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

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Experimental

General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) or a Bruker AV600 (600 MHz for ^{1}H and 150 MHz for ^{13}C) (chemical shifts quoted relative to CD_3OD). Elemental analyses of all synthesized compounds used in enzyme assays were performed at the Australian National University Microanalytical Facility. Flash chromatography was performed on BDH silica gel with the specified solvents. Thin-layer chromatography (TLC) was effected on Merck silica gel 60 F_{254} aluminium-backed plates that were stained by heating (>200 °C) with 5% sulfuric acid in EtOH. Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by column chromatography, and purity was assessed by TLC or ^{1}H NMR spectroscopy.

6-Amino-6-deoxycastanospermine (3)

A saturated solution of ammonia in MeOH (2 ml) was added to the amine **9**[1] (80 mg) in MeOH (3 ml) and the solution left to stand (r.t., 2 h). Concentration followed by flash chromatography of the residue (CH_2Cl_2/MeOH/NH_4OH 5:4:1) gave the desired triol **3** (45 mg, 95%). ^{1}H and ^{13}C NMR spectra consistent with that found in the literature.[1]

8-Azido-8-deoxyaustraline (10)

A saturated solution of ammonia in MeOH (2 ml) was added to the azide **8**[1] (105 mg) in MeOH (3 ml) and the solution left to stand (r.t., 2 h). Concentration followed by flash chromatography of the residue (MeOH/EtOAc 1:4) gave the desired triol **10** (60 mg, 92%). R_f 0.25 (MeOH/EtOAc 1:4); δ_H (600 MHz, CD_3OD): 4.24-4.22 (m, 2H, H1, H7), 3.84 (dd, J = 7.7, 8.9, 1H, H2), 3.56 (dd, J = 3.2, 12.7, 1H, H8) 3.27 (dd, J = 6.6, 1H, H8□), 3.18 (dd, J = 4.0, 6.8, 1H, H7a), 4.36-4.33 (m, 1H, H5), 2.75-2.68 (m, 2H, H3, H5□), 2.00-1.95 (m, 1H, H6), 1.92-1.85 (m, 1H, H6□). δ_C (150 MHz, $CDCl_3$): 81.3 (C2), 74.5 (C1), 73.8 (C7a), 71.2 (C7), 70.4 (C3), 54.4 (C5), 53.0 (C8), 37.3 (C6).

8-Amino-8-deoxyaustraline (4)
10% Palladium-on-carbon (20 mg) was added to solution of **10** (80 mg) in MeOH (5 ml) and the mixture stirred at room temperature under an atmosphere of nitrogen (1 atm, 3 h). The mixture was filtered through a pad of celite and concentrated to give **4** as a colourless oil (67 mg, 95%). R_f 0.45 (CH_2Cl_2/MeOH/NH_4OH 5:4:1); δ_H (500 MHz, CD_3OD): 4.26-4.23 (m, 2H, H1, H7), 3.84 (dd, *J* = 7.6, 8.7, 1H, H2), 3.18 (dd, *J* = 4.1, 6.7, 1H, H7a), 3.12-3.08 (m, 1H, H5), 2.80 (dd, *J* = 5.2, 13.2, 1H, H8), 2.76-2.70, 2.62-2.58 (2m, 3H, H3, H5□, H8□), (m, 1H, H3, H5□), 2.02-1.98 (m, 1H, H6), 1.94-1.86 (m, 1H, H6□). δ_C (125 MHz, $CDCl_3$): 82.5 (C2), 74.9 (C1), 74.0 (C7a), 73.4 (C7), 70.6 (C3), 53.1 (C5), 44.8 (C8), 37.3 (C6). Anal. calcd for $C_8H_{16}N_2O_3$: C, 51.05; H, 8.57. Found: C, 51.22; H, 8.65%.

Kinetic Analysis of CsxA
The CsxA protein was produced and purified as described previously.[2] The protein was buffer exchanged into 5 mM sodium acetate, pH 5.5, by repeated washing in a 10 ml stirred ultrafiltration device using a 5K MWCO membrane (Millipore). All assays were carried out in triplicate at 37 °C for 60 minutes by using a stopped assay procedure in which the enzymatic reactions (50 μL) were quenched by the addition of a 4-fold excess (200 μL) of quenching buffer (200 mM glycine, pH 10.75). Assays were initiated by the careful addition, *via* pipette, of enzyme (5 μL), and in all cases the final pH of the resulting quenched solution was greater than 10. Time-dependent assay of CsxA revealed that the enzyme was stable in its buffers over the period of the assay: 50 mM sodium acetate, pH 5.3. The progress of the reaction at the end of 60 minutes was determined by measuring the extent of 4-methylumbelliferone liberated as determined by fluorescence measurements using a BMG Labtech Spectrophotometer 96-well plate system and comparison to a standard curve of 4-methylumbelliferone under identical buffer conditions. Excitation and emission wavelengths of 368 and 450 nM were used, respectively, with 5 mm slit openings. CsxA was used in the inhibition assays at a concentration (μg/μL) of 0.0106, using 4-methylumbelliferyl 2-amino-2-deoxy-β-D-glucopyranoside[3] as substrate. Both inhibitors were tested at six concentrations and K_i values were determined by linear regression of data from Dixon plots.

