

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 5945

www.rsc.org/obc

COMMUNICATION

A potential fortuitous binding of inhibitors of an inverting family GH9 β -glycosidase derived from isofagomine†Solange Moréra,^{*a} Armelle Vigouroux^a and Keith A. Stubbs^{*b}

Received 16th May 2011, Accepted 7th June 2011

DOI: 10.1039/c1ob05766a

Using structural insight, the binding mode of isofagomine-derived inhibitors with family GH9 glycosidases is achieved via the study of *Alicyclobacillus acidocaldarius* (AaCel9A) endoglucanase. In contrast to what was observed in the first report using these compounds with inverting glycosidases from family GH6, these inhibitors do not adopt a distorted conformation in the active site.

The glycoside hydrolases are an important group of carbohydrate-processing enzymes found in nature as they have roles in the biosynthesis and degradation of glycoconjugates. One particular set of enzymes that are receiving increased attention due to biotechnological and environmental applications¹ are the cellulases which degrade the major plant cell wall polysaccharide, cellulose. These enzymes display a variety of biological activity² and are currently grouped into 11 of the 120 glycoside hydrolase sequence-based families (GHs) within the Carbohydrate-Active enZymes (CAZy) database.^{3–6}

One particular subset of cellulases are those from family GH9. This family of glycoside hydrolases contains enzymes that have endoglucanase, cellobiohydrolase, β -glucosidase and *exo*- β -glucosaminidase activity. Previous structural studies of enzymes in this family^{7–12} cover the four themes (A, B, C and D) describing the modular arrangement of the GH9 enzymes.¹³ As a result of the importance of these enzymes, much interest has been expressed in the synthesis and testing of compounds as putative inhibitors. The best known inhibitors of cellulases are the non-hydrolysable thio-oligosaccharides^{9,14–18} and the azasugar-based oligosaccharides^{19–21} with the former already being used to study the binding mode of substrates for family GH9 enzymes.⁹ However, to date no structural insight has been obtained for GH9 enzymes using iminosugar-based oligosaccharides which would be of interest to understand not only the binding mode of these compounds but would also allow insight into the protonation states of

putative transition-state mimics and the surrounding residues of the enzyme.

Previously, we reported the structural characterization of a novel endoglucanase, Cel9A, from *Alicyclobacillus acidocaldarius* (AaCel9A).²² This enzyme, like all other members of glycoside hydrolase family GH9, uses an inverting catalytic mechanism where, in the case of AaCel9A, Glu515 acts as the catalytic acid and Asp146, the catalytic base (Fig. 1). The enzyme has demonstrated cellobiohydrolase activity and acts on carboxy-methylcellulose (CMC), lichenan and *p*NP-cellooligosaccharides and displays a temperature optimum of 70 °C and a pH optimum of 5.5. Here, we present a detailed analysis of the inhibition and structural analysis of AaCel9A using a series of oligosaccharide inhibitors 1–3, derived from the parent azasugar, isofagomine 4.

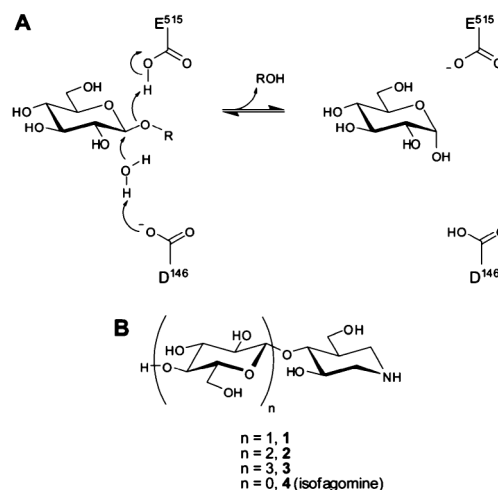


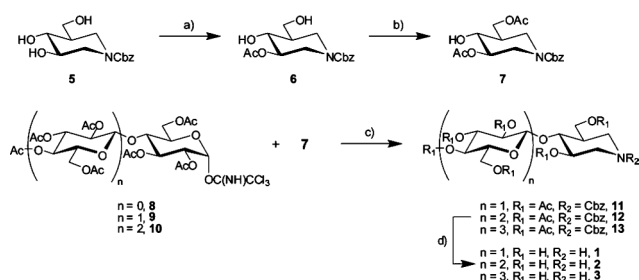
Fig. 1 (A) AaCel9A uses a catalytic mechanism involving an inversion of anomeric configuration. (B) Structures of the compounds 1–3 used in the structural study and the known glycosidase inhibitor isofagomine 4.

