Inhibition of the *exo*-β-D-glucosaminidase CsxA by a *glucosamine*-configured castanospermine and an *amino*-australine analogue†

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The synthesis of *amino*-derivatives of castanospermine and australine and their characterisation as inhibitors of the *exo*-β-D-glucosaminidase CsxA through enzyme kinetics and X-ray structural analysis is described.

Polysaccharides are well known for their roles in the storage of energy, in the provision of structural integrity and most notably, for their complexity. Due to the nature of their overall complex structures there are a wide variety of enzymes that are involved in their biosynthesis and degradation. The polysaccharides chitin and chitosan, for example, are widely distributed in living organisms, including insects, crustaceans, and fungi. These two macromolecules are similar in that chitin is a linear polysaccharide composed almost exclusively of β-1,4-*N*-acetyl-D-glucosamine residues while chitosan is a partly or totally *N*-deacetylated chitin derivative. Similarly, the enzymes that process these two polysaccharides are also abundant. *Endo*-chitinases and exo-chitinases (including chitobiohydrolases and *exo*-β-*N*-acetyl-D-glucosaminidases), are involved primarily in the degradation of chitin,[1–3] with *endo*-chitosanases (found in organisms including bacteria[4–7] and fungi[8–10]) and *exo*-chitosanases (*exo*-β-D-glucosaminidases)[11–14] acting primarily to degrade chitosan.

Due the biological importance of these enzymes in the breakdown of these polysaccharides it would be of interest to design small molecule inhibitors to be used as tools for establishing the role these enzymes have in biological processes. For the chitinases, there are examples of compounds that have been prepared that are inhibitors of both the enzymes that act on chitin,[3,15–18] but quite surprisingly little work has gone into the development of compounds as inhibitors for chitosanases, especially the *exo*-β-D-glucosaminidases. One reason for this is, in part, due to the lack of crystallographic information available that describes the molecular basis of substrate recognition by this class of enzyme. Recently an *exo*-β-D-glucosaminidase from *Amycolatopsis orientalis*, CsxA, which is a member of family 2 (GH2)[19,20] of the glycoside hydrolases, was crystallized in complex with a reaction product and a natural substrate, which has allowed insight into the catalytic mechanism of the enzyme and the basis for the enzyme's specificity for its substrate, chitosan.[21]

CsxA, like other members of GH2, uses a catalytic mechanism involving a glycosyl-enzyme intermediate with the overall reaction proceeding in two ordered steps, each involving inversion of stereochemistry at the anomeric centre such that the reaction proceeds with overall retention of stereochemistry (Fig. 1A). One key feature of the active site was the unique negatively charged pocket that specifically accommodates the nitrogen of non-reducing end D-glucosamine residues and which allows this enzyme to discriminate between D-glucose, D-glucosamine and *N*-acetyl-D-glucosamine. This feature was of interest to us as it formed a potential starting point for the rational design of inhibitors of CsxA. Another important consideration in the inhibitor design was the choice of inhibitor scaffold. Multiple crystal structures have been determined for enzymes in GH2[22–24] (other enzymes in this family include β-glucuronidases, β-mannosidases and β-galactosidases) both in the native form as well as in complex with several inhibitors.[25,26] From this information it was decided that an appropriately designed imino-sugar would be a good candidate for an inhibitor of CsxA. These compounds have proven to be good inhibitors of a wide range of enzymes, not only in GH2 but other GH families as well. We felt a stable inhibitor of CsxA could be based on the indolizidine imino-sugar castanospermine **1**[27,28] (Fig. 1B) with the choice of this scaffold being made for several reasons. Castanospermine **1** is a natural product readily obtained in large quantities from the seeds of the Moreton Bay Chestnut tree,[27] and it is a potent inhibitor of retaining β-glucosidases.[29] Furthermore, polyhydroxylated pyrrolizidine alkaloids such as australine **2** (Fig. 1B), another potential inhibitor scaffold for CsxA, can be readily obtained synthetically from castanospermine **1**. In consideration of this scaffold, we synthesized the novel castanospermine and australine analogues **3** and **4** (Fig. 1C) respectively from the known triacetate **5**[30] (Scheme 1).

