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# Exploiting $sp^2$-hybridization in the development of potent 1,5-α-L-arabinanase inhibitors

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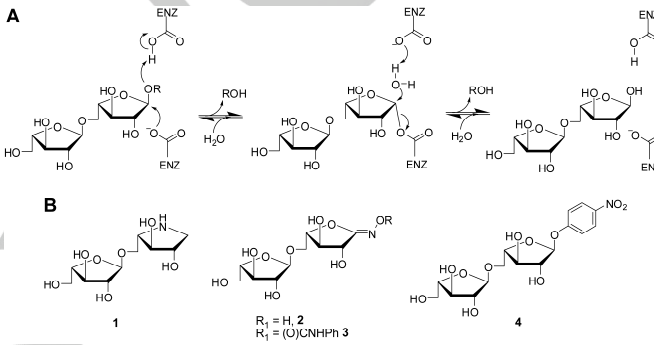
Dedication ((optional))

The synthesis of potent inhibitors of GH93 arabinanases as well as a synthesis of a chromogenic substrate to measure GH93 arabinanase activity is described. An insight into the reasons behind the potency of the inhibitors was gained through X-ray crystallographic analysis using the arabinanase Arb93A from *Fusarium graminearum*. These compounds lay a foundation for future inhibitor development as well as for the use of the chromogenic substrate in biochemical studies of GH93 arabinanases.

The monosaccharide L-arabinose is one of the fundamental carbohydrate building blocks of plant cell walls. The abundance of this material, which is usually found as α-L-arabinofuranosyl moieties, varies in different plant biomolecules but it is most commonly found as part of the structures of arabinans, arabinoxylans, and arabinogalactans.[1-5] The natural role of these large biomolecules, in concert with other plant-cell wall components, is to provide strength to the cell and a physical barrier against the external environment.[6]

Recent studies have suggested plant biomass as a source of materials for renewable biofuel production and chemical feedstocks.[7] However, efficient processing of the biomass is inhibited by L-arabinose containing materials and therefore these must first be broken down so that full utilisation can occur.[8-10] One method that is used in nature to break down oligo- and polysaccharides is enzymatic degradation through the action of glycoside hydrolases (GH).[11,12] It is not surprising then that enzymes that process L-arabinose and more specifically α-L-arabinofuranoside glycosidic linkages are of growing interest to industry.[13] These enzymes, termed α-L-arabinofuranosidases, display a range of activities and substrate specificities and have been classified, using the CAZy classification system, into GH families 3, 43, 51, 54, 62 and 93.[14] A specific subset of α-L-arabinofuranosidases, termed arabinanases, acts to cleave the linear portion of arabinan, made up of repeating 1,5-α-L-arabinofuranosyl units, into smaller pieces. Arabinanases are found in GH43 and GH93 with the enzymes from GH43 acting with an *endo*-mode of action[15-17] while the arabinanases from GH93 act to cleave discrete arabinobiose units from the non-reducing end of the arabinan polymer (Scheme 1A).[18-21]




**Scheme 1.** (A) GH93 arabinanases use a retaining catalytic mechanism involving a glycosyl-enzyme intermediate such that the overall reaction proceeds with net retention of stereochemistry (R = repeating 1,5-α-L-arabinofuranosyl units). (B) Structure of the known inhibitor of GH93 arabinanases **1** and structures of the corresponding hydroximolactones **2** and **3**, as well as the putative substrate **4**[22,23], relevant to the work described here.

