ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 9684–9686

www.rsc.org/chemcomm

COMMUNICATION

α-L-Arabinofuranosylated pyrrolidines as arabinanase inhibitors†‡

Ethan D. Goddard-Borger,§[a] Raphael Carapito,¶[b] Jean-Marc Jeltsch,[b] Vincent Phalip,[b] Robert V. Stick[a] and Annabelle Varrot*[c]

Received 20th June 2011, Accepted 5th July 2011
DOI: 10.1039/c1cc13675e

As part of continued efforts to understand the mechanisms of 1,5-α-L-arabinanases better, some arabinan-like iminosugar oligosaccharides were synthesized. An iminosugar analogue of arabinobiose was found to be a good inhibitor of the arabinanase Arb93A from *Fusarium graminearum*. Structures were determined for complexes of this inhibitor with wild-type Arb93A and a catalytically inactive mutant.

Plant arabinan, a component of pectin, is a polysaccharide possessing a backbone of repeating (1→5)-α-L-arabinofuranosyl units, known as linear arabinan, adorned with branching α-(1→2)- and α-(1→3)-linked L-arabinofuranosides. The saccharification of arabinan requires the co-operative action of many α-L-arabinofuranosidases, hailing from glycoside hydrolase (GH) families 3, 43, 51, 54, 62 and 93,[1,2] as defined by the Carbohydrate Active enZyme (CAZy) database (available at http://www.cazy.org/).[3] Glycoside hydrolases from families 43 and 93 are arabinanases that hydrolyze linear arabinan into smaller oligosaccharides. GH43 enzymes present an *endo* mode of action,[2] whilst GH93 enzymes are *exo*-hydrolases that liberate arabinobiose from the non-reducing end of linear arabinan.[4,5] These enzymes, like the many others recruited by various organisms to degrade plant cell-wall polysaccharides, are of great interest to those seeking to create renewable chemical commodities from plant biomass.

Enzyme-mediated hydrolysis of glycosidic bonds proceeds with either a net inversion or retention of the substrate's anomeric configuration.[6] GH43 arabinanases are well established as inverting hydrolases.[7] GH93 arabinanases are thought to be retaining hydrolases—an assignment that was initially made on the basis of transglycosylation experiments because rapid mutarotation of the α-arabinobiose product precluded assignment by ^{1}H NMR experiments.[4,8] Retention results from a double displacement mechanism where a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed *via* oxacarbenium-ion-like transition states. Two catalytic residues are usually involved, with one acting as an acid/base residue and the other as a nucleophile. Structures have been determined for the GH93 arabinanases Arb93A from *Fusarium graminearum* and Abnx from *Penicillium chrysogenum* 31B in complex with arabinobiose. They reveal two carboxylate residues that are ideally positioned to function as a catalytic acid/base and a nucleophile (Glu170 and Glu242 in Arb93A, respectively), thereby supporting the assignment of these enzymes as retaining hydrolases.[9] On the basis of this information, subsequent mutagenesis studies confirmed the essential role that these residues play in catalysis.[4,10]

Further studies of the mechanisms employed by arabinanases, particularly those from GH family 93, with regard to conformational itinerary and transition state stabilisation, would be aided by appropriate small molecule inhibitors, just as other inhibitors have helped to delineate the details of other GH mechanisms. Iminosugars have proven to be invaluable tools for the study of the glycoside hydrolase mechanisms, irrespective of debate as to whether or not they are true transition state mimics.[11–14] Simple iminosugars are relatively poor inhibitors of glycanases, as they occupy very little of these enzymes' large, catalytically active, substrate-binding cleft.[15] However, glycosylated iminosugars occupy much more of the substrate-binding site and have a greater affinity for the enzyme of interest.[15–17] To make such tools available for continuing mechanistic studies of arabinanases, compounds **1** and **2**, the deoxyiminosugar equivalents of arabinobiose and arabinotriose, respectively, were synthesised and evaluated as arabinanase inhibitors (Fig. 1).

