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An efficient synthesis of argifin: A natural product chitinase inhibitor with chemotherapeutic potential

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Abstract—The first synthesis of the cyclopentapeptide family 18 chitinase inhibitor argifin has been achieved by a combination of solid phase and solution chemistry. Synthetic argifin is a nanomolar inhibitor of chitinase B1 from *Aspergillus fumigatus* and the high-resolution X-ray structure of the synthesized material in complex with the same enzyme is identical to that previously obtained for the natural product.

The glycopolymer chitin, a homopolymer of β(1,4)-linked *N*-acetyl-D-glucosamine residues, is a key constituent of many organisms that are pathogenic to humans. Chitin is the main structural component of the cell wall of fungi[1] and the exoskeletons of insects,[2] and is also found in the eggshells of nematodes.[3] Since these organisms depend on the ability to hydrolyse chitin at key-points in their lifecycles, inhibitors of chitinases are of considerable interest as potential fungicides and insecticides.[4] Furthermore, although chitin is not a component of mammalian cells, recent studies have shown that mammalian chitinases may be implicated in the allergic response associated with asthma and related diseases,[5] as well as glycolipid storage disorders, such as Gaucher's disease.[6]

Chitin metabolism is a very attractive target for drug design, but until recently relatively few effective inhibitors of chitinases were known. The pseudotrisaccharide natural product allosamidin[7] has been most widely studied and is a potent inhibitor of many chitinases of glycosyl hydrolase family 18 that contains enzymes from mammals, insects, nematodes and fungi.[8] However, the synthesis of allosamidin is complex,[8] which limits its availability as a biological tool and potential as a drug lead, and thus has prompted the search for other novel inhibitors that are more synthetically accessible and amenable to rational structure-based optimisation.[9]

Argifin (**1**, Fig. 1), originally isolated from a *Gliocladium* fungal culture,[9c–e] is a cyclopentapeptide composed of two β-linked Asp residues, an *N*-methyl Phe residue, a D-Ala residue and an unusual *N*-methyl carbamoyl-derivatised Arg residue. Although **1** shows low micromolar inhibition of several family 18 chitinases,[9c,10] no synthesis has so far been achieved, thus preventing a more detailed evaluation of the potential of such peptide-based inhibitors. In this paper, we therefore describe the first preparation of **1** and its characterisation against a representative family 18 chitinase.

Our synthetic approach to **1** first involved Fmoc-based solid-phase synthesis of the orthogonally protected linear pentapeptide **2** (Scheme 1). Peptide **2** was assembled

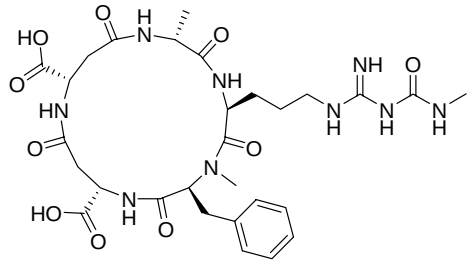

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Figure 1. Argifin **1**.

Keywords: Natural product; Chitinase inhibitor; Cyclic peptide; Solid phase synthesis.
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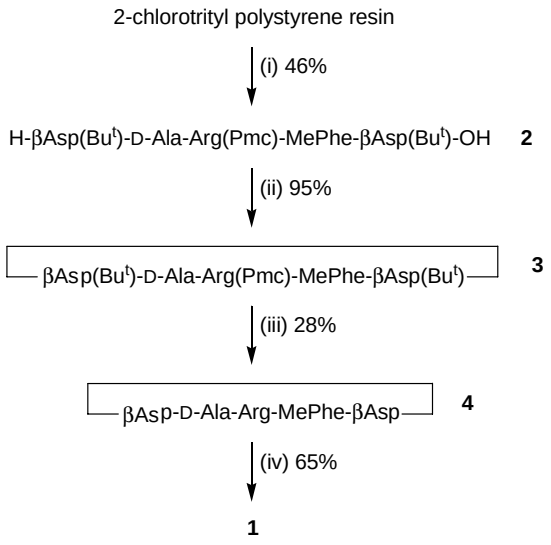