The triacetate **5** was prepared from castanospermine **1** according to a known procedure.[30] Reaction of **5** with methanesulfonyl chloride in pyridine afforded cleanly the mesylate **6**. Treatment of **6** with NaN_3 in DMSO, using a known procedure,[30] gave a separable mixture of the desired *azido*-castanospermine **7** and the *azido*-australine analogue **8**. The preparation of the desired *amino*-castanospermine **3** followed a literature procedure[30] but instead of removing the acetyl protecting groups from **9** with methanolic sodium methoxide solution, we found that in our hands a saturated solution of ammonia in methanol gave a far better result. For the *amino*-australine derivative **4**, care had to be taken due to the known acetyl migration from nitrogen to oxygen that occurs when **8** is placed under reducing conditions.[30] Removal of the acetyl protecting groups first with a saturated solution of ammonia in

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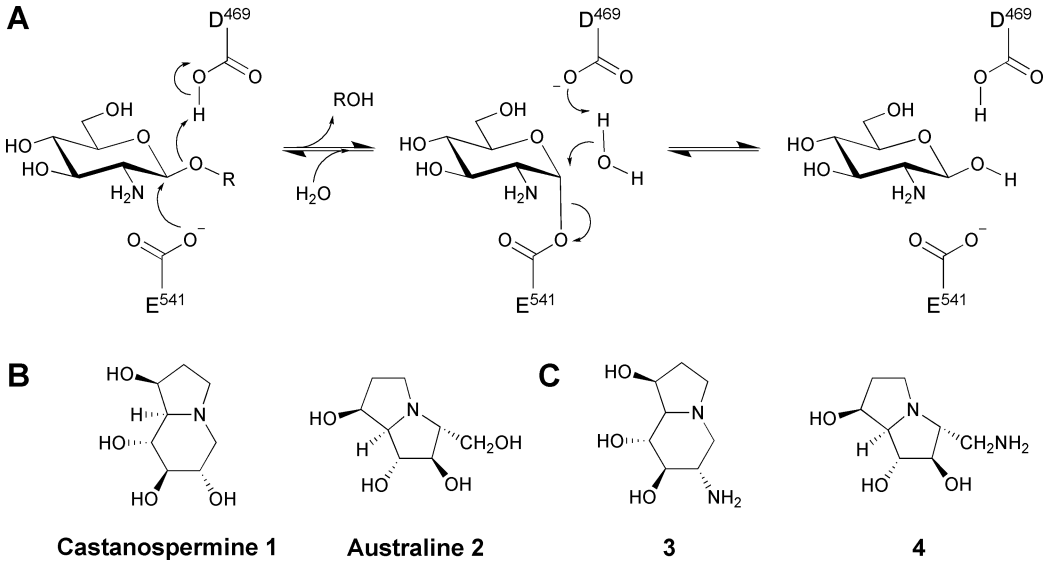
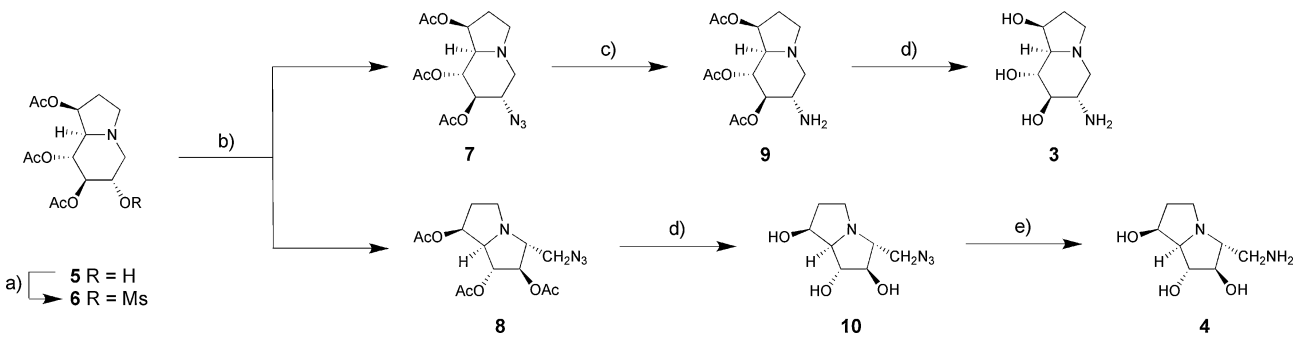