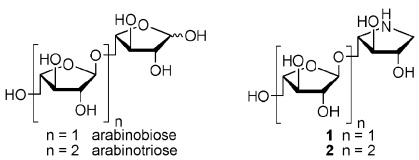


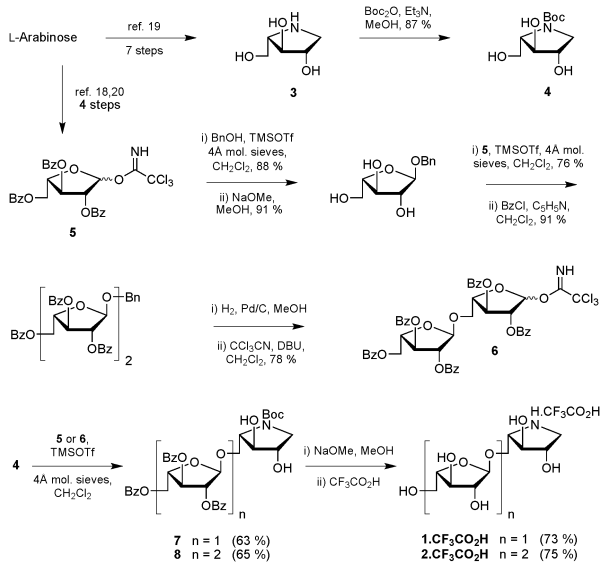
Fig. 1 Arabinan-like oligosaccharides and their corresponding iminosugar analogues.

[a] *School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia. E-mail: rvs@cyllene.uwa.edu.au*
[b] *Institut de recherche de l'Ecole de biotechnologie de Strasbourg, Boulevard Sébastien Brandt-BP 10413, 67412 ILLKIRCH Cedex, France. E-mail: phalip@unistra.fr; Fax: +33 368855330; Tel: +33 368854820*
[c] *CERMAV-CNRS, affiliated with Université Joseph Fourier and ICMG, BP 53, 38041 Grenoble cedex 9, France. E-mail: varrot@cermav.cnrs.fr; Fax: +33 476547203; Tel: +33 476037634*

† Figures were drawn with the PyMOL Molecular Graphics System, Schrödinger, LLC.

‡ Electronic supplementary information (ESI) available: Experimental data and a table of data collection and refinement statistics are provided. See DOI: 10.1039/c1cc13675e

§ Current address: Department of Chemistry, 2036 Main Mall, University of British Columbia, Vancouver, Canada V6T 1Z1.
¶ Current address: Physiopathologie et Médecine Translationnelle.4 Rue Kirschleger, 67085 Strasbourg cedex, France.



Scheme 1 Synthesis of the iminosugar oligosaccharides **1** and **2**.

The syntheses of compounds **1** and **2** were realized by extending a selective glycosylation strategy reported by Du *et al.*,[18] where unprotected arabinoside acceptors were glycosylated preferentially at the C5 primary hydroxyl group. Iminosugar **3** was prepared from L-arabinose in seven steps, as previously described,[19] then converted into the corresponding *tert*-butyl carbamate **4** (Scheme 1). The donor required for the assembly of pseudo-disaccharide **1**, the trichloroacetimidate **5**, was prepared according to literature procedures.[18,20] This imidate was used to synthesise the disaccharide donor **6**, necessary for the assembly of pseudo-trisaccharide **2**, using conventional approaches.

Glycosylation of carbamate **4** using the monosaccharide donor **5** proceeded well, providing the pseudo-disaccharide **7** in good yield (Scheme 1). Structural assignment of **7**, a *tert*-butyl carbamate, was complicated by severe broadening of both the ^{1}H and ^{13}C NMR spectra; acetylation of the free hydroxyl groups and removal of the carbamoyl group were necessary to confirm the stereo- and regio-chemical outcome of the glycosylation reaction (see ESI‡).[21,22] Similarly, glycosylation of carbamate **4** with the disaccharide donor **6** provided the pseudo-trisaccharide **8**, the structure of which was confirmed by chemical modification (see ESI‡).

