aziridine 13.

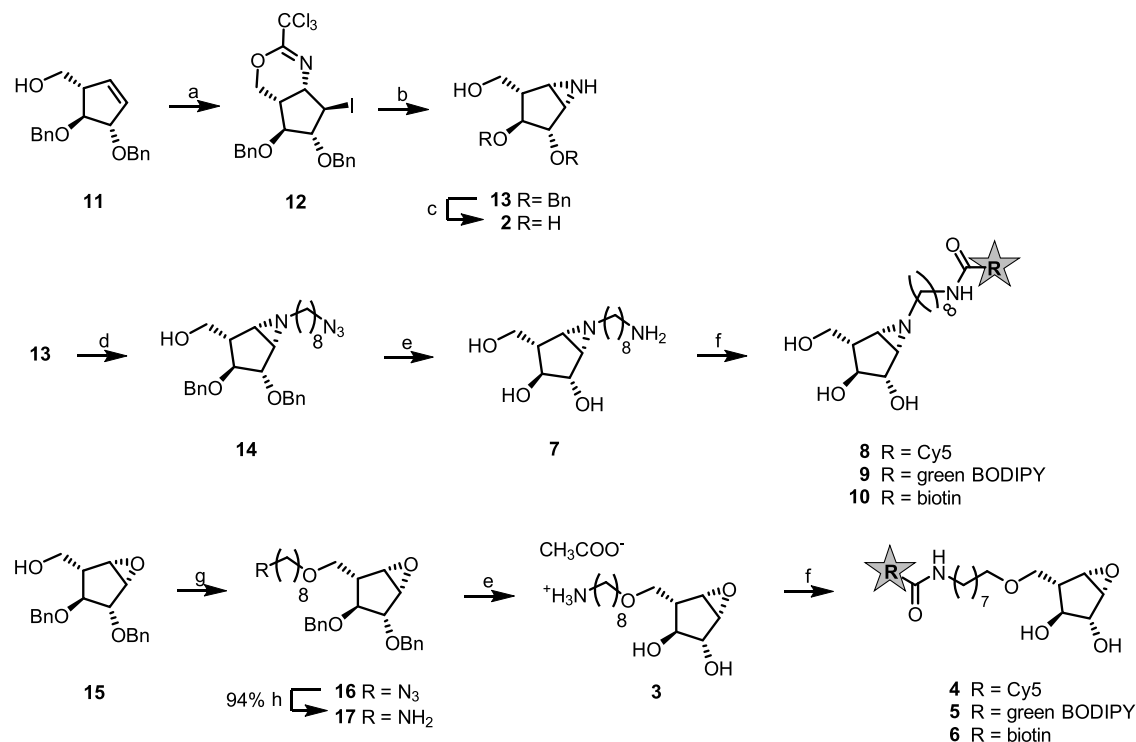
With bisbenzylated aziridine 13 available, this intermediate was globally deprotected under Birch conditions generating the final β -L-arabinofuranosyl cyclophellitol aziridine 2, the aziridine equivalent to unmodified epoxide 1.¹⁵ Unsubstituted secondary aziridine 13 was modified by *N*-alkylation with freshly prepared 8-azido-octyl-1-*O*-triflate.²³ This step was followed by Birch reduction of *N*-alkyl aziridine 14, which was converted into the water-soluble primary amine 7 by azide reduction and concomitant debenzoylation. Probes 8–10 were obtained upon formation of an amide linkage between amine 3 and the desired reporter carboxylic acid prior to activation with pentafluorophenyl-trifluoroacetate (Pfp-TFA). The final compounds were purified by HPLC, with the exception of green BODIPY probe 9, whose relative lipophilicity allowed purification by silica gel flash chromatography.

The synthesis of epoxides 4–6 commenced from late-stage epoxide 15 obtained according to the published procedures.¹⁵ The azido-octyl linker was introduced on the primary hydroxyl functionality by *O*-alkylation with 8-azido-octyl-1-*O*-triflate²³ under basic conditions, generating azide 16, which was reduced to primary amine 17 by Staudinger reduction. Reductive cleavage of the benzyl protecting groups in 17 under Birch conditions afforded primary amine 3, which served both as a prospective inhibitor and as a starting point for the synthesis of ABPs 4–6. Following the same protocol used for the installation of reporters onto aziridine warhead 7, ABPs 4–6 were obtained.

Inhibition Assays. To assess the effects of the two different electrophilic traps on the inhibitory kinetics of β -L-arabinofuranoside-mimicking scaffolds against retaining β -L-arabinofuranosidases, the inhibitory activity of newly developed inhibitors 2 and 7 and probe 4 was assessed and compared with the findings reported for unmodified epoxide 1. Inhibition kinetics were measured for recombinant HypBA1 and BtGH146 at their optimal pH for catalysis (pH 4.5 for rHypBA1 and pH 7.5 for rBtGH146), using hydrolysis of chromogenic *p*-nitrophenyl β -L-arabinofuranoside²⁵ as a measure of residual enzymatic activity (Table 1).

