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First Synthesis of Argadin: A Nanomolar Inhibitor of Family-18 Chitinases

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The first synthesis of the cyclic peptide natural product, argadin is reported. Use of a solid-phase approach featuring side-chain resin attachment through histidine and a novel protecting group strategy allows rapid and efficient access to the argadin backbone, whereupon the unusual 3-amino-5-hydroxy-2-pyrrolidone moiety of the peptide is introduced by oxidative cyclisation of a homoserine residue. Argadin is shown to exist as a 5:1 mixture of diastereoisomers at the 5-hydroxy centre of the pyrrolidone ring, and inhibits a representative family-18 chitinase (ChiB1 from *Aspergillus fumigatus*) with K_i = 33 nM. The high-resolution X-ray crystal structure of synthetic argadin in complex with the same enzyme shows the binding of a single diastereoisomer as previously observed with the authentic natural product.

Introduction

Chitin, a homopolymer of β(1,4)-linked *N*-acetyl-D-glucosamine, is found in a number of organisms that are pathogenic to humans. Chitin is the main structural component of the exoskeleton of insects,[1] the cell wall of fungi[2] and is also found in the eggshells of nematodes.[3] These organisms rely on the ability of enzymes termed chitinases to hydrolyse chitin at key points in their life cycles and consequently chitinase inhibitors have attracted interest as molecules with chemotherapeutic potential. Although chitin is absent from mammalian physiology, two human chitinases[4,5] and several chitin-binding proteins (chi-lectins[6]) have been identified. The over-expression of such proteins has been implicated in several human diseases (e.g. Gaucher's disease,[4] osteoarthritis[6a]), and so chitinase inhibitors are also of interest as selective chemical probes to investigate the role of human chitinases in these disorders.

The most widely studied chitinase inhibitor to date is the pseudotrisaccharide natural product allosamidin (Figure 1),[7] which shows nM inhibition against a range of family-18 chitinases and produces phenotypic effects consistent with chitinase inhibition in fungal and insect pathogens. Very recently, allosamidin has also been shown to inhibit chitinase activity and alleviate pulmonary inflammation in a mouse asthma model,[8] suggesting the exciting prospect of a new approach to the design of anti-asthma drugs. Although total syntheses of allosamidin have been reported by several laboratories,[9] their complexity severely limits the availability of the latter, as well as precluding rapid analogue development. Several other classes of natural product chitinase inhibitors have been reported,[10] but so far they have tended to be relatively weak inhibitors in the mM range and are therefore not obvious surrogates for allosamidin, either as starting points for drug design or biochemical tools.

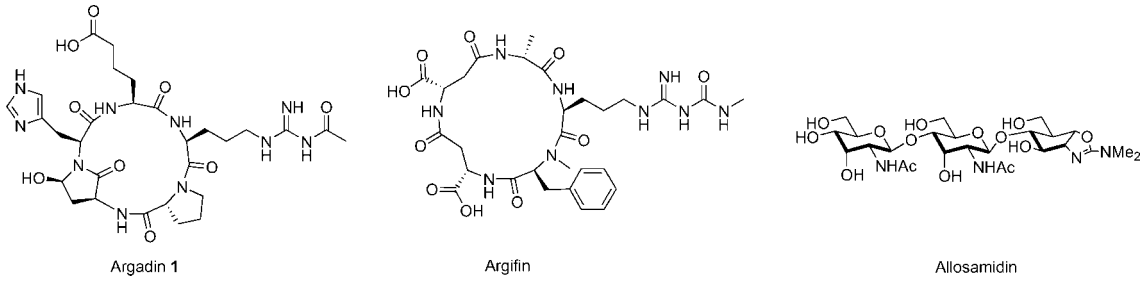

Figure 1. Argadin, argifin and allosamidin.