Deprotection of the pseudo-disaccharide **7** began with a Zemplén transesterification, which presumably gave the corresponding pentol. This product was treated with dilute hydrochloric acid and lyophilized, with the intention that this would remove the carbamoyl protecting group. Unfortunately, this protocol produced a mixture of products, which, by ^{13}C NMR spectroscopy, appeared to contain **1**·**HCl**, L-arabinose and pyrrolidinium chloride **3**·**HCl**. More dilute hydrochloric acid and/or lower temperatures failed to prevent the partial hydrolysis of the pseudo-disaccharide's glycosidic bond. To avoid this problem, anhydrous trifluoroacetic acid was used to remove the carbamoyl group; the pseudo-disaccharide **1** was obtained as the trifluoroacetic acid salt **1**·$\mathbf{CF_3CO_2H}$ in high yield. The pseudo-trisaccharide **8** was deprotected in the same manner as the pseudo-disaccharide **7**; debenzolyation and removal of the carbamoyl protecting group with neat trifluoroacetic acid provided the desired compound as its trifluoroacetic acid salt **2**·$\mathbf{CF_3CO_2H}$. The rapid assembly of compounds **1** and **2** is a fine demonstration of the utility of this selective glycosylation strategy for the assembly of linear arabinan-like oligosaccharides.

The pseudo-disaccharide **1**·$\mathbf{CF_3CO_2H}$ was evaluated as an inhibitor of the GH93 *exo*-1,5-arabinanase Arb93A; it inhibited this enzyme competitively with a K_i of 3.0 ± 0.3 μM. A comparison of this dissociation constant to K_M values previously determined for the natural substrates arabinotriose (3.6 mM), arabinotetraose (9.3 mM), arabinopentaose (4.0 mM), arabinohexaose (2.0 mM) and linear arabinan (0.75 mM) indicates that the inhibitor binds some 200–2500 times better than known substrates, making it a relatively good inhibitor.[4]

To gain some insight into the nature of the interactions between Arb93A and **1**, the 3-D structures of pseudo-disaccharide **1** in complex with the wild-type enzyme and its acid/base mutant E242A were determined at 1.60 Å and 1.85 Å resolution, respectively. The small variations observed among the three molecules of the mutant structure or between the mutant and wild-type structures result from differences in crystal contacts. Examination of the first electron density difference map revealed without ambiguity that compound **1** occupied the −1 and −2 subsites of the enzyme (Fig. 2). As is typical of GHs, complexation of Arb93A with a ligand did not result in any major local or global conformational changes. Very small readjustments of several amino acid side-chains were observed between the complex of arabinobiose and compound **1**, presumably to optimise the hydrogen bonding network of each complex.

The L-arabinosyl moiety of **1** is found within the −2 subsite and makes similar interactions to those previously described for the Arb93A–arabinobiose complex.[4] Within this subsite, C5 is surrounded by several residues, including Tyr62, Tyr100 and Glu60, which permits recognition of the non-reducing end of arabinan and appears to be a determinant of the enzyme's *exo*-hydrolase activity. The iminosugar moiety of **1** occupies the −1 subsite, as expected, and exists in a $^{4}T_{N}$ conformation. The side-chain carboxylate of the catalytic nucleophile

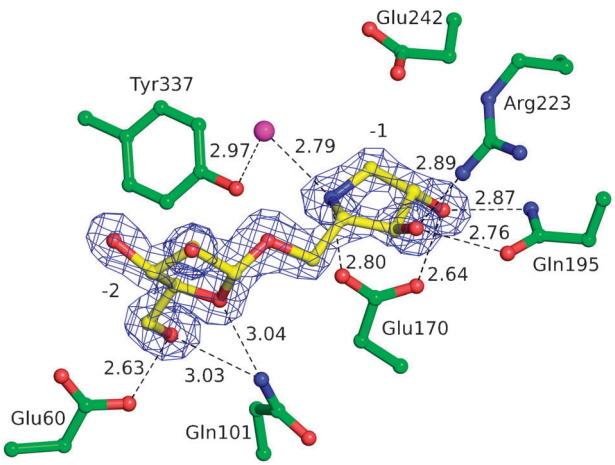


Fig. 2 Electron density for compound **1** bound to wild-type Arb93A. 2Fobs–Fcalc electron density at 0.95 eÅ^{-3}. Interactions are represented by dashed lines.