Aziridine 2 displayed the highest inhibitory efficacy toward both rHypBA1 and rBtGH146, indicating the broad-spectrum reactivity of β -L-arabinofuranosyl cyclophellitol aziridines toward β -L-arabinofuranosidases. The different degrees of inhibition rates of 2 with rHypBA1 (GH127) and rBtGH146 may be attributed to structural differences of their binding sites but also to the different pH values at which these enzymes act optimally (and at which the inhibition assays were executed) in relation to the pK_{aH} of the aziridine. This pK_{aH} is expected to be around pH 7.4, as aziridines in general are at about three points lower pK_{aH} compared to nonconstrained aliphatic secondary amines. Also, the presence of three electronegative hydroxyls appended to the pentane ring will lower the pK_{aH} . Therefore, the expected aziridine pK_{aH} likely lies within the physiological pH range (6.0–7.5)²⁶ of the human intestine. The intestinal tract represents the predominant colonization site for *B. thetaiotaomicron* and *Bifidobacterium*, which are reported to optimally grow *in vitro* at pH 6.5–7.0.^{27,28} Recently, in growing *B. longum* cultures, a two-unit pH reduction from the typical growth conditions (pH 6.5–7.0) for *Bifidobacterium* was reported upon supplementation of fructooligosaccharide (FOS) in various sugar systems, as a function of FOS concentration and bacterial strain.²⁸ This growth habit of *B. longum* suggests that the optimal pH for HypBA1 enzymatic activity may be achieved by the addition of FOS or equivalent nutrients in bacterial growth medium. Irrespective of this hypothesis, the enhanced rate of inhibition of HypBA1 over BtGH146 in the study presented here (Table 1) may also be due to the greater degree of protonation (and so activation) of the aziridine, already outside the enzyme active site, under the significantly more acidic conditions at which HypBA1 (pH optimum of 4.5) activity is measured than that of BtGH146 (pH optimum of 7.5).

To complement the kinetic assessment, epoxides 3–6 and aziridines 2 and 7 were analyzed by intact mass spectrometry (Supporting Figure S2) following incubation with rHypBA1 and rBtGH146. Covalent adduct formation was observed for all tested compounds after 16 h of incubation, and a covalent adduct was detected with aziridines 2 and 7 after 1 h for rHypBA1. rHypBA1 thus reacted faster with β -L-arabinofuranosyl cyclitol aziridines compared with the epoxides. For aziridines 2 and 7, intact MS analysis upon incubation with rHypBA1 resulted in an observed mass difference (+146.2 for 2 and +273.8 for 7) differing by one unit from the expected values (+145 for 2 and +272 for 7). The HypBA1 binding cleft differs from that of BtGH146 in having a nonconserved cysteine residue (C415) positioned in proximity to the catalytic nucleophile C417. The reported mass difference for rHypBA1 might account for a change in the protonation state of this additional nonconserved thiol, which may undergo protonation under the test conditions. An alternative hypothesis is that one of the noncovalently modified conserved

Scheme 1. Synthesis of 2–10^a

^aReagents and conditions: (a) i. CCl₃CN, DBU, DCM, 0 °C → rt, 3 h; ii. NIS, CHCl₃, 0 °C → rt, 17 h, and quant. over two steps. (b) i. HCl in CH₃OH, DCM/CH₃OH, and 6 h and ii. Amberlite IRA-67, 1 day; and 63% over 2 steps. (c) Na(s), NH₃, THF/^tBuOH, -60 °C, 45 min, and 23%; (d) 8-azido-octyl-1-*O*-triflate,²³ DIPEA, DCM, -15 °C → rt, 20 h, and 44%; (e) Na(s), NH₃, THF/^tBuOH, -60 °C, 45 min, 3: 84%, and 7: 77%; (f) reporter-COOH, Pfp-TFA, DMF; then 3 or 7, DIPEA, DMF, 24 h. 4: 7%; 5: 41%; 6: 23%; 8: 8%; 9: 45%; and 10: 8%; (g) 8-azido-octyl-1-*O*-triflate,²³ NaH, THF, -15 °C → rt, 28 h, and 84%; and (h) polymer-bound PPh₃, AcCN/H₂O, 70 °C, 5h, and 94%.