Recently, the cyclic pentapeptides argadin and argifin (Figure 1), which were isolated from *Clonostachys*[11] and

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Gliocladium[12] fungal cultures respectively, were shown to be potent inhibitors of family-18 chitinases, with argadin typically displaying more potent inhibition. High-resolution X-ray crystallographic structures have been determined for argifin and argadin in complex with several family-18 chitinases, which reveal that these peptide inhibitors precisely mimic the binding of the natural carbohydrate substrates.[13]

Importantly, both compounds are significantly more synthetically accessible than the current benchmark inhibitor allosamidin. We have recently described the first synthesis of argifin by a combined solid phase and solution peptide synthesis approach,[14] and in this paper we now describe the first total synthesis of argadin using methodology suitable for the rapid generation of analogues of this biologically important natural product.

Results and Discussion

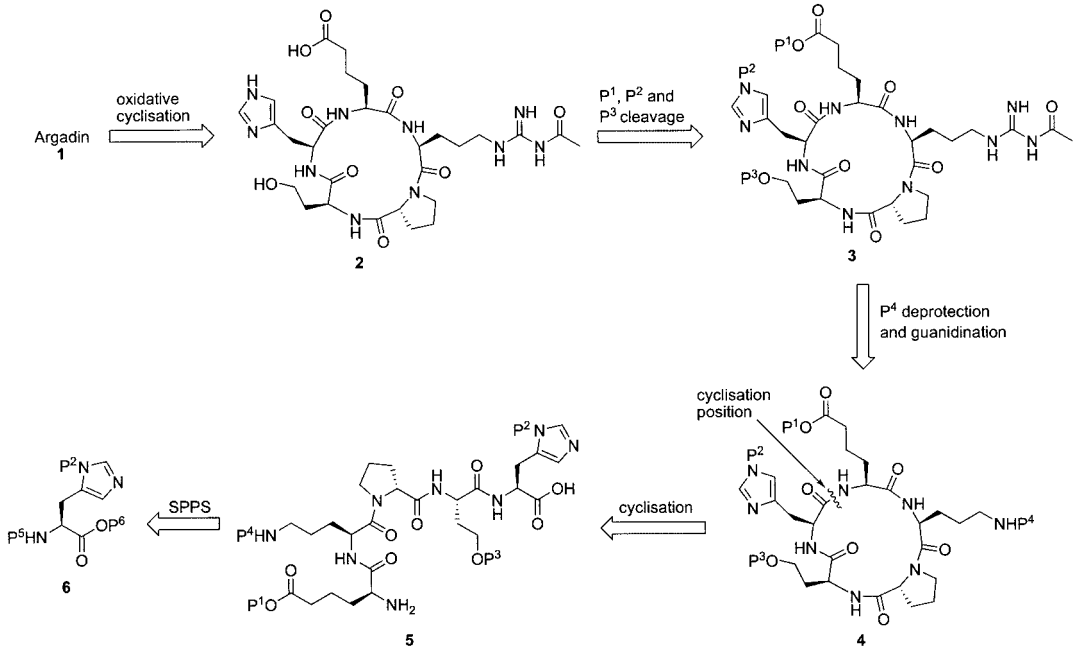
Our retrosynthetic analysis for argadin is outlined in Scheme 1.

For maximum efficiency and flexibility, we envisaged a synthesis in which as many transformations as possible, including peptide cyclisation, were performed on solid phase. The approach features anchoring of the growing peptide precursor to the solid support through the histidine side chain[15] (via group P^2), one-step incorporation of the modified arginine side chain by guanidination of an ornithine residue,[16] and final introduction of the sensitive cyclic hemiaminal moiety by oxidative cyclisation of a fully deprotected homoserine-containing derivative. Cyclisation at the point indicated was chosen so as to place the potentially turn-inducing proline residue mid-way along the precursor linear sequence.[17]