Crystallization and Structure Solution of CsxA
Crystallization experiments were performed as described by previously.[4] Briefly, crystals were obtained at 18 °C in a crystallization solution containing 18% (v/v) polyethylene glycol 2K monomethylether, 20 mM $CdSO_4$ and 100 mM Tris-HCl, pH 7.5. Crystals were then soaked in the mother liquor lacking $CdSO_4$ for 2 hours followed by transfer of the crystals into the soaking solution supplemented with 5 mM inhibitor (**3** or **4**) for 1 hour. All crystals were cryoprotected by the addition of a progressive concentration of glycerol until 20% (v/v) and frozen at 113 K prior to X-ray diffraction analysis. Diffraction data were collected on a home detector (Rigaku R- 15Axis 4++ area detector coupled to an MM-002 X-ray generator with Osmic Blue optics and an Oxford Cryostream 700) or on beamline 9.1 at the Stanford Synchrotron Radiation Laboratory (*SSRL*) and processed with MOSFLM/SCALA.[5] The complex structures were determined using the native CsxA structure as a starting point (PDB ID 2VZO). Coordinate files and restraint libraries for the inhibitors were produced using the prodrg2 server.[6,7] The final

structures of the complexes were obtained by manual correction using COOT and refinement with REFMAC.[8,9] Waters were added automatically using COOT and inspected manually. 5% of the reflections were flagged as "free" to monitor refinement procedures.[10] Model validation was performed with SFCHECK and PROCHECK in the CCP4 suite.[11,12] All statistics concerning the data collection and structure refinements are shown in Table 1.

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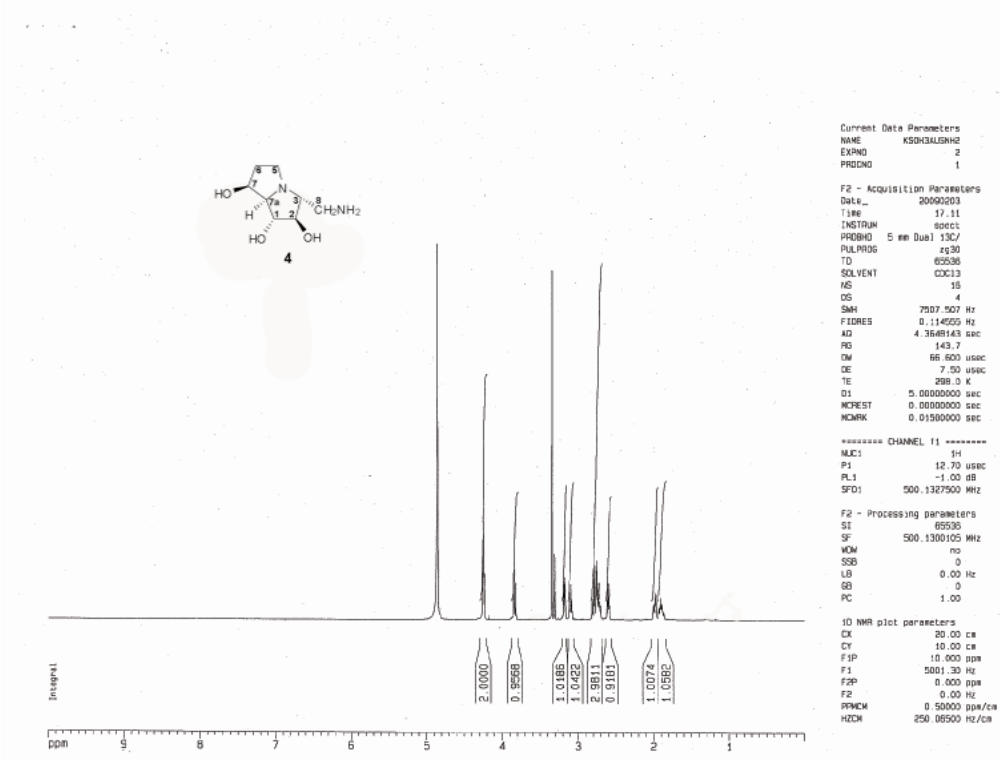
Table. Data collection, phasing and refinement statistics for the structure solution of CsxA with **3** and **4.**