Table 1. rHypBA1 and rBtGH146 Inactivation Rates and Inhibition Constants (k_{inact} and K_{I}) of β -L-Arabinofuranosyl compounds 1, 2, 4, and 7

compound	rBtGH146			rHypBA1		
	k_{inact} (min ⁻¹)	K_{I} (μ M)	$k_{\text{inact}}/K_{\text{I}}$ (M ⁻¹ ·s ⁻¹)	k_{inact} (min ⁻¹)	K_{I} (μ M)	$k_{\text{inact}}/K_{\text{I}}$ (M ⁻¹ ·s ⁻¹)
1 ¹⁵	n.d. ^a	n.d. ^a	<0.5	n.d. ^a	n.d. ^a	14.7
2	79 ± 6	350 ± 40	62	>1	n.d. ^b	>4200
4	3.2 × 10 ⁻² ± 0.3 × 10 ⁻²	140 ± 10	3.8	n.d. ^a	n.d. ^a	<0.1
7	8.3 ± 0.4	190 ± 20	12	1.3 ± 0.3	280 ± 90	76

^an.d.: not determined due to the limited inhibition observed. ^bn.d.: not determined due to the rapid and complete inactivation observed with the lowest concentration of inhibitor tested under the experimental conditions of the assay. To ensure the validity of the pseudo-first-order inhibition kinetic assumption, the lowest concentration of the inhibitor tested was maintained as a ~ 50-fold excess of inhibitor over enzyme.

cysteines (C418, C340) may lose coordination to the zinc cation, as a result of protein denaturation during the intact mass spectrometry analysis.

Ligand–Enzyme Interactions by 2 with rHypBA1 and rBtGH146. In order to gain structural insights into the reactivity of aziridine 2 with rHypBA1 and rBtGH146, it was cocrystallized with both enzymes, and the resulting complexes were studied by X-ray diffraction. Electron density for the carbon emulating the anomeric center (C1) in aziridine 2 was observed at covalent bond distance from the catalytic nucleophile residue in both isozymes (C416 for rBtGH146 in Figure 3A; C417 for rHypBA1 in Figure 3B). Inspection of the network of H-bond interactions by 2 in complex with rHypBA1 and rBtGH146 revealed that the primary hydroxyl O5 and the transdiaxially opened aziridine group interact differently with the active sites of the two isozymes. In particular, the primary hydroxyl O5 in 2 was involved in H-

bond interactions with distinct H-bond acceptor residues within the binding pocket—these are E217 in rBtGH146:2 (Figure 3C) and H142 in rHypBA1:2 (Figure 3D). The amino group of the opened aziridine in 2 displayed H-bond interactions with the thiol group of nonconserved cysteine C415 in rHypBA1:2. However, no cysteine equivalent to C415 of rHypBA1 is present in the active site of rBtGH146. In the rBtGH146:2 complex, N1 of 2 interacts exclusively with a proximal glutamine (Q689) in the active site. The presence of a nonconserved cysteine in rHypBA1, which interacts with the amino moiety of the opened aziridine, may explain the superior reactivity of 2 with rHypBA1 over rBtGH146.

The covalent complex of rBtGH146 with aziridine 2 displayed the anomeric-mimicking carbon C1 at covalent bond distance to the thiol group of C416. This points to the expected regiochemical outcome as for conventional retaining glycosidases^{29–33} and provides further evidence supporting