A great deal of information about the function of glycoside hydrolases, in terms of substrate recognition and catalytic mechanism, has come from the use of chemically synthesized small molecule substrates and inhibitors in concert with X-ray crystallography.[24,25] With regards to GH93 arabinanases, studies have revealed a retaining catalytic mechanism since the stereochemistry at the anomeric centre is retained in the product (Scheme 1A). Little attention has been given to developing inhibitors for GH93 arabinanases, even though understanding the biochemistry of these enzymes is critical to their potential use in the biofuel production and chemical feedstock industries.[26] Most development of competitive inhibitors of glycoside hydrolases centres on preparing compounds that either mimic the charge or shape of the putative oxocarbenium ion-like transition state of the enzyme-catalysed reaction.[24] In terms of charge-based mimics, the iminosugar **1** (Scheme 1B) has been prepared and found to be a potent competitive inhibitor of Arb93A ($K_i$ = 3.0 μM), a known GH93 arabinanase.[26] From these as well as other biochemical studies,[19-21] it has been suggested that the conformational itinerary for the enzyme-catalysed reaction may involve an $E_3$ transition state placing the itinerary in the northern hemisphere of the L-furanose pseudorotational wheel.[27,28]

For inhibitors that mimic shape, efforts have been mainly focused on developing compounds for glycoside hydrolases that

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act on glycopyranosides. These compounds usually possess a $sp^2$-hybridised anomeric carbon that acts to flatten the pyranose ring into a conformation that mimics the shape of the ring at the transition state of the enzyme-catalysed reaction.[29] Some of the best inhibitors that have this synthon present are PUGNAc[30,31] and its derivatives,[32-36] which act on retaining β-*N*-acetylglucosaminidases. To date however, little effort has been made toward developing this type of compound as inhibitors[37] not only for arabinanases but for glycoside hydrolases that process furanosyl glycosides in general[38] with only some efforts towards inhibitors of nucleoside hydrolases being conducted.[39] With regards to substrates and methods to specifically study GH93 arabinanase activity, these have been limited to degradation assays that take advantage of reducing ends of fragment products,[21] or separation of products using chromatographic[40,41] or gel electrophoresis methods.[19,42]

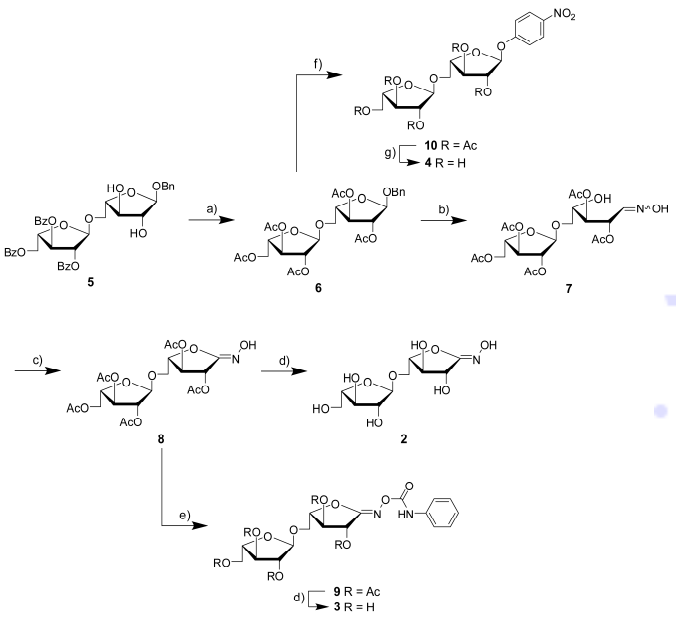
With these points in mind we felt, based on the putative conformational itinerary analysis that has been described, that molecules bearing an $sp^2$-hybridized anomeric carbon such as in **2** and **3** may act as potent inhibitors of GH93 arabinanases. In addition, by utilizing the chemistry employed for the synthesis of **2** and **3**, the development of a more efficient preparation of the 4-nitrophenyl glycoside **4**, prepared previously using a chemoenzymatic[23] and chemical methods,[22] might yield a new substrate for measuring GH93 arabinanase activity directly. A more robust synthesis of **4** would make it easier to use and amenable to further biochemical studies of these enzymes due to the easy readout with molecules of this type.[25]

To that end the disaccharide **5**,[26] prepared using modified literature procedures,[43] was converted into the pentaacetate **6** by first removing the benzoyl protecting groups and then in situ acetylation (Scheme 2). Removal of the benzyl protecting group from **6** followed by treatment with hydroxylamine hydrochloride gave the presumed oxime **7** as a mixture of E and Z isomers in good yield. Treatment of **7** with DBU and NCS allowed for oxidative ring closure to give **8**. The configuration of the oxime **8** was expected to be Z, as this is known to be the preferred configuration for hydroximolactones.[37] Removal of the acetyl groups by treatment with saturated ammonia in methanol proceeded smoothly and provided the 1,4-hydroximolactone **2**.