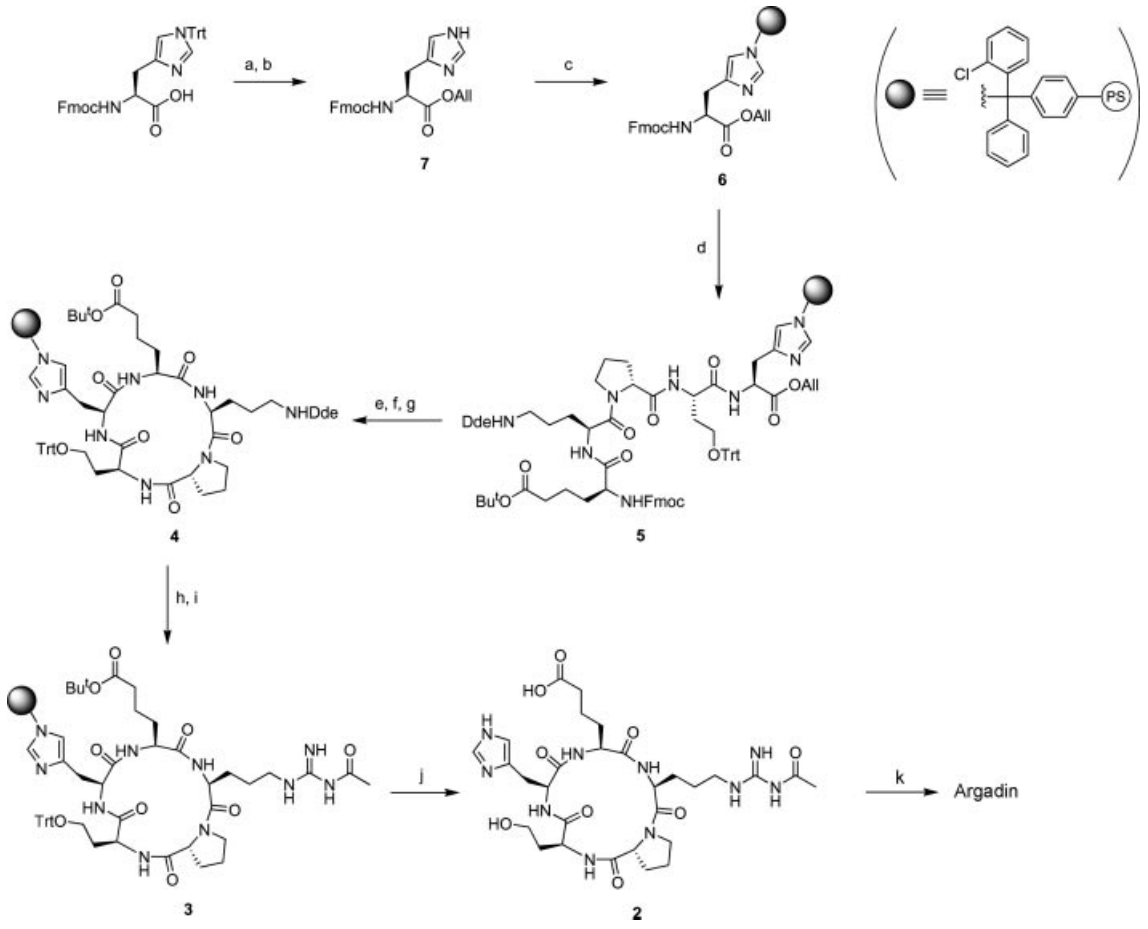
The required level of orthogonality between the solid-phase linker (P^2) and the various protections (P^1, P^3–P^6) necessary was achieved as follows. Acid-labile 2-chlorotrityl chloride polystyrene resin[15] was chosen as solid support (P^2), with Fmoc as temporary N^{α}-protection (P^5), and allyl ester protection, removable under neutral conditions, selected for P^6 so as to be orthogonal to both P^2 and P^5. The Dde group,[18] normally cleaved with hydrazine, and stable to both Fmoc and allyl ester deprotection conditions, was chosen to protect the ornithine side chain of **4**, while further acid-labile protecting groups were chosen for the amino-adipic acid (P^1) and homoserine (P^3) residues (*tert*-butyl ester and trityl ether, respectively).