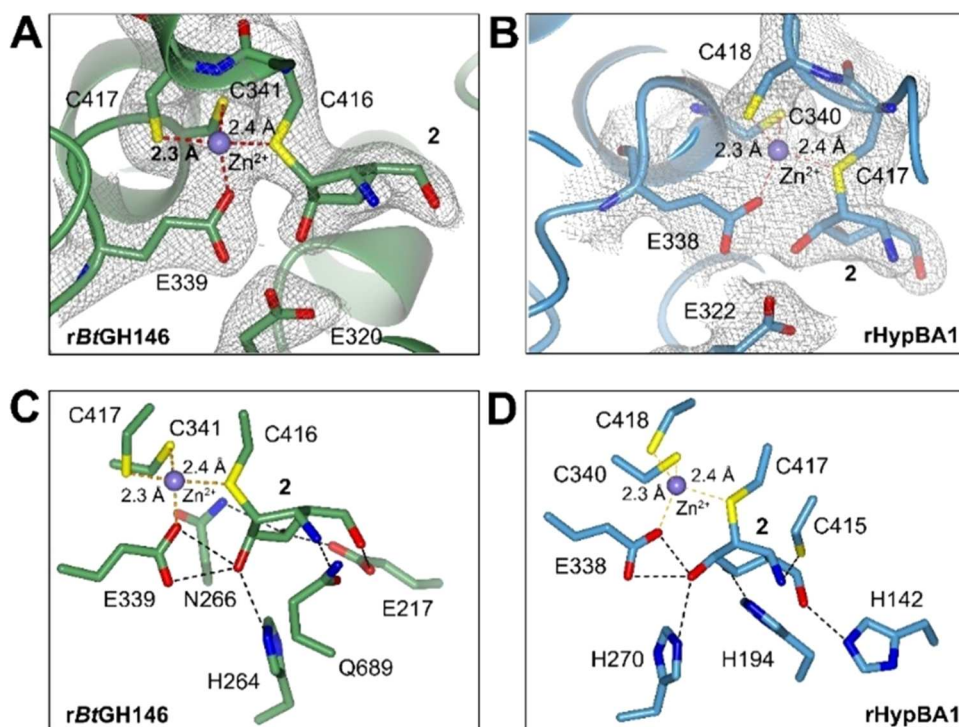


Figure 3. (A) Active site structure of rBtGH146 upon reaction with **2** revealing electron density indicative of a covalent bond between C1 and C416. The $2F_o - F_c$ map is shown contoured to 1σ for key residues, and bonds coordinating the zinc ion are depicted as dashed orange lines. (Please see notes on residue numbering for rBtGH146 in the Supporting Information.) (B) Active site structure of rHypBA1 bound to **2**, with density calculated for **2** and C417, and coordination shown as in panel A. (C) Active site structure of rBtGH146 upon reaction with **2** showing hydrogen bond interactions between **2** and the active site of rBtGH146, with hydrogen bonds shown as black dashed lines and coordination to the zinc ion as dashed orange lines with distances in angstroms. (D) Active site structure of rHypBA1 bound to **2**, with hydrogen bonds and coordination shown as in panel C.

annotation of this enzyme, like rHypBA1, as a retaining β -L-arabinofuranosidase utilizing a cysteine catalytic site nucleophile. However, there is a significant difference in the mode of action toward aziridine **2** and epoxide **1**. When combining the inhibitory kinetic findings with the predicted *in silico* conformational analysis, we conclude that the observed differences in inactivation kinetics and regiochemical outcomes between **1** and **2** with rBtGH146 cannot be ascribed to their conformational behavior. Finally and as also discussed above (enzyme inhibition assay section), the difference in reactivity may also originate in the pK_{aH} values of the respective inhibitors, in combination with the enzyme pH optimum.

Activity-Based Protein Profiling. We next investigated the labeling efficiency of both recombinant isozymes (Figure 4A) for Cy5 aziridine **8** and Cy5 epoxide ABP **4**. For this purpose, samples of the recombinant enzyme were treated with either of the probes at varying concentrations and times, after which the samples were denatured and resolved on SDS-PAGE and the ABP-labeled proteins visualized by fluorescence scanning of the wet gel slabs. In agreement with the difference in kinetic parameters determined for aziridine **7** and epoxide **4**, ABP **8** labeled both tested isozymes more rapidly and more potently than ABP **4**. ABP **4** labeled rBtGH146 effectively after 4 h of ABP incubation, whereas no equivalent band was detected for rHypBA1 (74 kDa) within the tested incubation time window. These results are in accordance with the *in vitro* inhibition assessments. As shown in Figure 4B, complete fluorescent labeling of rBtGH146 (92 kDa) can be achieved both by Cy5 ABP **8** and green BODIPY ABP **9** upon 4 h of ABP incubation at $1 \mu\text{M}$ ABP, and fluorescence bands could be

detected after as little as 3 min of incubation with either ABPs by increasing the ABP concentration 10-fold. In contrast, no significant fluorescence band corresponding to the expected mass of rBtGH146 (92 kDa) was observed after 4 h of incubation with epoxide ABPs **4** or **5** at $10 \mu\text{M}$ concentration using lower amounts than 1000 ng of enzyme. However, increasing the ABP concentration by 1 order of magnitude ($100 \mu\text{M}$) afforded an approximately 10-fold decrease in the detection limit (100 ng rBtGH146). Green BODIPY-tagged aziridine **9** ($1 \mu\text{M}$ ABP) enabled fluorescence detection of rBtGH146 down to 100 ng of enzyme (Figure 4D), that is, 10-fold lower enzyme amounts than with an epoxide ABP. This points to the utility of aziridine-type chemical tools for profiling of retaining β -L-arabinofuranosidases, where no selectivity for either GH127 or GH146 isozymes needs to be applied. In contrast, β -L-arabinofuranosyl-configured epoxide-type probes were found to react preferentially with rBtGH146, which may be due to the different reactivity (or pK_{aH}) of the epoxide versus that of the aziridine but also because of the position of the reporter tag (O5 versus aziridine).