For the preparation of **3**, the pentaacetate **8** was treated with phenylisocyanate, providing **9** in good yield. Subsequent removal of the acetyl protecting groups using a similar method as for the preparation of **2** gave the phenyl carbamate **3** in excellent yield. To prepare the putative substrate **4**, the pentacetate **6** was again used. Removal of the benzyl protecting group followed by treatment with trichloroacetonitrile gave an *in situ* trichloroacetimidate donor which was then treated with 4-nitrophenol under glycosylation conditions to yield the glycoside **10**. Removal of the acetyl groups using sodium methoxide in methanol provided the disaccharide **4** which had spectral properties consistent to that found in the literature.[23] The overall yield of **4** (65% from **6**) makes this method far more useful in preparing this compound compared to earlier preparations which require enzymes or are synthetically less tractable.[22,23]

Next we evaluated the inhibitors **2** and **3** against Arb93A from *F. graminearum*, using the known gel electrophoresis method, PACE[19,42] and found them both to be competitive inhibitors with a $K_i$ value of 66 ± 4 μM for **2** and the phenyl carbamate **3** being more potent with a $K_i$ value of 14 ± 0.5 μM.

With this knowledge in hand we then evaluated the disaccharide **4** as a substrate for Arb93A. We found **4** to indeed be a substrate for the enzyme and obtained kinetic parameters of Arb93A toward this compound of $k_{cat}$ = 3.9 ± 0.08 x $10^3$ $s^{-1}$, $K_M$ = 2.2 ± 0.15 mM and $k_{cat}/K_M$ = 1.8 ± 0.16 x $10^3$ $s^{-1}$ $mM^{-1}$. In addition, we also obtained $K_i$ values for **2** and **3** of 72 ± 3 μM and 12 ± 2 μM respectively using **4** as a substrate. These results demonstrate that the chromogenic substrate **4** is very suitable for use as a tool to detect GH93 arabinanase activity.




**Scheme 2.** a) i) NaOMe, MeOH; ii) $Ac_2O$, pyr.; b) i) $H_2$, Pd/C, MeOH, EtOAc; ii) $NH_2OH.HCl$, pyr., MeOH; c) NCS, DBU, $CH_2Cl_2$; d) $NH_3$/MeOH; e) PhNCO, $Et_3N$, $CH_2Cl_2$; f) i) $H_2$, Pd/C, MeOH, EtOAc; ii) $Cl_3CCN$, $K_2CO_3$, $CH_2Cl_2$; iii) 4-nitrophenol, $BF_3.OEt_2$, 4A MS, $CH_2Cl_2$; g) NaOMe, MeOH.

To our knowledge, **2** and **3** are the first rationally designed inhibitors of GH93 arabinanases that utilize shape as their mode of inhibition. The greater potency of **3** compared to **2** is in correlation with what has been observed for other hydroximolactones and their associated carbamates.[44] In addition, the potency of **3** was similar to that observed for the iminosugar **1**.[26]

To gain a more detailed understanding of the molecular basis for the inhibition of Arb93A, co-crystallization trials with **2** and **3** were attempted. Trials to obtain Arb93A in complex with **3** were unsuccessful but we determined the crystal structure of Arb93A in complex with **2** at 2 Å resolution. Initial inspection of the electron density maps showed clearly the arabinose moiety whilst the one for the hydroximolactone was limited but became unambiguous during the refinement process (Figure 1). The interactions made by the arabinosyl moiety in the -2 subsite are similar to those described previously.[19,26] The L-arabinohydroximo-1,4-lactone moiety is found in the -1 subsite where it also makes interactions with amino acid sidechains. The O2 hydroxyl is hydrogen bonded to Glu170 and Gln195 and the O3 hydroxyl to Gln195 and Arg223. The ring oxygen makes a weak interaction (3.14 Å) with the hydroxyl of Tyr337 and is found at 2.77 Å from the nucleophile, Glu170. Finally, the acid/base Glu242 interacts directly with both of the hydroximo atoms (2.55 Å for the nitrogen and 2.81 Å for the hydroxyl) whilst a water mediated interaction with the hydroxyl group is also observed. As previously found for the pyrrolidine **1**, the