For the synthesis, Fmoc-His-OAll (**7**) was loaded onto 2-chlorotrityl chloride polystyrene resin to give **6** with a loading of 0.35 mmol/g (Scheme 2).[15]

The loading was deliberately kept low so as to minimise potential dimerisation/oligomerisation at the backbone cyclisation step. Resin-bound pentapeptide **5** was then assembled from **6** by standard Fmoc SPPS using PyBOP activation for coupling,[19] except for the coupling of Fmoc-Orn(Dde)-OH to D-Pro residue, for which PyBrOP[20] was employed. Removal of the *C*-terminal allyl ester ($Pd(Ph_3P)_4/PhSiH_3$[21]) and N-terminal Fmoc protection was followed by cyclisation upon treatment with 2 equiv. of PyBOP for 2×2 h to give the fully protected resin-bound cyclic peptide **4** (confirmed by HPLC/ES-MS analysis of cleaved resin). Selective removal of the Dde group was achieved by brief treatment with hydrazine monohydrate in DMF prior to introduction of the derivatised Arg side chain through reaction with 10 equiv. of the known reagent acetyl methylthiourea hydroiodide[22] in DMF for 2×8 h to give **3**. Once more, analysis of partially protected cleaved



Scheme 1. Retrosynthesis of argadin.



Scheme 2. a) allyl alcohol, DCC, DCM, 65%; b) TFA/DCM/TIPS (10:9:1), 95%; c) 2-chlorotrityl chloride polystyrene resin, DIPEA, DCM; d) Fmoc SPPS; e) $Pd(Ph_3P)_4$, $PhSiH_3$, DCM; f) piperidine/DMF (1:4); g) PyBOP, DIPEA, DCM; h) H_2NNH_2/DMF (1:49); i) acetyl methylthiourea hydroiodide, DIPEA, DMF; j) TFA/DCM/TIPS (10:9:1), 10% from **6**; k) IBX, DMSO, 71%.

peptide confirmed essentially quantitative conversion in the on-resin guanidination cycle. Final cleavage from the resin to yield the desired precursor **2** with concomitant removal of the trityl and *tert*-butyl ester side-chain protection was achieved by treatment with TFA/DCM/TIPS (10:9:1) for 30 min. HPLC and ES-MS analysis of the crude product revealed **2** to be the major species (40% by peak area) present. Purification by reverse-phase semi-prep HPLC then gave **2** in 10% overall yield for the 15 solid-phase steps, on the basis of the original resin loading. On prolonged exposure to the cleavage cocktail, significant quantities of products resulting from cleavage of the cyclic peptide backbone to the *C*-terminal side of homoserine and accompanying loss of the histidine residue were also obtained. This side reaction, which is currently under investigation, was also observed on cleavage of the intermediate peptides **3**–**5**.

For the remaining critical oxidative cyclisation step, purified **2** was treated with 4 equiv. of iodoxybenzoic acid (IBX)[23] in DMSO solution. HPLC analysis after 12 h revealed complete consumption of starting material, and the formation of two new products in an approximate 5:1 ratio (Figure 2).

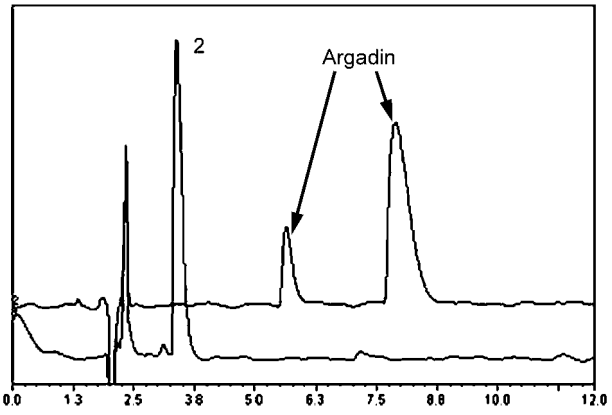


Figure 2. HPLC profiles of **2** (lower trace) and synthetic argadin **1** (upper trace) showing 5:1 mixture of diastereoisomers (for HPLC conditions see supporting information).