The synthesis of the desired compounds proceeded using the known Cbz-protected isofagomine **5**²³ (Scheme 1) which can be readily prepared from isofagomine **4**²⁴ or prepared *in situ* from **4**. Using various protecting group manipulations the diol **6** could be obtained in good yield. The diol was selectively acetylated to yield the alcohol **7** which was then treated with the appropriate trichloroacetimidate **8–10** to give the corresponding desired products **11–13**, all of which had ¹H and ¹³C NMR characteristics consistent with the chemoenzymatic preparation

^aLaboratoire d'Enzymologie et Biochimie Structurales (LEBS), CNRS, Avenue de la Terrasse, 91198, Gif-sur-Yvette, France. E-mail: morera@lebs.cnrs-gif.fr; Tel: +33 1 69823470

^bChemistry M313, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA, Australia, 6009. E-mail: kstubbs@cyllene.uwa.edu.au; Tel: +61 8 6488 2725

† Electronic supplementary information (ESI) available: Materials and methods, refinement statistics and supplemental figures and tables. See DOI: 10.1039/c1ob05766a



Scheme 1 a) i. PhCH(OMe)₂, CSA, CHCl₃; ii. Ac₂O, pyridine; iii. AcOH, H₂O; b) AcCl, pyridine, CH₂Cl₂; c) TMSOTf, CH₂Cl₂; d) NaOH, CH₃OH, H₂O.

of these compounds.²³ Removal of the protecting groups gave the desired materials **1–3**²³ in good yields.

Compounds **1–4** were determined to be competitive inhibitors of *Aa*Cel9A with *K_i* values, determined at the pH optimum for catalysis (pH = 5.5), of 610 ± 14 nM (**1**), 540 ± 11 nM (**2**), 940 ± 12 nM (**3**) and 87 ± 4 μM (**4**) (see ESI†). These data reveal some interesting changes in *K_i* values across the series. The monosaccharide analogue, isofagomine **4**, is a modest inhibitor of the enzyme but the addition of a single β-glucosyl moiety residue to the 4-position (yielding **1**) improves binding dramatically. However, each successive addition of β-glucosyl residues provides little binding enhancement. These results though are in line with what has been observed previously for this enzyme with it being demonstrated that the –1²⁵ and –2²² subsites are the strongest binding subsites and only weak interactions are observed for glucosyl moieties found in the –3 and –4 subsites. These observations suggest that the enzyme has, primarily, cellobiohydrolase activity. Notably, the *K_i* value obtained for **1** is consistent with that obtained for other cellulases, from GH5²⁰ and GH6.¹⁹

To gain a more detailed understanding of the molecular basis for the binding of the compounds to *Aa*Cel9A, we determined the three-dimensional structure of *Aa*Cel9A in complex with **1**, **2** and **3** (Fig. 2 and Fig. S1 and S2, ESI†) by soaking experiments at 2.56 Å, 1.99 Å and 2.02 Å resolution respectively (see Table, ESI†). Following maximum-likelihood refinement, *F_{obs}* – *F_{calc}* “difference” electron density unambiguously reveals the position of all carbohydrate associated hydrogen atoms for each structure. Compound **1** binds in the –2/–1 subsites, and in the +1, +2 subsites with the isofagomine moiety in the catalytic –1 and +1 subsites. This, to our knowledge, is the first reported structure of isofagomine bound to the +1 subsite of a cellulase and it is of note. Both the isofagomine and glucose residue in the aglycone subsites bind in a somewhat ‘twisted-chair’ conformation with the twisted

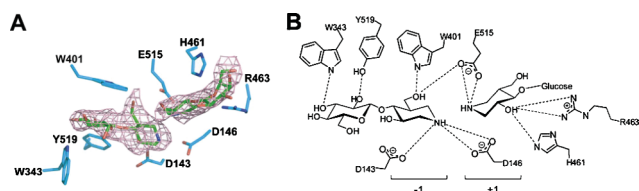


Fig. 2 (A) *F_{obs}* – *F_{calc}* electron density map contoured at 3σ for **1** bound in the active site cleft of *Aa*Cel9A, with the important active site residues displayed. (B) Interactions between *Aa*Cel9A and the two molecules of **1**. Only the –2, –1 and +1 subsites are shown for simplicity. The catalytic water molecule is not found in the structure of **1** with *Aa*Cel9A due to the interaction of the ring nitrogen of isofagomine with Asp146.