	Complex with **3**	Complex with **4**
Data Collection		
Wavelength (Å)	0.98	1.54
Space Group	$P2_1$	$P2_1$
Cell Dimensions		
a, *b* , *c* (Å)	86.1, 121.8, 91.6	86.8, 122.0, 92.1
α, β, γ (Å)	90.0, 90.5, 90.0	90.0, 90.7, 90.0
Resolution (Å)	50-2.30 (2.42-2.30)	20.00-2.40 (2.53-2.40)
R_{sym} or R_{merge}	0.154 (0.395)	0.135 (0.319)
I/σI	4.9 (2.5)	5.5 (3.3)
Completeness (%)	99.1 (99.8)	97.5 (94.9)
Redundancy	3.1 (3.1)	2.6 (2.5)
No. of reflections	261,451	192,716
No. unique	83,030	72,891
Refinement		
Resolution (Å)	50-2.30	20.00-2.40
R_{work}/R_{free}	0.184/0.248	0.176/0.240
No. Of atoms		
Protein	6520 (A); 6532 (B)	6518 (A); 6512 (B)
Ligand	19 Cd^{2+}; 13 inhibitor (A) 13 inhibitor (B)	3 Cd^{2+}; 13 inhibitor (A) 13 inhibitor (B)
Water	1206	1446
B-factors		
Protein	18.6 (A); 18.1 (B)	16.5 (A); 15.4 (B)
Ligand	89.9 (Cd^{2+}); 17.6 inhibitor (A) 17.3 inhibitor (B)	29.1 (Cd^{2+}); 17.6 inhibitor (A) 14.5 inhibitor (B)
Water	23.3	23.6
r.m.s.d		
Bond lengths (Å)	0.011	0.011
Bond angles (Å)	1.357	1.352
Ramachandran (%)		
Preferred	99.5	99.4
Generously allowed	0.4	0.5
Disallowed	0.1	0.1

Values in parentheses are for the highest-resolution shell

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TD                65536
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1D NMR plot parameters
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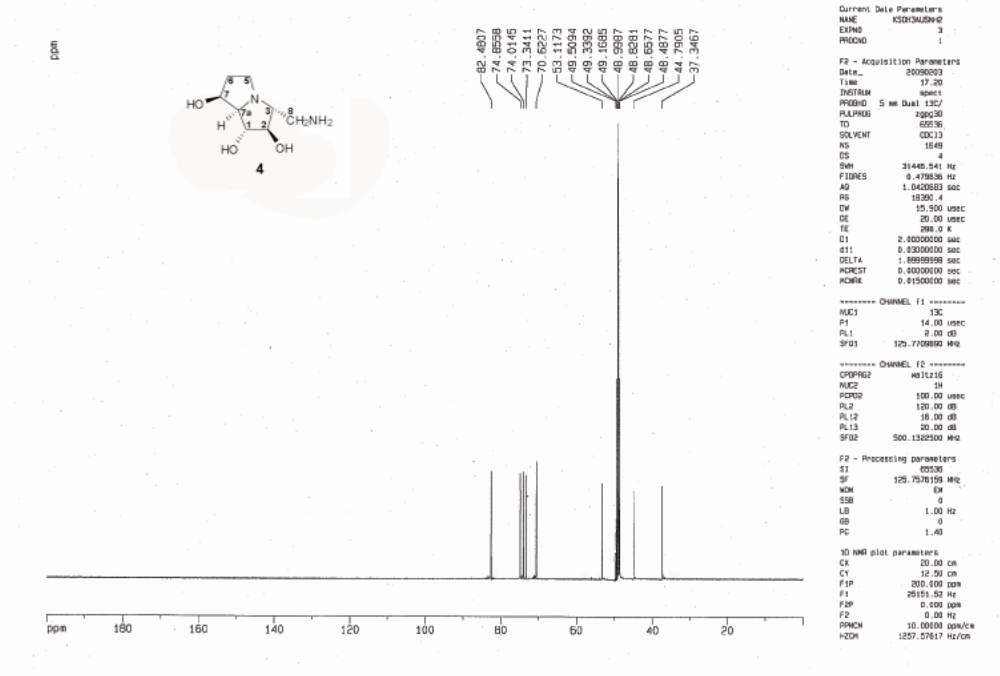


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======== CHANNEL f1 ========
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======== CHANNEL f2 ========
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PL12               16.00 dB
PL13               20.00 dB
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F2 - Processing parameters
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1D NMR plot parameters
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F2P                0.000 ppm
F2                 0.00 Hz
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HZCM          1257.57617 Hz/cm
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