MS analysis was consistent with the expected formation of two diastereoisomeric hemiaminals, with ^1H and ^{13}C NMR spectroscopic data for the major diastereoisomer being indistinguishable from those reported by Omura et

al.[11] While numerous examples of peptide natural products containing a homologous glutamic acid-derived hemiaminal have been reported,[24] to the best of our knowledge, this is the first example of the synthesis of such a molecule incorporating the five-membered variant. The oxidation could also be performed using polystyrene-supported IBX,[25] however the rate of conversion was very slow (approx. 10% conversion after 5 d). Although separable by HPLC, the individual argadin diastereoisomers were found to rapidly interconvert to the same equilibrium mixture, which was therefore isolated as such in 71% yield from **2**. It should be noted that HPLC analysis of an authentic sample of argadin also reveals the same equilibrium composition of diastereoisomers.

Synthetic argadin was evaluated against a representative family-18 chitinase, namely the secreted chitinase B1 from *Aspergillus fumigatus* (AfChiB1), using a fluorometric assay with 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobiose as substrate.[13b] Steady-state kinetics measurements in the presence of increasing amounts of synthetic argadin show that it inhibits the enzyme competitively with a K_i of 33 ± 2 nM.

A complex of the synthetic inhibitor with *Af*ChiB1 was also obtained by soaking *Af*ChiB1 crystals with a solution of the diastereoisomeric mixture. Diffraction data on a soaked crystal were collected to 1.7 Å resolution, and the structure was partially refined starting from the previously published *Af*ChiB1-argadin complex (PDB entry 1W9U). Inspection of the unbiased F_o-F_c map reveals that the structure displays a single-bound diastereoisomer, with *R* configuration at the hemiaminal centre, and the hydroxy group occupying a pseudoaxial orientation on the five-membered ring where it is able to form an intramolecular hydrogen bond with the nitrogen of the aminoadipic acid residue. In this way, binding of synthetic argadin to *Af*ChiB1 is identical in both structure and conformation to the *Af*ChiB1-natural product complex previously described (Figure 3).[13b]

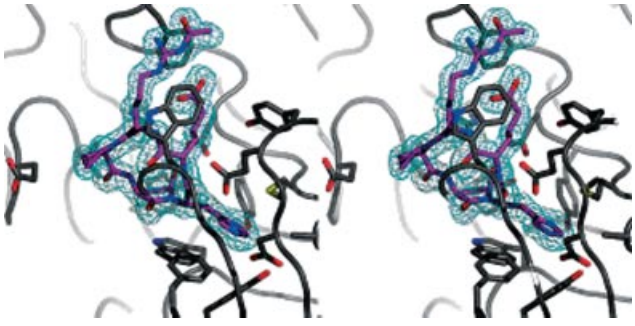


Figure 3. Stereo image of the partially refined crystal structure of *Af*ChiB1 (grey ribbon and sticks with grey carbon atoms) in complex with synthetic argadin (sticks representing carbon atoms, in magenta, model extracted from the previously published *Af*ChiB1-argadin complex, PDB entry 1W9U). Unbiased $|F_o||F_c|[\text{phiv}]_{\text{calcd.}}$ electron-density maps are shown in cyan, contoured at 2.5σ.

As has been previously observed, it would appear that the (2*R*)-hemiaminal configuration allows the inhibitor to adopt a more rigid structure that is more favourable for binding to bacterial family-18 chitinases. This is partly supported by analysis of the more flexible non-cyclised precursor **2**, which shows no inhibition of *Af*ChiB1 up to a concentration of 1 mM.

Conclusions

The first synthesis of the natural product family-18 chitinase inhibitor, argadin, has been achieved by an efficient and highly flexible solid-phase approach. This synthesis allows rapid access to a potent family-18 chitinase inhibitor which may now be used both as a tool for fundamental investigations into the role of family-18 chitinases in human disorders, as well as a potential starting point for the development of new drugs against chitin-containing pathogens. Further studies on the synthesis and screening of analogues of this natural product are currently being pursued and will be reported in due course.

Supporting Information (see also the footnote on the first page of this article): Details of synthesis for **1** and **2**; NMR spectroscopic data of **1**; details of enzymology and X-ray crystallographic experiments.

Acknowledgments

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