Scheme 1. Reagents and conditions:[15] (i) Fmoc solid-phase peptide synthesis; (ii) PyBOP, DIPEA, DCM, 16 h; (iii) TFA/thioanisole/DCM/H_2O (16:2:1:1), 2 h; (iv) *N*-Succinimidyl-*N*-methylcarbamate (3 equiv), DBU (6 equiv), DMF, 40 °C, 2 h.

on 2-chlorotrityl polystyrene resin using 2 equiv of Fmoc-protected amino acids and PyBOP activation,[11] except for the coupling of Fmoc-Arg(Pmc)-OH to the hindered MePhe residue that required the use of PyBrOP.[12] Cleavage from the resin with 1% TFA/DCM gave **2** in 46% yield based on the original resin loading. The choice of Asp protecting group was found to be critical in the assembly of the linear precursor; in preliminary studies with the alternative sequence H-D-Ala-Arg(Pmc)-MePhe-βAsp(OBn)-βAsp(OBn)-OH, HPLC and ESMS analysis revealed an essentially quantitative formation of a product resulting from aspartimide formation at the non-terminal Asp residue. The subsequent choice of **2** as linear precursor limited the exposure of the sensitive Asp residue to a single cycle of Fmoc synthesis, while the use of *tert*-butyl rather than benzyl ester protection was expected to significantly reduce base-induced aspartimide formation.[13]

The cyclisation of **2** to the fully protected cyclic pentapeptide **3** proceeded remarkably efficiently in dilute DCM solution using PyBOP activation and DIPEA as base. Optimum results were obtained with a single equivalent of PyBOP and a final peptide concentration of 1 mM giving an isolated yield of 96% after a simple extractive work-up. Under these conditions, HPLC and ESMS analysis revealed almost quantitative formation of **3** after 16 h, with no evidence of oligomeric side products (Fig. 2). The remarkably efficient cyclisation presumably stems from the judicious choice of linear precursor sequence that maximises the possibility of favourable 'precyclised' conformations by placing potential turn-inducing residues (MePhe, D-Ala) in the second and fourth positions of the sequence, respectively.[14]

To complete the synthesis, removal of the Pmc and *tert*-butyl protecting groups from **3** was first effected using a cleavage cocktail of TFA/thioanisole/DCM/water (16:2:1:1) for 2 h. HPLC analysis of the crude reaction product revealed several impurities and the rather low isolated yield (28%) of this step therefore partly reflects difficulties experienced in the HPLC purification of **4**. Introduction of the *N*-methyl carbamoyl group onto the N^{ω} position of the Arg side chain was then achieved by the reaction of **4** with 3 equiv of *N*-succinimidyl-*N*-methylcarbamate in DMF at 40 °C with DBU as base. This gave **1** in 65% yield after HPLC isolation and in 17% overall yield from linear peptide **2**. No diacylated or isomeric products were observed. The identity of **1** was confirmed by ESMS (calcd mass 676.3049 Da, observed mass 676.3048 Da), and ^1H and ^{13}C NMR spectra, which were identical to those reported by Arai et al.[9d] for the natural product.[15]