Fig. 1 (A) CsxA uses a catalytic mechanism involving a glycosyl–enzyme intermediate. Both steps of the reaction occur with inversion of stereochemistry at the anomeric centre such that the overall reaction proceeds with net retention of stereochemistry. (B) Structures of the known glycosidase inhibitors castanospermine **1** and australine **2**. (C) Structures of the corresponding amino analogues **3** and **4**, relevant to the work described here.



Scheme 1 a) i. MsCl, pyridine; b) NaN_3, DMSO; c) H_2, Pd/C, toluene; d) NH_3, MeOH; e) H_2, Pd/C, MeOH.

methanol yielded **10**, which was then reduced using an atmosphere of hydrogen to give **4** in excellent yield.

Next we prepared a known substrate for *exo*-β-D-glucosaminidases, 4-methylumbelliferyl 2-amino-2-deoxy-β-D-glucopyranoside,[31] and evaluated the inhibitors **3** and **4** against CsxA. We found in both cases a clear pattern of competitive inhibition (Fig. 2A and B). To our knowledge these compounds are the first rationally designed inhibitors of *exo*-β-D-glucosaminidases. We found **3** to be a potent competitive inhibitor of CsxA, with a K_i value of 610 ± 12 nM (Fig. 2A). The *amino*-australine derivative **4** on the other hand was only a modest inhibitor of CsxA with a K_i value of 175 ± 8 μM. Complementing this data was the fact that neither **1** nor **2** were capable of inhibiting the enzyme at a concentration of 1 mM.

In light of these results we considered that the *amino*-australine derivative **4** may not bind as favourably to CsxA due to the aminomethyl arm not fully utilizing the negatively charged pocket that in the case of the natural substrate, chitosan, specifically accommodates the nitrogen atom of D-glucosamine. Alternatively, the conformational rigidity imposed by the ring system may not allow **4** to adopt a conformation that maximises adventitious interactions with the enzyme.

To gain a more detailed understanding of the molecular basis for these differences, we determined the three-dimensional structure of CsxA in complex with **3** and **4**. At 2.3 and 2.4 Å resolutions the electron densities of **3** and **4**, respectively, were unambiguous, allowing the inhibitors to be easily modelled into the active site of CsxA (Fig. 3A and B). Notably, the structure of **4** with CsxA is, to our knowledge, the first X-ray complex of a derivative of australine with any enzyme.

A comparison of these inhibitor complexes with the previously determined complex of CsxA with D-glucosamine revealed a virtually identical set of protein–carbohydrate interactions (Fig. 3C). Within the error limitations imparted by the resolutions of these structures, there are no apparent large differences in the inter-atomic distances. Indeed, all of these compounds also result in the burial of ~107 Å^2 of surface area in the active site, which is divided roughly 1:1 between polar to apolar surface area. In particular, the amino groups of **3** and **4** are found in virtually identical positions in the acidic pocket formed by E394, E591, and D649 of the CsxA active site. This interaction is thought to be particularly important in substrate recognition, which is supported by our observation that **1** and **2** were incapable of inhibiting the enzyme.[21] Given the very