conformation being more pronounced in the isofagomine residue. These distortions appear to be driven through opportunistic interactions with the enzyme. Indeed, the isofagomine in the +1 subsite makes five interactions with the active site residues, Glu515, His461 and Arg463, which is two additional interactions compared to a glucose at this position.²²

Both the glucose and isofagomine moieties in the –2 and –1 subsite respectively lie in undistorted ⁴C₁ (chair) conformations which is different to the distorted ^{2,5}B (boat) conformation observed for isofagomine for the inverting GH6 enzymes.^{19,26} The observed binding mode has only been reported so far for retaining GH5 enzymes.^{20,21} Similarly for **2** and **3**, one inhibitor molecule binds at the distal subsite with the isofagomine moiety in the catalytic –1 subsite lying in an undistorted ⁴C₁ conformation (see Fig. S3, ESI†). For each structure, the isofagomine in the catalytic –1 subsite makes the same interactions with *Aa*Cel9A. For the structure of *Aa*Cel9A with **2**, a cellobiose molecule, which results from the cleavage of **2** by *Aa*Cel9A into isofagomine and cellobiose, binds in the +1 and +2 subsites. For the structure of *Aa*Cel9A with **3**, compound **1**, which results from the cleavage of **3**, is bound in the +1/+2 subsites but is bound, quite distinctly, in the opposite orientation to that observed for the parent compound **1** structure. This observation can be potentially rationalised by the cleavage of **3** between the second and third glucose residues, which liberates cellobiose and **1**. Release of the cellobiose moiety and binding of a second molecule of **3** is then able to occur in the –4 to –1 subsites. This observation is consistent with what has been reported for the catalytic activity and preferred binding mode of substrates for this enzyme.²⁷ Overall the structural results observed here give credence to previous reports that the occupancy of the –1/–2 binding sites are crucial to the catalytic activity of the enzyme.²²

The ring nitrogen of the isofagomine moiety in **1**, **2** and **3** interacts with the side chain of Asp143 and Asp146 (Fig. 2), consistent with electrostatic interactions of a protonated amine. It overlaps with the O1 atom of the α-glucose (–1) observed in the previously reported enzyme-product structures (PDB code 3H2W and 3H3K).²² Furthermore, the catalytic water molecule which is very well defined in the unliganded structure of *Aa*Cel9A (PDB code 3GZK²²) is not found in the structures of *Aa*Cel9A with **1–3** or α-glucose. Both the nitrogen atom and the O1 of the α-glucose are located only 1 Å away from the position of the catalytic water molecule in the unliganded structure and so overall this occupation of the binding space by these atoms presumably prevents binding of the catalytic water molecule. Interestingly, for all the compounds tested, distortion of the isofagomine moiety toward an oxocarbenium-like half chair is not observed although a ⁴H₃ half chair conformation has been observed for a cellotetraose-bound active site mutant GH9 cellulase from *Clostridium thermocellum* (CbhA).²⁸ The half chair conformation is thought to facilitate oxocarbenium ion formation and subsequent substrate cleavage. Both the position and ⁴C₁ conformation of isofagomine in *Aa*Cel9A resemble a product intermediate so far observed only for retaining glycosidases.^{20,21,29}

In conclusion, *Aa*Cel9A is another example of an inverting enzyme obtained in complex with inhibitors-derived from isofagomine. In contrast to the binding mode with inverting GH6 enzymes^{19,26} these inhibitors are not tight-binding inhibitors of inverting GH9 enzymes by virtue of their bound conformation

in the active site, which does not reflect any potential transition-state shape or “transition-state mimic” (as seen in GH6). They potentially only rely on the putative transition state-mimicking positive charge on the ring nitrogen. These subtle differences in binding observed for these inhibitors will be critical for the future development of selective inhibitors of these enzymes. Overall, the structural details observed here for *AaCel9A* with **1–3** can potentially be extrapolated to all family GH9 members.

SM and AV are grateful to Beatriz Guimarez for help in data collection on PROXIMA I at SOLEIL and KAS thanks the Australian Research Council for funding.