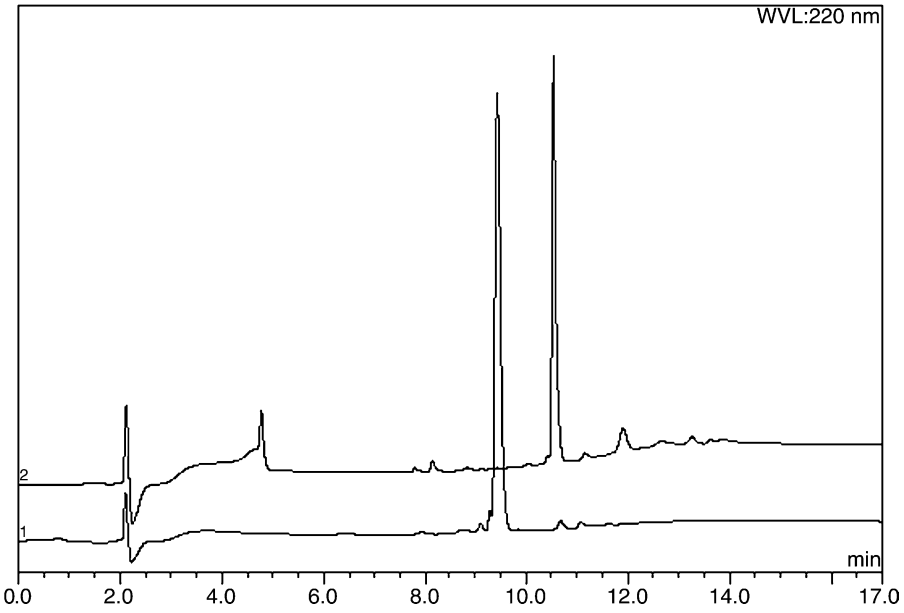

Figure 2. HPLC traces of linear partially protected peptide **2** (lower trace) and crude cyclised peptide **3** (upper trace).[18]

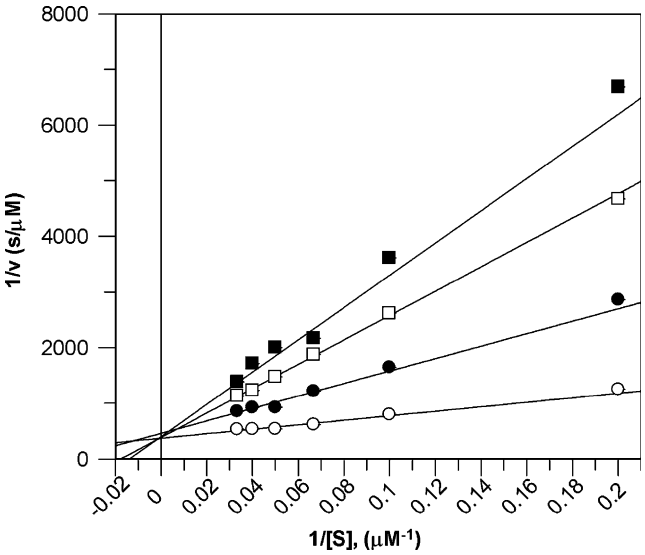

Figure 3. Lineweaver–Burk plots of synthetic **1** measured against *Af*ChiB1 at different concentrations of the inhibitor: no inhibitor (○); 40 nM (●); 80 nM (□); 120 nM (■). A fit of all the data against a competitive inhibition model resulted in a K_i of 17 ± 1 nM with a V_{max} of 2.7 ± 0.1 nM/s and a K_m of 10.4 ± 1.1 μM.

The inhibitory properties of synthetic **1** against the secreted chitinase B1 from *Aspergillus fumigatus* (*Af*-ChiB1) were also investigated using a fluorometric assay with 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobiose as substrate.[16] Steady-state kinetic measurements in the presence of increasing amounts of synthetic **1** show that it inhibits the enzyme competitively with a K_i of 17 nM (Fig. 3). Significantly, and in accordance with X-ray structure data (see below), when **4**, which differs from **1** only in the absence of the *N*-methyl carbamoyl moiety, was evaluated against *Af*ChiB1, no inhibition was observed up to a concentration of 1 mM. This highlights the importance of the derivatised Arg residue for binding of **1** to family 18 chitinases and hence the inhibitory potency of the natural product.

As a further verification of the correct structure and stereochemistry of synthetic **1**, a complex with *Af*ChiB1 was obtained by soaking *Af*ChiB1 crystals in a solution of **1**, as described previously.[17] Diffraction data on a soaked crystal were collected to 1.95 Å resolution, and the unbiased F_o-F_c map was inspected together with the partially refined structure, starting from the previously published *Af*ChiB1–**1** complex (PDB entry 1W9V).[10] This shows that synthetic **1** is identical in both structure and conformation to the *Af*ChiB1–natural product complex previously described by Rao et al. (Fig. 4).[10] As **1** binds to *Af*ChiB1 mainly through active site residues that are strongly conserved in all family 18 chitinases, most notably through interactions of the *N*-methyl carbamoyl moiety with the Glu, Asp, and Trp side chains highlighted in Figure 4, it therefore offers a very attractive new scaffold for the development of novel inhibitors of this class of enzymes.