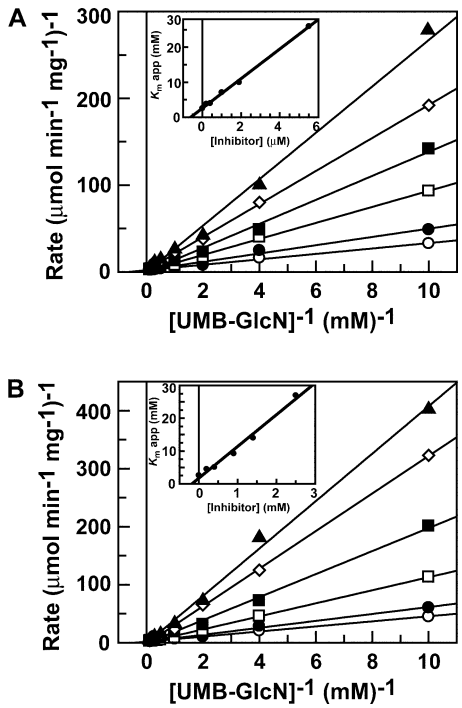


Fig. 2 (A) Inhibition of CsxA catalyzed hydrolysis of UMB-GlcN by **3** shows a pattern of competitive inhibition. The concentrations of **3** (μM) used were 5.5 (▲), 1.9 (◊), 1.0 (■), 0.4 (□), 0.2 (●), and 0.0 (○). Inset, graphical analysis of K_i from plotting values of K_M apparent against concentration of **3**. (B) Similarly for **4**, a pattern of competitive inhibition is observed. The concentrations of **4** (mM) used were 2.5 (▲), 1.4 (◊), 0.9 (■), 0.4 (□), 0.2 (●), and 0.0 (○).

similar binding modes of **3** and **4** and the capacity of the amino groups of these compounds to make a potentially key charge–charge interaction with the active site it is somewhat surprising that the two compounds bind with an approximate difference of 3.5 kcal/mole in their change in Gibbs free energy.

However, closer examination of the interactions made by the amino groups on **3** and **4** provided some insight into the selectivity CsxA shows between these two inhibitors. Though the amino groups of **3** and **4** are positioned similarly, only the amino group of **3** has the appropriate geometry in its protonated NH_3^+ form to make three hydrogen bonds with all of the residues in the acidic pocket (Fig. 3D). This is directly analogous to the key interaction noted between CsxA and a D-glucosamine substrate that sits in the -1 subsite.[21] In contrast, due to the bend in the aminomethyl arm of **4**, the geometry of the amino group is only suitable for making a single hydrogen bond with E394 (Fig. 3E). The aminomethyl arm of **4** has the potential for some flexibility, possibly allowing it to make numerous transient interactions in the acidic pocket. Analysis of the B-factor of this nitrogen atom revealed it to be very similar to that of all of the atoms in **4** and the surrounding atoms of the protein, indicating only one major conformation of **4** in the active site. These observations imply that charge–charge complementarity in the acidic pocket is not the major driving force between the interaction of substrates and inhibitors with CsxA and that the primary difference in the effectiveness of **3** and **4** lies in their different hydrogen bonding patterns in the acidic pocket of the active site. We cannot, however, rule out the possible influences that the different ring structures of **3** and **4** and the flexibilities imparted therein might have on the energetic contributions to their recognition by CsxA. Nevertheless, our observations here are