Notes and references

- 1 M. E. Himmel and E. A. Bayer, *Curr. Opin. Biotechnol.*, 2009, **20**, 316.
- 2 H. J. Gilbert, *Plant Physiol.*, 2010, **153**, 444.
- 3 B. Henrissat and A. Bairoch, *Biochem. J.*, 1993, **293**, 781.
- 4 B. Henrissat and A. Bairoch, *Biochem. J.*, 1996, **316**, 695.
- 5 B. Henrissat and G. Davies, *Curr. Opin. Struct. Biol.*, 1997, **7**, 637.
- 6 B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard and B. Henrissat, *Nucleic Acids Res.*, 2009, **37**, D233.
- 7 G. Parsiegla, A. Belaich, J. P. Belaich and R. Haser, *Biochemistry*, 2002, **41**, 11134.
- 8 J. Sakon, D. Irwin, D. B. Wilson and P. A. Karplus, *Nat. Struct. Biol.*, 1997, **4**, 810.
- 9 D. Mandelman, A. Belaich, J. P. Belaich, N. Aghajari, H. Driguez and R. Haser, *J. Bacteriol.*, 2003, **185**, 4127.
- 10 M. Juy, A. G. Amit, P. M. Alzari, R. J. Poljak, M. Claeysens, P. Beguin and J. P. Aubert, *Nature*, 1992, **357**, 89.
- 11 F. D. Schubot, I. A. Kataeva, J. Chang, A. K. Shah, L. G. Ljungdahl, J. P. Rose and B. C. Wang, *Biochemistry*, 2004, **43**, 1163.
- 12 S. Khademi, L. A. Guarino, H. Watanabe, G. Tokuda and E. F. Meyer, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2002, **58**, 653.
- 13 E. A. Bayer, Y. Shoham and R. Lamed, in *In The Prokaryotes: An Involving Electronic Resource for the Microbial Community*, ed. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt, Springer Verlag, New York, NY, 2000.
- 14 G. Parsiegla, M. Juy, C. Reverbel-Leroy, C. Tardif, J. P. Belaich, H. Driguez and R. Haser, *EMBO J.*, 1998, **17**, 5551.
- 15 J. Zou, G. J. Kleywegt, J. Ståhlberg, H. Driguez, W. Nerinckx, M. Claeysens, A. Koivula, T. T. Teeri and T. A. Jones, *Structure*, 1999, **7**, 1035.
- 16 A. Varrot, T. Frandsen, H. Driguez and G. J. Davies, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2002, **58**, 2201.
- 17 A. Varrot, M. Schülein, S. Fruchard, H. Driguez and G. J. Davies, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2001, **57**, 1739.
- 18 G. Parsiegla, C. Reverbel, C. Tardif, H. Driguez and R. Haser, *J. Mol. Biol.*, 2008, **375**, 499.
- 19 A. Varrot, J. Macdonald, R. V. Stick, G. Pell, H. J. Gilbert and G. J. Davies, *Chem. Commun.*, 2003, 946.
- 20 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.
- 21 M. E. C. Caines, S. M. Hancock, C. A. Tarling, T. M. Wrodnigg, R. V. Stick, A. E. Stutz, A. Vasella, S. G. Withers and N. C. J. Strynadka, *Angew. Chem., Int. Ed.*, 2007, **46**, 4474.
- 22 K. Eckert, A. Vigouroux, L. Leggio and S. Moréra, *J. Mol. Biol.*, 2009, **394**, 61.
- 23 J. M. Macdonald, R. V. Stick, D. M. G. Tilbrook and S. G. Withers, *Aust. J. Chem.*, 2002, **55**, 747.
- 24 E. D. Goddard-Borger and R. V. Stick, *Aust. J. Chem.*, 2007, **60**, 211.
- 25 G. J. Davies, K. S. Wilson and B. Henrissat, *Biochem. J.*, 1997, **321**, 557.
- 26 A. Varrot, S. Leydier, G. Pell, J. M. Macdonald, R. V. Stick, B. Henrissat, H. J. Gilbert and G. J. Davies, *J. Biol. Chem.*, 2005, **280**, 20181.
- 27 K. Eckert, F. Zielinski, L. L. Leggio and E. Schneider, *Appl. Microbiol. Biotechnol.*, 2002, **60**, 428.
- 28 F. D. Schubot, I. A. Kataeva, J. Chang, A. K. Shah, L. G. Ljungdahl, J. P. Rose and B. Wang, *Biochemistry*, 2004, **43**, 1163.
- 29 J. Sakon, D. Irwin, D. B. Wilson and P. A. Karplus, *Nat. Struct. Biol.*, 1997, **4**, 810.