In summary, the first total synthesis of the natural product family 18 chitinase inhibitor argifin has been achieved utilising a combination of solid- and solution-phase chemistry, and the synthetic compound has been shown to be a low nanomolar, competitive inhibitor of a representative family 18 chitinase. The synthesis is significantly simpler than currently available routes to other potent family 18 chitinase inhibitors and lends itself to the rapid generation of analogues via straightforward solid-phase peptide synthesis. Efficient access to **1** and related compounds should greatly facilitate further investigation into both the role of family 18 chitinases in human conditions (such as asthma and Gaucher's disease), as well as the development of new drugs against a range of human pathogens. Further studies on the synthesis and screening

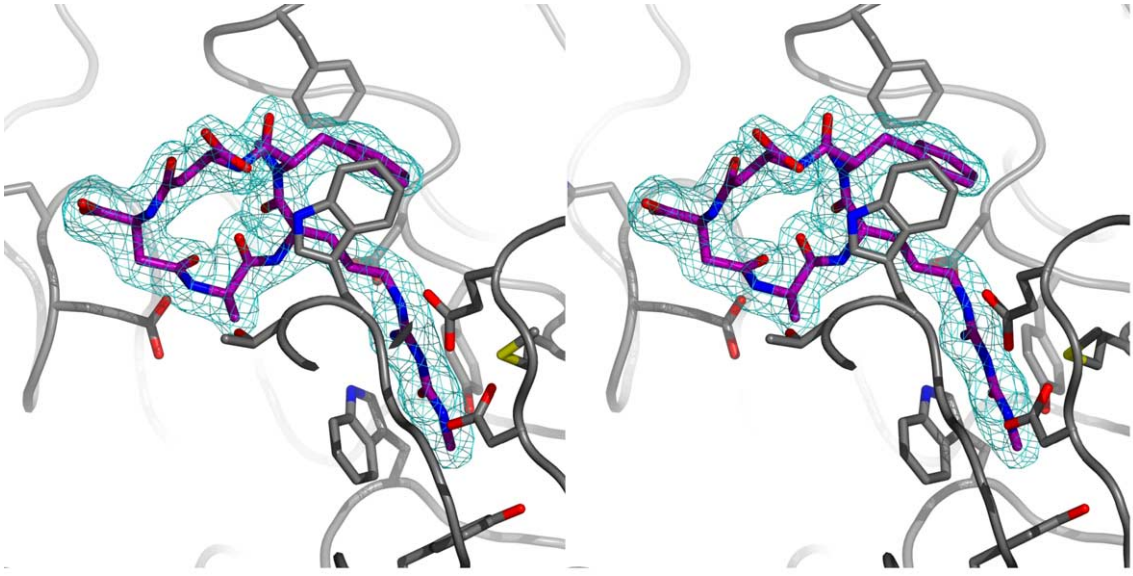

Figure 4. Stereo image of the partially refined crystal structure of *Af*ChiB1 (grey ribbon and sticks with grey carbon atoms) in complex with synthetic **1** (sticks with magenta carbon atoms, model extracted from the previously published *Af*ChiB1–argifin complex, PDB entry 1W9V).[10] Unbiased $|F_o| - |F_c|$, ϕ_{calcd} electron density maps are shown in cyan, contoured at 2.5σ.

of analogues of this natural product are currently being pursued and will be reported in due course.