CONCLUSIONS

This work reports on the synthesis and *in vitro* biochemical evaluation of a panel of β -L-arabinofuranosyl cyclophellitol epoxides and aziridines as covalent inhibitors and activity-based probes for retaining β -L-arabinofuranosidases from GH families 127 and 146. Development of these chemical scaffolds builds on the recent discovery¹⁵ of the enzymatic mechanism adopted by rBtGH146 and rHypBA1, as elucidated by the use of β -L-arabinofuranosyl cyclophellitol epoxide **1**. Building on

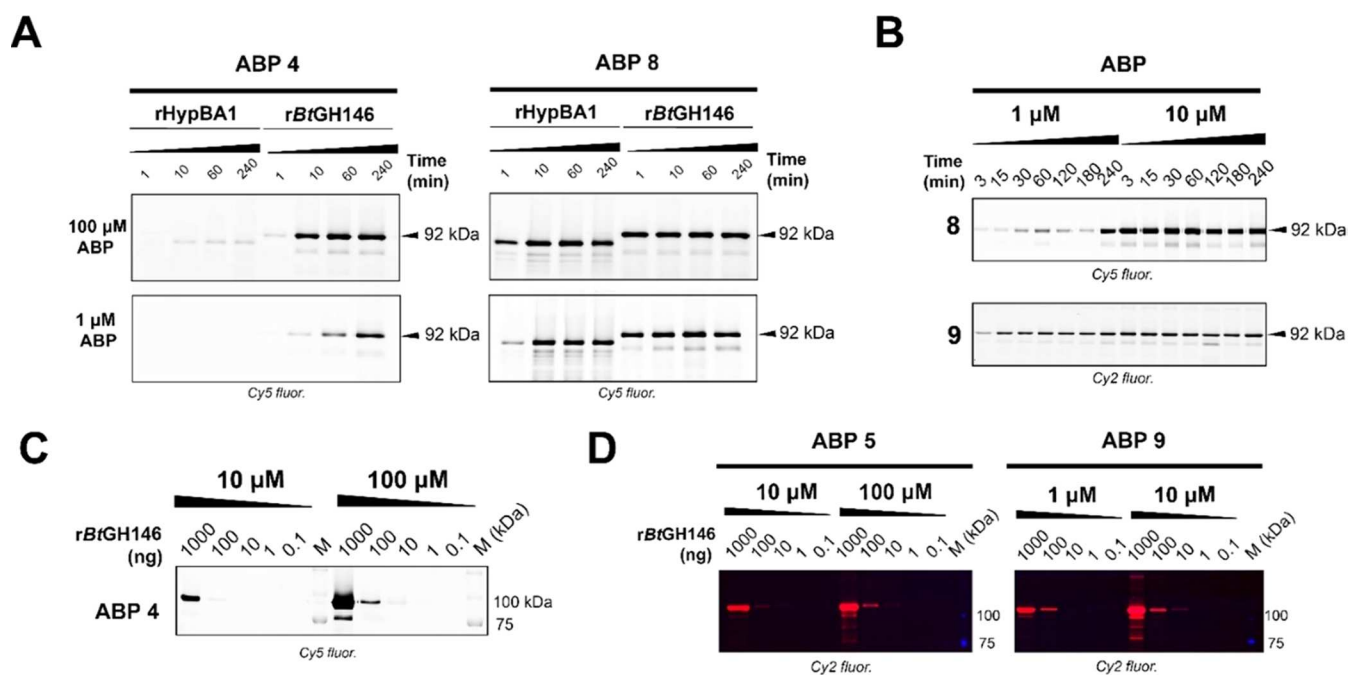


Figure 4. (A) Time-dependent fluorescence labeling of rHypBA1 and rBtGH146 by epoxide-armed Cy5-tagged ABP 4 (left) or aziridine-armed Cy5-tagged ABP 8 (right) at 1 or 100 μM final ABP concentration during 4 h of incubation at optimal enzyme pH (rHypBA1: pH 4.5 NaOAc, 1 mM DTT; rBtGH146: pH 7.0 NaPi, 1 mM DTT), showing a faster and more effective labeling of both rHypBA1 and rBtGH146 by 8 compared to epoxide-based ABP 4 within the tested time window. (B) Fluorescence labeling of rBtGH146 (100 ng) by Cy5-tagged ABP 8 (above) and green Bodipy-tagged ABP 9 (below) after 1 h of incubation at pH 7.4 (PBS/NaCl) and 1 or 10 μM final probe concentration, with ABP 9 displaying faster and more effective labeling of the tested enzyme than 8 at 1 μM ABP concentration. (C) Detection sensitivity of Cy5-tagged ABP 4 toward rBtGH146 after 1 h of incubation at pH 7.5 (HEPES/NaCl), with 10 and 100 μM final probe concentrations. (D) Detection sensitivity of green Bodipy-tagged epoxide-type ABP 5 and aziridine-type ABP 9 toward rBtGH146 after 1 h of incubation (with 5: at pH 7.5 HEPES/NaCl; with 9: pH 7.4 PBS/NaCl), showing the lower rBtGH146 detection limit of aziridine-type probe 9 with respect to 5 under the tested conditions. We note the presence of a lower running (kDa of about 75) band in the rBtGH146 labeling experiments. While we have no evidence, we hypothesize that these bands may be partially proteolyzed (by the expression host, *E. coli*) yet still active enzyme, considering that the full-length enzyme has a floppy, proteolysis-prone lid domain.