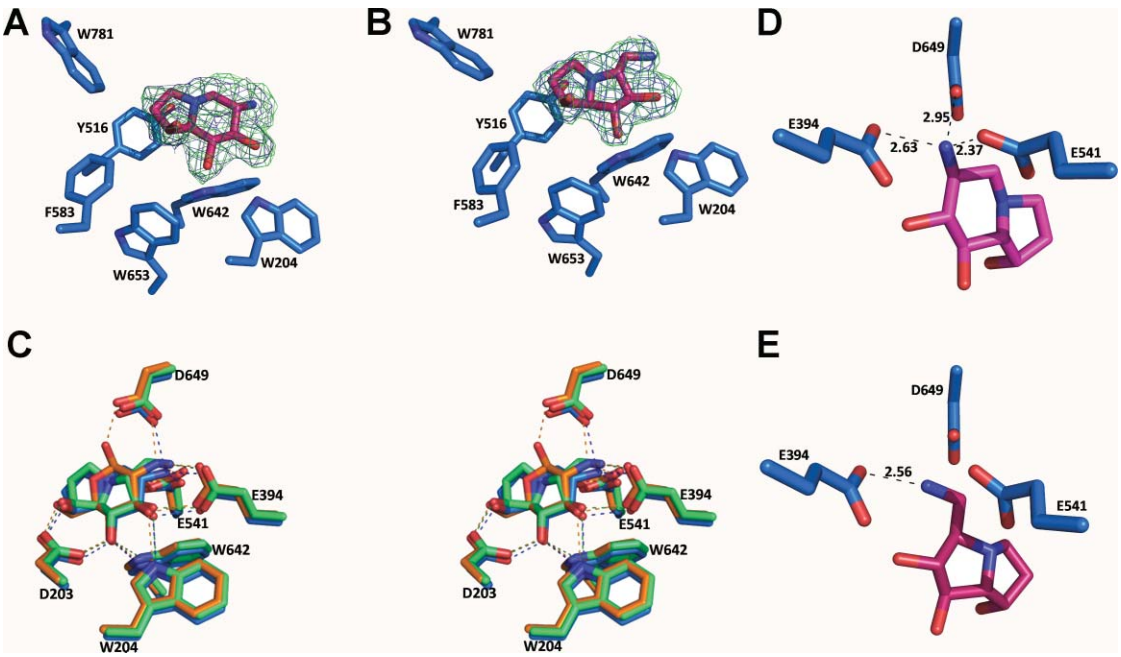


Fig. 3 Electron density of (A) **3** and (B) **4** shown with the aromatic amino acids in the CsxA active site in stick representation. The blue mesh shows maximum-likelihood/σ_a-weighted $2F_{obs}$-F_{calc} electron density maps at 1σ (0.35 $e^-/Å^3$ for both inhibitors). Green mesh shows maximum-likelihood/σ_a-weighted F_{obs}-F_{calc} electron density maps at 3σ (0.13 $e^-/Å^3$ for **3** and 0.18 $e^-/Å^3$ for **4**) produced prior to modelling the inhibitors. (C) A divergent stereo view of an active site overlap of the complexes of **3** (green), **4** (blue), and glucosamine (orange; PDB ID 2VZS). Hydrogen bonds are shown as dashed lines. The catalytic nucleophile is E541 and the acid/base D469. (D) Interactions between the amino group of **3** and the acidic pocket of CsxA. (E) Interactions between the amino group of **4** and the acidic pocket of CsxA. Hydrogen bonds are shown in panels D and E as dashed lines and distances are shown in Å.

consistent with our previous supposition that the unique hydrogen bond accepting acidic pocket in the active site of CsxA and the distinctive hydrogen bonding donor capacity of protonated amine sugars are critical components of substrate, and now inhibitor, recognition by CsxA.

In conclusion, we have developed two compounds one of which is a potent inhibitor of CsxA, the *exo*-β-D-glucosaminidase from *A. orientalis*. Collectively, the results obtained here suggest that compounds having a suitably placed amino moiety, which take full advantage of the unique, negatively charged pocket found in these enzymes, will potentially also be potent inhibitors. Overall, these inhibitors and further derivatives will be useful molecules for studying *exo*-β-D-glucosaminidases.