Acknowledgments

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15. *Experimental conditions, general information*: NMR spectra were acquired on a Bruker Avance DPX 300 spectrometer, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. All coupling constants (J values) were measured in hertz. Low-resolution ES mass spectra were recorded on an ABI Mariner TOF Electrospray Ionisation mass spectrometer in the University of Dundee Post-Genomics and Molecular Interactions Centre. High-resolution ES mass spectra were recorded at the EPSRC National Mass Spectrometry Service (University of Wales, Swansea). Analytical RP-HPLC was performed on a Dionex HPLC system equipped with a Dionex Acclaim 3 μm C-18 (150 × 4.6 mm) column with a flow rate of 1 mL/min. Semi-preparative RP-HPLC was performed on a Dionex HPLC system equipped with a Phenomenex Gemini 5 μm C-18 (250 × 10 mm) column with a flow rate of 2.5 mL/min. Mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in acetonitrile. Gradient 1 was $T = 0$ min, $B = 5\%$; $T = 10$ min, $B = 95\%$; $T = 15$ min, $B = 95\%$; $T = 15.1$ min, $B = 5\%$. Gradient 2 was $T = 0$ min, $B = 5\%$; $T = 20$ min, $B = 60\%$; $T = 22$ min, $B = 95\%$; $T = 27$ min, $B = 95\%$; $T = 27.1$, $B = 5\%$; $T = 32$ min, $B = 5\%$. Gradient 3 was $T = 0$ min, $B = 5\%$; $T = 30$ min, $B = 30\%$; $T = 32$ min, $B = 95\%$; $T = 37$ min, $B = 95\%$; $T = 37.1$ min, $B = 5\%$; $T = 42$ min, $B = 5\%$.
Synthesis of **2**: Loading of the resin was achieved by treating 2-chlorotrityl polystyrene resin (300 mg, 0.51 mmol) with a solution of Fmoc-Asp-OBu^{t} (2 equiv) and DIPEA (8 equiv) in DCM for 90 min. Fmoc deprotection was achieved by treatment with piperidine/DMF (v/v, 1:4) for 4 × 3 min, except in the final deprotection step where HOBt (0.1 M) was also included. Peptide couplings were performed using Fmoc-amino acid (2 equiv), PyBOP (1.9 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in DCM/DMF (v/v, 3:1) for 90 min, except in the case of coupling to MePhe when Fmoc-Arg(Pmc)-OH (2 equiv), PyBrOP (2 equiv) and DIPEA (4 equiv) in DCM/DMF (v/v, 3:1) for 3 × 90 min were used. Cleavage from the resin was achieved by treatment with TFA/DCM (v/v, 1:99) for 10 × 2 min. The cleavage solutions were immediately neutralised by addition to pyridine/MeOH (v/v, 1:9). Solid-phase reactions were monitored by use of a qualitative Kaiser test[19] for the detection of primary amines and the chloranil test[20] for detection of secondary amines. The crude peptide was isolated by precipitation from cold water, collected by centrifugation, dissolved in DCM (20 mL), washed with water (20 mL), dried ($MgSO_4$) and concentrated in vacuo to yield a white solid (238 mg, 0.24 mmol, 46%). RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $t_R = 9.5$ min (100%). MS (ES+) *m/z*: 508.3 (100%) $[M+2H]^{2+}$, 1015.6 (30%) $[M+H]^{+}$.
Synthesis of **3**: Peptide **2** (113 mg, 0.11 mmol) and PyBOP (60 mg, 0.11 mmol) were dissolved in DCM (110 mL), and the solution was adjusted to pH 8 by the addition of DIPEA and stirred for 16 h. The reaction mixture was concentrated in vacuo, the residue taken into EtOAc (20 mL) and washed with 5% aq citric acid (15 mL), 5% aq $NaHCO_3$ (3× 15 mL), satd aq $NaHCO_3$ (3× 15 mL), dried ($MgSO_4$) and concentrated in vacuo to yield a white solid (107 mg, 0.11 mmol, 96%). RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $t_R = 10.6$ min (100%). MS (ES+) *m/z*: 997.7 (100%) $[M+H]^{+}$.
Synthesis of **4**: Peptide **3** (107 mg, 0.11 mmol) was dissolved in TFA/thioanisole/DCM/H_2O (16:2:1:1, 5 mL) and stirred for 2 h. The reaction mixture was concentrated in vacuo, the resulting residue was dissolved in TFA and precipitated by a dropwise addition to ice-cold Et_2O. The precipitate was collected by centrifugation and purified by semi-preparative HPLC (gradient 3, $t_R = 23.1$ min) to

yield a white solid (20 mg, 0.03 mmol, 28%). RP-HPLC (analytical, gradient 2, λ = 220 nm): t_R = 7.9 min (100%). MS (ES+) *m/z*: 619.4 (100%) $[M+H]^+$.