the mechanism of action inferred from the previous crystallographic studies conducted with epoxide 1 reacted with rBtGH146 and rHypBA1,¹⁵ the regiochemical outcome of the catalytic reactions of both enzymes with aziridine 2 was explored by 3D crystallographic studies. We found that aziridine 2 reacts at the conventional position, that is, the carbon equivalent to the anomeric center in β -L-arabinofuranoside, with rBtGH146 as well as rHypBA1. This result contrasts with our previous results on epoxide 1, which when reacted with rBtGH146 was found to ring-open at the “wrong” epoxide carbon. We believe the enhanced reactivity of the aziridines, when compared to epoxide 1, may be behind these findings, and while we have no satisfactory explanation for this finding, we do believe that the BtGH146 reaction with the aziridines better reflects its mode of action on natural substrates. Our gel-based ABPP experiments further show the utility of our β -L-arabinofuranosyl cyclophellitol aziridines as *bonafide* β -L-arabinofuranosidase probes and form, in our opinion, a valuable addition to the growing activity-based glycosidase probe portfolio for the discovery of such enzymes in an unbiased fashion from various biological sources.

METHODS

Chemical Synthesis. All chemicals were purchased from Sigma-Aldrich unless otherwise specified. L-Arabinocyclopentene 11²¹ and bisbenzylated β -L-arabinofuranosyl epoxide 15¹⁵ and were synthesized at the Bio-organic Synthesis, Leiden Institute of Chemistry at Leiden University, according to the published methods. Synthetic methods

and NMR characterization of newly synthesized compounds can be found in the [Supporting Information](#).

Recombinant Enzyme Production. BtGH146 and HypBA1 were produced and purified according to the published procedures.^{13,15}

Intact Mass Spectrometry. rBtGH146 was diluted to 1 mg mL⁻¹ in an SEC buffer. Compounds 2–7 (0.1 mM final concentration) were added to the enzyme solution, and the reactions were incubated at 37 °C for different time periods. 2 μL samples were taken after 1 or 16 h. These samples (2 μL) were then diluted with 48 μL of 1% formic acid and 10% acetonitrile and analyzed as described previously. rHypBA1 was diluted to 0.1 mg mL⁻¹ in 50 mM NaPi (pH 4.5), and compounds 2–7 (0.1 mM final concentration) were added. 10 μL of samples was taken after 60 and 960 min. These aliquots (10 μL) were diluted with 10 μL of 1% formic acid and 10% acetonitrile and stored at -20 °C until intact MS could be performed in the same manner as for the samples treated with rBtGH146.

Enzyme Inhibition Kinetics. rHypBA1 (20 mg mL⁻¹ stored at -80 °C) was freshly thawed and diluted to 1 mg mL⁻¹ in 50 mM acetate (pH 4.5) supplemented with 1 mM DTT (assay buffer). Inhibitors 2, 4, and 7 were dissolved in water at 5 mM concentration and used to prepare a dilution series from 1 mM to 16 μM in the assay buffer alongside a buffer control without an inhibitor. A working enzyme solution was prepared at 20 $\mu\text{g}/\text{mL}$ in assay buffer. 35 μL of enzyme solution was added to 35 μL of prewarmed inhibitor solution, and the inhibition reactions were incubated at 37 °C. Aliquots (7.5 μL) of these inactivation mixtures were removed at time intervals (5, 10, 20, 30, 40, and 60 min of incubation) and diluted with 142.5 μL of prewarmed 0.25 mM 4-nitrophenyl- β -L-arabinofuranoside²⁵ in the assay buffer and then incubated at 37 °C. Aliquots (40 μL) of the resulting samples were taken from each substrate hydrolysis reaction

at defined time intervals (1, 3, and 8 min of incubation) and mixed with 40 μL of stop solution (200 μM Na_2CO_3) in a 384-well plate. Absorbance change at 405 nm wavelength (A405) was read by using an Epoch plate reader (Biotek). Hydrolysis rates were determined as the slope of a linear fit of A405 versus time. Slope values were converted into rates using a 40 μL calibration series of 4-nitrophenol in assay buffer mixed with 40 μL of stop solution. To account for slow enzyme activity loss in the no-inhibitor control, rates were converted into residual activity by dividing each measured rate by the inhibitor-free hydrolysis rate at that incubation point. Residual activities were then plotted against incubation time using OriginPro graphing software (OriginLab) for each inhibitor concentration and fitted with exponential decay curves having y offset (y_0) values fixed at 0 (with the exception of the uninhibited rates where $y_0 = 1$). Extracted apparent decay constant (k_{app}) values were then plotted against the concentration of compounds **2**, **4**, and **7** in each inhibition reaction with error estimates taken as the standard error from the exponential decay fit. Since no inflection was observed in the k_{app} vs $[I]$ curve, an error-weighted linear fit was performed to determine k_{inact}/K_i .