(Glu170) bridges the pyrrolidine ring, making interactions with both the pyrrolidine nitrogen (N4–OE2, 2.80 Å) and the hydroxyl group at C2 (O2–OE1, 2.64 Å). The pyrrolidine nitrogen also interacts indirectly with the hydroxyl group of Tyr337 by way of a water molecule. The catalytic acid/base residue Glu242 does not interact directly with the inhibitor and its mutation to alanine does not appear to perturb the binding of compound **1** to Arb93A, since the inhibitor's position, orientation and conformation are equivalent to those in the wild-type structure. It is interesting to note that in the mutant complex two water molecules are found in proximity to the positions previously occupied by OE1 and OE2 of the carboxylate side-chain of Glu242. The pyrrolidine likely exists in a protonated state on enzyme, in line with previous structural and kinetic studies of GH iminosugar inhibitors, but since the pK_a of 1,4-dideoxy-1,4-imino-L-arabinitol is 7.6, and crystallisation occurred at pH 7.5, this cannot be stated with certainty.[17,23,24]

The distortion that a pyranoside ring undergoes during electrophilic migration, which proceeds in accordance with Stoddart's pseudo-rotational itinerary, has been demonstrated for a number of GHs.[24,25] Some structural studies of enzyme–iminosugar complexes have shown that these inhibitors can adopt ring conformations similar to those expected at the transition state.[24] Nevertheless, owing to the lack of good mechanistic probes, little is known about the reaction coordinates utilised by *O*-furanosidases, which can be described by the furanose pseudorotational wheel of Altona and Sundaralingam.[26] Thus, it is worth considering the relevance of the present enzyme–inhibitor complex to the conformational itinerary of Arb93A.

The ^4T_N twist conformation of the pyrrolidine (Fig. 3) within the −1 subsite is a low energy conformer on the pseudorotational energy map calculated for α-L-arabinofuranosides that present a *gt* orientation of the *exo*-cyclic hydroxymethyl group.[27] This is similar to the conformation that might be expected for the Michaelis complex, as seen for several retaining GH51 α-L-arabinofuranosidases, such as AbfA from *Geobacillus stearothermophilus* T-6.[28] This conformation places the glycosidic bond in an axial orientation and therefore allows in-line nucleophilic attack at the anomeric centre. In structures of the product complex of both Arb93A and Abnx with arabinobiose the ring conformation of the L-arabinose residue in the −1 subsite is close to an E_3 conformation with the anomeric carbon slightly above the plane.[4,10] The transition state would then be expected to have a southern conformation close to E_3, since it should have significant oxacarbenium ion character, which requires C4–O4–C1–C2 to be close to coplanar.

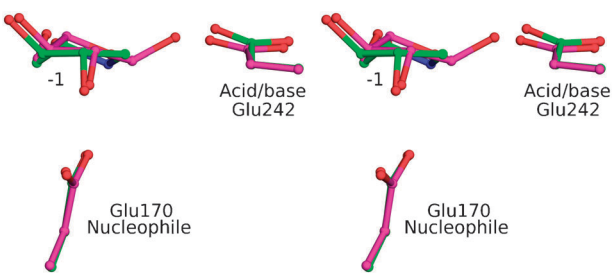


Fig. 3 Overlay of the active site of Arb93A in wall-eye stereo. The structures shown are wild-type Arb93A in complex with arabinobiose (magenta) or compound **1** (green).

The iminosugar oligosaccharides presented here represent the first small molecule arabinanase inhibitors and are an important step towards the development of further tools to delineate the mechanisms by which these enzymes effect catalysis. Other competitive inhibitors comprising at least two L-arabinose units and possessing an amine or sp^2 hybridisation at the anomeric position, as well as mechanism-based inhibitors that lead to an accumulation of the glycosyl-enzyme intermediate, will be important tools in future mechanistic studies of these enzymes.