Synthesis of **1**: Peptide **4** (20 mg, 0.03 mmol) and DBU (29 μL, 0.19 mmol) were dissolved in DMF (600 μL) and stirred at 40 °C. A solution of *N*-succinimidyl-*N*-methylcarbamate (17 mg, 0.1 mmol) in DMF (100 μL) was added and the reaction mixture was stirred at 40 °C for 2 h. The crude product was purified by semi-preparative HPLC (gradient 3, t_R = 26.7 min) to yield the natural product as the TFA salt as a white solid (14 mg, 0.02 mmol, 65%). δ_H (300 MHz, D_2O): 8.59 (1H, d, *J* 5, Asp NH), 8.33 (1H, d, *J* 6.5, Arg α-N*H*), 7.38–7.21 (5H, m, MePhe 2 × δC*H*,2 × εC*H*, ζC*H*), 5.12 (1H, dd, *J* 11, 3, MePhe αC*H*), 4.73 (1H, m, Asp αC*H*), 4.52 (1H, dd, *J* 12, 2, Asp αC*H*), 4.31 (1H, m, Arg αC*H*), 4.15 (1H, q, *J* 7, Ala αC*H*), 3.18–2.89 (6H, m, MePhe βCH_2, Arg δCH_2, Asp βCH_2) 2.87 (3H, s, MePhe NCH_3), 2.81 (1H, m, Asp βC*H*H), 2.75 (3H, s, MeCbmNCH_3), 2.50 (1H, t, *J* 12, Asp βCH*H*), 1.40 (1H, m, Arg γCH*H*), 1.30 (3H, d, *J* 7, Ala βCH_3), 1.25–1.04 (2H, m, Arg γCH*H*, Arg βCH*H*), −0.31 (1H, m, Arg βCH*H*). δ_C (75 MHz, D_2O): 175.2 (C), 174.2 (C), 171.4 (C), 171.1 (C), 170.2 (C), 155.0 (C), 153.5 (C), 137.3 (C), 129.6 (CH), 129.0 (CH), 127.2 (CH), 62.2 (CH), 50.7 (CH), 50.6 (CH), 49.5 (CH), 48.6 (CH), 40.6 (CH_2), 37.8 (CH_2), 35.0 (CH_2), 33.2 (CH_2), 29.8 (CH_3), 26.5 (CH_2), 25.9 (CH_3), 23.9 (CH_2), 16.7 (CH_3). RP-HPLC (analytical, gradient 2, λ = 220 nm): t_R = 9.1 min (100%). MS (ES+) *m/z*: 676.4 (100%) $[M+H]^+$. HRMS (ES+) $C_{29}H_{42}N_9O_{10}$ (calcd) 676.3049; (found) 676.3048 $[M+H]^+$.

16. AfChiB1 inhibition was studied using the fluorogenic substrate 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobiose (Sigma), as described previously.[10] Briefly, in a final volume of 50 μL, 2 nM of enzyme was incubated with 5–30 μM substrate in McIlvain buffer (100 mM citric acid, 200 mM sodium phosphate, pH 5.5) containing 0.1 mg/ml BSA, for 10 min at 37 °C in the presence of different concentrations of the inhibitor. After the addition of 25 μL of 3 M glycine–NaOH, pH 10.3, the fluorescence of the liberated 4-methylumbelliferone was quantified using a Flx 800 microtitreplate fluorescence reader (Bio-Tek Instruments Inc.), with excitation and emission wavelengths of 360 nm and 460 nm, respectively, using 40 nm slits. Experiments were performed in triplicate. Production of 4-methylumbelliferone was linear with time for the incubation period used, and less than 10% of available substrate was hydrolysed. The mode of action was determined by plotting the data as Lineweaver–Burk plots, and by fitting all data to the standard competitive inhibition equation with GraFit® software.[21]
17. *Af*ChiB1 was expressed, purified and crystallised, as previously described.[10] Crystals used for soaking experiments were washed three times in 0.1 M sodium citrate, pH 5.5, and 1.4 M Li_2SO_4, and thereafter soaked for 3 h in mother liquor containing 1 mM **1**. Crystals were cryoprotected by a 10 s immersion in 3 M Li_2SO_4 and frozen in a nitrogen cryostream for data collection. Data were collected on a rotating anode (1.95 Å resolution with 97% overall completeness and an overall R_{merge} of 0.063), processed with the HKL suite of programs[22] and partially refined with CNS[23] to an *R*-factor of 0.201 (R_{free} = 0.224). Images were generated with PyMol (www.pymol.org).
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