rBtGH146 (20 mg mL^{-1} stored at -80°C) was freshly thawed and diluted to 1 mg mL^{-1} in 50 mM NaPi at pH 7.0 with the assay buffer (1 mM DTT, 20 mM HEPES (pH 7.5) supplemented with 200 mM NaCl). Inhibitors **2**, **4**, and **7** were dissolved in water at 5 mM concentration and used to prepare a dilution series from 1 mM to 16 μM in the assay buffer alongside a buffer control without an inhibitor. A working enzyme solution was prepared at 20 $\mu\text{g/mL}$ in assay buffer. The subsequent steps were the same as those for the procedure described for HypBA1 mentioned above.

ABP Labeling of rHypBA1 and rBtGH146 by 4. *rHypBA1* and *rBtGH146* were labeled under optimum conditions. ABP **4** (10 mM stock, 100% w/v DMSO) was diluted with Milli-Q water to prepare a 1 mM ABP working solution (10% w/v). The enzyme stock (HypBA1:20 mg mL^{-1} HypBA1 in 20 mM MOPS, pH 7.0, 1 mM DTT; *BtGH146*:1 mg mL^{-1} in 20 mM MOPS, pH 7.5) was thawed on ice. The assay buffer for *rHypBA1* was prepared by mixing 500 μL of 1 M NaOAc, 50 μL of 1 M DTT, and 9.45 mL of deionized water. For *BtGH146* labeling, the assay buffer (pH 7.0, NaPi, 1 mM DTT) was prepared with deionized water. The enzyme stock (10 μL) was diluted to 0.01 mg mL^{-1} enzyme concentration with the assay buffer (990 μL assay buffer). Aliquots (100 μL) of the enzyme working solution were heated to 95°C for 5 min to inactivate the enzyme solution. To both the working and inactivated enzyme solutions (90 μL each) was added the ABP working solution (**4**, 10 μL) to achieve a final ABP concentration of 0.1 mM. The samples were incubated at 37°C . At regular time intervals (0, 10, 60, 240 min), 15 μL samples were taken and mixed with 5 μL of 4 \times SDS-PAGE loading buffer (40% glycerol, 4% SDS, 250 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 0.2 mg of mL^{-1} bromophenol blue). The samples were immediately heated to 95°C for 2 min and then stored frozen (-20°C) until ready for analysis. Wet slab gels were scanned for fluorescence using a Typhoon FLA 9500 (GE Healthcare) at λ_{EX} 635 nm and $\lambda_{\text{EM}} \geq 665$ nm for Cy5-tagged ABP **4**.

ABP Labeling of rBtGH146 by 4, 5, or 9. To prepare for labeling, recombinant *BtGH146* stock (1 mg mL^{-1} in 20 mM MOPS pH 7.5) was diluted with assay buffer (for ABPs **4**: 20 mM HEPES 150 mM NaCl, pH 7.5; for ABPs **5** and **9**: PBS 150 mM NaCl, pH 7.4) to varying enzyme concentrations (111, 11, 1, 0.1, and 0.01 $\mu\text{g/mL}$). ABP **4**, **5**, and **9** stocks (10 mM in DMSO) were diluted with assay buffer to 1 mM and 0.1 mM ABP concentrations (for **4** and **5**) or to 100 μM and 10 μM (for ABP **9**). For labeling, 9 μL of each of the enzyme working solutions was loaded in separate Eppendorf tubes on ice to yield a total final amount of 1000, 100, 10, 1, and 0.1 ng of *BtGH146* per reaction. The enzyme solution was thus incubated with 1 μL of ABP (**4**, **5**, or **9**) at 37°C for 1 h (Figure 4B and 4C). Samples were then denatured with 2.66 μL of sample buffer (5 \times Laemmli buffer, containing 50% (v/v) 1 M Tris-HCl pH 6.8, 50% (v/v) glycerol, 10% (w/v) dithiothreitol (DTT), 10% (w/v) sodium dodecyl sulfate (SDS), 0.01% bromophenol blue) and heated at 98°C for 5 min. Proteins were resolved by electrophoresis in sodium dodecyl sulfate (SDS-PAGE) 10% polyacrylamide gels, and wet slab

gels were scanned as described above. Wet slab gels were scanned for fluorescence using a Typhoon FLA 9500 (GE Healthcare) at $\lambda_{\text{EX}} = 635$ and $\lambda_{\text{EM}} \geq 665$ nm for Cy5-tagged ABP **4** and **9** and at $\lambda_{\text{EX}} = 473$ and $\lambda_{\text{EM}} \geq 510$ nm for green BODIPY-tagged ABP **5**.