Notes and references

1 N. Matsuo, S. Kaneko, A. Kuno, H. Kobayashi and I. Kusakabe, *Biochem. J.*, 2000, **346**, 9.
2 V. A. McKie, G. W. Black, S. J. Millward-Sadler, G. P. Hazlewood, J. I. Laurie and H. J. Gilbert, *Biochem. J.*, 1997, **323**, 547.
3 B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard and B. Henrissat, *Nucleic Acids Res.*, 2009, **37**, D233.
4 R. Carapito, A. Imberty, J. M. Jeltsch, S. C. Byrns, P. H. Tam, T. L. Lowary, A. Varrot and V. Phalip, *J. Biol. Chem.*, 2009, **284**, 12285.
5 T. Sakamoto and J. F. Thibault, *Appl. Environ. Microbiol.*, 2001, **67**, 3319.
6 D. E. Koshland, *Biol. Rev.*, 1953, **28**, 416.
7 S. M. Pitson, A. G. Voragen and G. Beldman, *FEBS Lett.*, 1996, **398**, 7.
8 T. Sakamoto, T. Fujita and H. Kawasaki, *Biochim. Biophys. Acta*, 2004, **1674**, 85.
9 B. Henrissat and G. Davies, *Curr. Opin. Struct. Biol.*, 1997, **7**, 637.
10 Y. Sogabe, T. Kitatani, A. Yamaguchi, T. Kinoshita, H. Adachi, K. Takano, T. Inoue, Y. Mori, H. Matsumura, T. Sakamoto and T. Tada, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2011, **67**, 415.
11 N. Asano, R. J. Nash, R. J. Molyneux and G. W. J. Fleet, *Tetrahedron: Asymmetry*, 2000, **11**, 1645.
12 T. M. Gloster and G. J. Davies, *Org. Biomol. Chem.*, 2010, **8**, 305.
13 V. H. Lillelund, H. H. Jensen, X. Liang and M. Bols, *Chem. Rev.*, 2002, **102**, 515.
14 S. G. Withers, M. Namchuk and R. Mosi, in *Iminosugars as glycosidase inhibitors: nojirimycin and beyond*, ed. A. E. Stütz, Wiley-VCH, Weinheim, New-York, 1999, p. 188.
15 S. J. Williams, R. Hoos and S. G. Withers, *J. Am. Chem. Soc.*, 2000, **122**, 2223.
16 T. Kawaguchi, K. Sugimoto, H. Hayashi and M. Arai, *Biosci., Biotechnol., Biochem.*, 1996, **60**, 344.
17 A. Varrot, C. A. Tarling, J. M. Macdonald, R. V. Stick, D. L. Zechel, S. G. Withers and G. J. Davies, *J. Am. Chem. Soc.*, 2003, **125**, 7496.
18 Y. Du, Q. Pan and F. Kong, *Carbohydr. Res.*, 2000, **329**, 17.
19 D. W. C. Jones, R. J. Nash, E. A. Bell and J. M. Williams, *Tetrahedron Lett.*, 1985, **26**, 3125.
20 R. K. Ness and H. G. Fletcher, *J. Am. Chem. Soc.*, 1958, **80**, 2007.
21 M. Joe, Y. Bai, R. C. Nacario and T. L. Lowary, *J. Am. Chem. Soc.*, 2007, **129**, 9885.
22 K. Mizutani, R. Kasai, M. Nakamura, O. Tanaka and H. Matsuura, *Carbohydr. Res.*, 1989, **185**, 27.
23 M. T. Axamawaty, G. W. Fleet, K. A. Hannah, S. K. Namgoong and M. L. Sinnott, *Biochem. J.*, 1990, **266**, 245.
24 T. M. Gloster, P. Meloncelli, R. V. Stick, D. Zechel, A. Vasella and G. J. Davies, *J. Am. Chem. Soc.*, 2007, **129**, 2345.
25 D. J. Vocadlo and G. J. Davies, *Curr. Opin. Chem. Biol.*, 2008, **12**, 539.
26 C. Altona and M. Sundaralingam, *J. Am. Chem. Soc.*, 1972, **94**, 8205.
27 S. Cros, C. H. du Penhoat, S. Pérez and A. Imberty, *Carbohydr. Res.*, 1993, **248**, 81.
28 K. Hovel, D. Shallom, K. Niefind, V. Belakhov, G. Shoham, T. Baasov, Y. Shoham and D. Schomburg, *EMBO J.*, 2003, **22**, 4922.