hydroximolactone moiety in the -1 subsite presents a $^4T_O$ twist conformation (See Supporting Figure).[26]

Coplanarity of the O4-C1-C2 atoms seems to be a prerequisite for inhibitors of Arb93A and $sp^2$-hybridization of the anomeric carbon is a good way to obtain this desirable geometry. The close contact between the nucleophile Glu170 and the ring oxygen could explain the reduced potency of **2** which are compensated for in **3** presumably by additional interactions with the protein such as hydrophobic interactions with its phenyl ring and hydrogen bonds with the atoms of the carbamate, which have been observed in X-ray crystallographic studies of β-*N*-acetylglucosaminidases when using PUGNAc.[45,46]

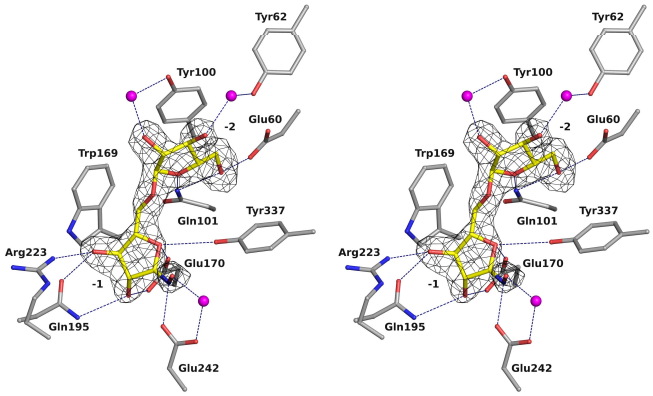



**Figure 1.** a) Electron density for compound **2** bound to Arb93A. $2F_{obs}–F_{calc}$ electron density displayed at 1 sigma (0.35 eA˚3). Interactions are represented by blue dashed lines and water molecules as spheres.

In conclusion, we have developed new tools to study L-arabinanases. The hydroximolactone **2** and the phenyl carbamate **3** are potent inhibitors of Arb93A, an arabinanase from *F. graminearum*. In addition, the development of a more robust synthesis of the 4-nitrophenyl glycoside **4** was achieved and the compound has been shown to be the first chromogenic substrate for measuring GH93 arabinanase activity. Collectively, the results obtained here suggest that inhibitors of this type, that mimic the shape of the ring at the transition state, have a place in the development of rational inhibitors of not only arabinanases but also α-L-arabinofuranosidases in general. Overall, these inhibitors and further derivatives, such as those which exhibit both shape and charge-based characteristics could be useful tools to study these types of enzymes in various biological settings. Moreover, the substrate **4**, with its easy use and readout, has the potential to not only be used to measure potency of GH93 arabinanase inhibitors but could also be used as a substrate in future biochemical studies of these enzymes. It could as well become a tool for gaining insights into enzyme robustness and substrate turnover in directed evolution experiments. Increased turnover and robustness of these enzymes may indeed allow for more efficient biomass processing and have direct implications in the use of these enzymes in the biofuel production and chemical feedstock industries.

## Acknowledgements

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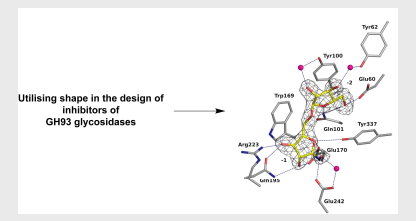
Layout 1:

# COMMUNICATION

Development of tools to study GH93 arabinanases, important enzymes in the biofuel production and livestock industries, are described.

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