Time-Dependent Labeling of rBtGH146 by 8. Recombinant *BtGH146* stock (1 mg mL^{-1} in 20 mM MOPS pH 7.5) was diluted with assay buffer (PBS 150 mM NaCl, pH 7.4), giving a 11 $\mu\text{g/mL}$ working enzyme solution. Cy5-tagged ABP **8** (10 mM in DMSO) was diluted with the assay buffer to 100 and 10 μM ABP concentrations. For labeling, 9 μL of enzyme working solution (100 ng of total *BtGH146* in the final reaction mixture) was loaded in separate Eppendorf tubes on ice, and 1 μL of ABP working solution was added to each tube. Reactions were incubated at 37°C while shaking. At time intervals (3, 15, 30, 60, 120, 180, 240 min), samples were denatured with 5 \times Laemmli buffer, boiled at 98°C for 5 min, and subjected to SDS-PAGE and fluorescence scan as described above.

Crystallization of rHypBA1 or rBtGH146 and Soaking with 2. Information on the crystallization of the purified enzymes and soaking of the crystals with inhibitor **2** can be found in the Supporting Information, together with the procedures used for X-ray data collection and structure solution.^{34–47}

Conformational Free Energy Landscape of 2 in Vacuum. The conformational free energy landscape (FEL) was computed for β -*L*-arabinofuranose cyclophellitol aziridine (**2**) using density functional theory-based molecular dynamics (MD), according to the Car–Parrinello (CP) method.⁴⁸ The molecule was enclosed in an isolated cubic box of 12.5 \AA^3 . A fictitious electron mass of 500 atomic units (au) was used for the CP Lagrangian, and a time step of 0.12 fs was used in all CPMD simulations. This is the same setup used in previous work on β - and α -*L*-arabinofuranose inhibitors.^{15,21} The Kohn–Sham orbitals were expanded in a plane wave basis set with a kinetic energy cutoff of 70 Ry. Ab initio pseudopotentials, generated within the Troullier–Martins scheme,⁴⁹ were employed. The Perdew, Burke, and Ernzerhoff (PBE)-generalized gradient-corrected approximation⁵⁰ was selected. The metadynamics algorithm,⁵¹ provided by the Plumed 2 plugin,⁵² was used to explore the conformational FEL of the systems, taking as collective variables (CVs) the pseudorotational-phase (φ) puckering coordinate⁵³ as well as a dihedral angle accounting for the rotation of the sugar hydroxymethyl group. The energy was projected into the φ coordinate for representation purposes. The height of these Gaussian terms was set at 0.6 kcal/mol, and a new Gaussian-like potential was added every 500 MD steps. To ensure convergence at the last 100 ps, the Gaussian height was decreased to 0.2 kcal/mol and the pace was set to 1000 MD steps. The widths of the CVs were set to 0.035 and 0.1 rad for φ and the hydroxymethyl dihedral angle, respectively, according to the oscillations of the CVs in the free dynamics. The simulations were stopped when energy differences among wells remain constant and a diffusive behavior was observed in both CVs, which was further confirmed by a time-independent free energy estimator.⁵⁴ The energy error, taken from the standard deviation within the last 30 ps, is below 0.6 kcal/mol. The exploration of the phase space was extended up to 300 ps, which corresponds to 4000 added Gaussian functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.3c00558>.

Synthetic procedures, characterization of compounds, biochemical inhibition data, intact mass spectrometry, ^1H and ^{13}C NMR spectra for all newly synthesized compounds, X-ray data processing, and refinement statistics (PDF)

Accession Codes

Coordinates and structure factors have been deposited with the Protein Data Bank under the accession numbers 8QF2 and 8QF8.

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V.B., L.B., A.I., and M.A. synthesized all compounds; A.N.-H. and C.R. performed computational studies; V.B., N.M., and Z.A. performed enzyme inhibition studies and enzyme labeling experiments; O.M., W.O., N.M., and Z.A. performed structural studies; V.B., N.M., G.J.D., and H.S.O. wrote the article; and G.J.D. and H.S.O. supervised the work. All authors have given approval to the final version of the article.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Bt, *Bacteroides thetaiotaomicron*; GH, glycosyl hydrolase; RG-II, rhamnogalacturonan II; FOS, fructooligosaccharide

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