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References

1 T. Fukamizo and K. J. Kramer, *Insect Biochem.*, 1985, **15**, 141–145.
2 V. G. Eijsink, G. Vaaje-Kolstad, K. M. Vårum and S. J. Horn, *Trends Biotechnol.*, 2008, **26**, 228–235.
3 M. Horsch, C. Mayer, U. Sennhauser and D. M. Rast, *Pharmacol. Ther.*, 1997, **76**, 187–218.
4 T. Fukamizo and R. Brzezinski, *Biochem. Cell Biol.*, 1997, **75**, 687–696.
5 Y. Y. Jo, K. J. Jo, Y. L. Jin, K. Y. Kim, J. H. Shim, Y. W. Kim and R. D. Park, *Biosci., Biotechnol., Biochem.*, 2003, **67**, 1875–1882.
6 J. S. Price and R. Storck, *J. Bacteriol.*, 1975, **124**, 1574–1585.
7 H. Seino, K. Tsukuda and Y. Shimasue, *Agric. Biol. Chem.*, 1991, **55**, 2421–2423.
8 D. M. Fenton and D. E. Eveleigh, *J. Gen. Microbiol.*, 1981, **126**, 151–165.
9 M. Shimosaka, M. Kumehara, X. Y. Zhang, M. Nogawa and M. Okazaki, *J. Ferment. Bioeng.*, 1996, **82**, 426–431.
10 D. Somashekar and R. Joseph, *Lett. Appl. Microbiol.*, 1992, **14**, 1–4.
11 X. Y. Zhang, A. L. Dai, X. K. Zhang, K. Kuroiwa, R. Kodaira, M. Shimosaka and M. Okazaki, *Biosci., Biotechnol., Biochem.*, 2000, **64**, 1896–1902.
12 M. Nogawa, H. Takahashi, A. Kashigawa, K. Ohshima, H. Okada and Y. Morikawa, *Appl. Environ. Microbiol.*, 1998, **64**, 890–895.
13 F. Nanjo, R. Katsumi and K. Sakai, *J. Biol. Chem.*, 1990, **265**, 10088–10094.
14 N. Côté, A. Fleury, E. Dumont-Blanchette, T. Fukamizo, M. Mitsutomi and R. Brzezinski, *Biochem. J.*, 2006, **394**, 675–686.
15 N. Arai, K. Shiomi, Y. Yamaguchi, R. Masuma, Y. Iwai, A. Turberg, H. Koelbl and S. Omura, *Chem. Pharm. Bull.*, 2000, **48**, 1442–1446.
16 S. Sakuda, A. Isogai, S. Matsumoto, A. Suzuki and K. Koseki, *Tetrahedron Lett.*, 1986, **27**, 2475–2478.
17 K. Shiomi, N. Arai, Y. Iwai, A. Turberg, H. Koelbl and S. Omura, *Tetrahedron Lett.*, 2000, **41**, 2141–2143.
18 M. J. Dixon, A. Nathubhai, O. A. Andersen, D. M. van Aalten and I. M. Eggleston, *Adv. Exp. Med. Biol.*, 2009, **611**, 525–526.
19 B. Henrissat and A. Bairoch, *Biochem. J.*, 1993, **293**, 781–788.
20 B. Henrissat and A. Bairoch, *Biochem. J.*, 1996, **316**, 695–696.
21 A. L. van Bueren, M. G. Ghinet, K. Gregg, A. Fleury, R. Brzezinski and A. B. Boraston, *J. Mol. Biol.*, 2009, **385**, 131–139.
22 R. H. Jacobson, X. J. Zhang, R. F. DuBose and B. W. Matthews, *Nature*, 1994, **369**, 761–766.
23 L. E. Tailford, V. A. Money, N. L. Smith, C. Dumon, G. J. Davies and H. J. Gilbert, *J. Biol. Chem.*, 2007, **282**, 11291–11299.
24 S. Jain, W. B. Drendel, Z. W. Chen, F. S. Mathews, W. S. Sly and J. H. Grubb, *Nat. Struct. Biol.*, 1996, **3**, 375–381.
25 D. H. Juers, T. D. Heightman, A. Vasella, J. D. McCarter, L. Mackenzie, S. G. Withers and B. W. Matthews, *Biochemistry*, 2001, **40**, 14781–14794.
26 L. E. Tailford, W. A. Offen, N. L. Smith, C. Dumon, C. Morland, J. Gratien, M. Heck, R. V. Stick, Y. Bleriot, A. Vasella, H. J. Gilbert and G. J. Davies, *Nat. Chem. Biol.*, 2008, **4**, 306–312.
27 L. D. Hohenschutz, E. A. Bell, P. J. Jewess, D. P. Leworthy, R. J. Pryce, E. Arnold and J. Clardy, *Phytochemistry*, 1981, **20**, 811–814.
28 R. J. Nash, L. E. Fellows, J. V. Dring, C. H. Stirton, D. Carter, M. P. Hegarty and E. A. Bell, *Phytochemistry*, 1988, **27**, 1403–1404.
29 R. Saul, J. P. Chambers, R. J. Molyneux and A. D. Elbein, *Arch. Biochem. Biophys.*, 1983, **221**, 593–597.
30 R. H. Furneaux, G. J. Gainsford, J. M. Mason and P. C. Tyler, *Tetrahedron*, 1994, **50**, 2131–2160.
31 K. R. Roeser and G. Legler, *Biochim. Biophys. Acta, Enzymol.*, 1981, **657**, 321–333.

