RESEARCH COMMUNICATION

The cyclic dipeptide CI-4 [*cyclo*-(L-Arg-D-Pro)] inhibits family 18 chitinases by structural mimicry of a reaction intermediate

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Family 18 chitinases are attractive targets for the development of new inhibitors with chemotherapeutic potential against fungi, insects and protozoan/nematodal parasites. Although several inhibitors have been identified, these are based on complex chemistry, which hampers iterative structure-based optimization. Here we report the details of chitinase inhibition by the natural product peptide CI-4 [*cyclo*-(L-Arg-D-Pro)], which possesses activity against the human pathogenic fungus *Candida albicans*, and describe a 1.7 Å (0.17 nm) crystal structure of CI-4 in complex with the enzyme. The structure reveals that the cyclic dipeptide inhibits chitinases by structurally mimicking a reaction intermediate, and could, on the basis of its accessible chemistry, be a candidate for further optimization.

Key words: allosamidin, *Candida albicans*, glycoside hydrolase, *Plasmodium falciparum*, X-ray crystallography.

INTRODUCTION

Over the past years, glycoside hydrolases have been validated as attractive targets for the design of novel antibiotics against human pathogens [1]. For example, inhibitors of family 18 glycoside hydrolases [which hydrolyse chitin, a polymer of β(1,4)-linked *N*-acetylglucosamine] affect the life cycles of several fungi [2–4] and block transmission of the malaria parasite (*Plasmodium falciparum*) from host to insect vector [5,6]. The most potent inhibitor available, the pseudo-trisaccharide allosamidin, inhibits all family 18 chitinases, yet it is expensive and difficult to synthesize [7–10]. Although carbohydrate oligomers and their derivatives are good candidates for the design of glycoside hydrolase inhibitors, they are often difficult to synthesize and too large to cross cell membranes. An alternative class of compounds for the construction of such inhibitors are peptides. Two glycoside hydrolase peptide inhibitors, argifin and argadin, have recently been co-crystallized with a family 18 chitinase and have been shown to inhibit these enzymes with nano- to micro-molar affinity [11–13]. The complexes revealed that the peptides bind the enzyme by directly contacting residues in the active site and occupying the subsites −1, +1 and +2 [13]. Although these inhibitors are peptides, they contain several unusual side-chain modifications, such as acetylation and cyclization, which negate the synthetic advantages of the otherwise more accessible peptide chemistry. Thus while being remarkably good inhibitors, argifin and argadin may not be ideal leads for further structure-based optimization.

Recently, a much simpler peptide, *cyclo*-(L-Arg-D-Pro) (CI-4), was identified as a natural-product chitinase inhibitor produced by the marine bacterium *Pseudomonas* IZ208 [3] (Figure 1). Although initial analysis showed that the affinity of

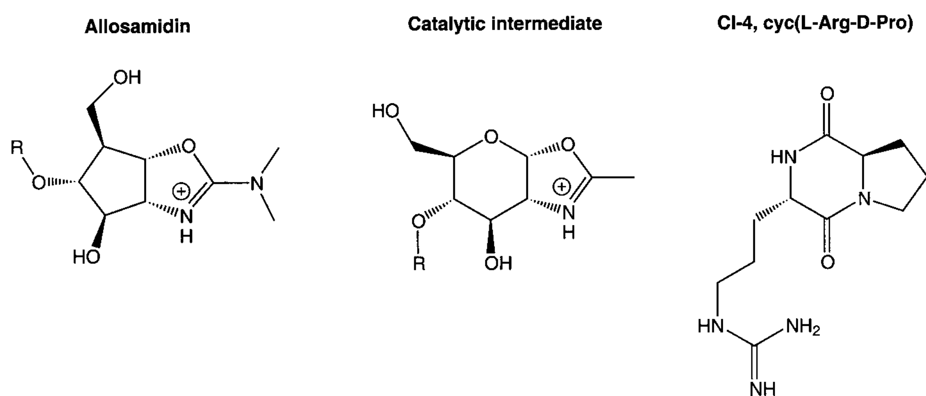

Figure 1 Comparison of the CI-4, allosamidin and catalytic intermediate chemical structures

The chemical structure of CI-4 ['CI-4, cyc(L-Arg-D-Pro)'] is shown, together with the chemical structures of allosamidin and the catalytic intermediate [16,18,19,20] in the −1 subsite. For the intermediate, R indicates the *N*-acetylglucosamine sugars in the −2, −3, etc. subsites. For allosamidin, the allosamizoline unit is shown, with R indicating the allosamine sugars in the −2 and −3 subsites.

Abbreviations used: CI-4, *cyclo*-(L-Arg-D-Pro); ChiB, chitinase B; 4-MU, 4-methylumbelliferone; PDB, Protein Data Bank (Rutgers University); Y214F etc., $Tyr^{214}\rightarrow$ Phe mutant; WT, wild-type.

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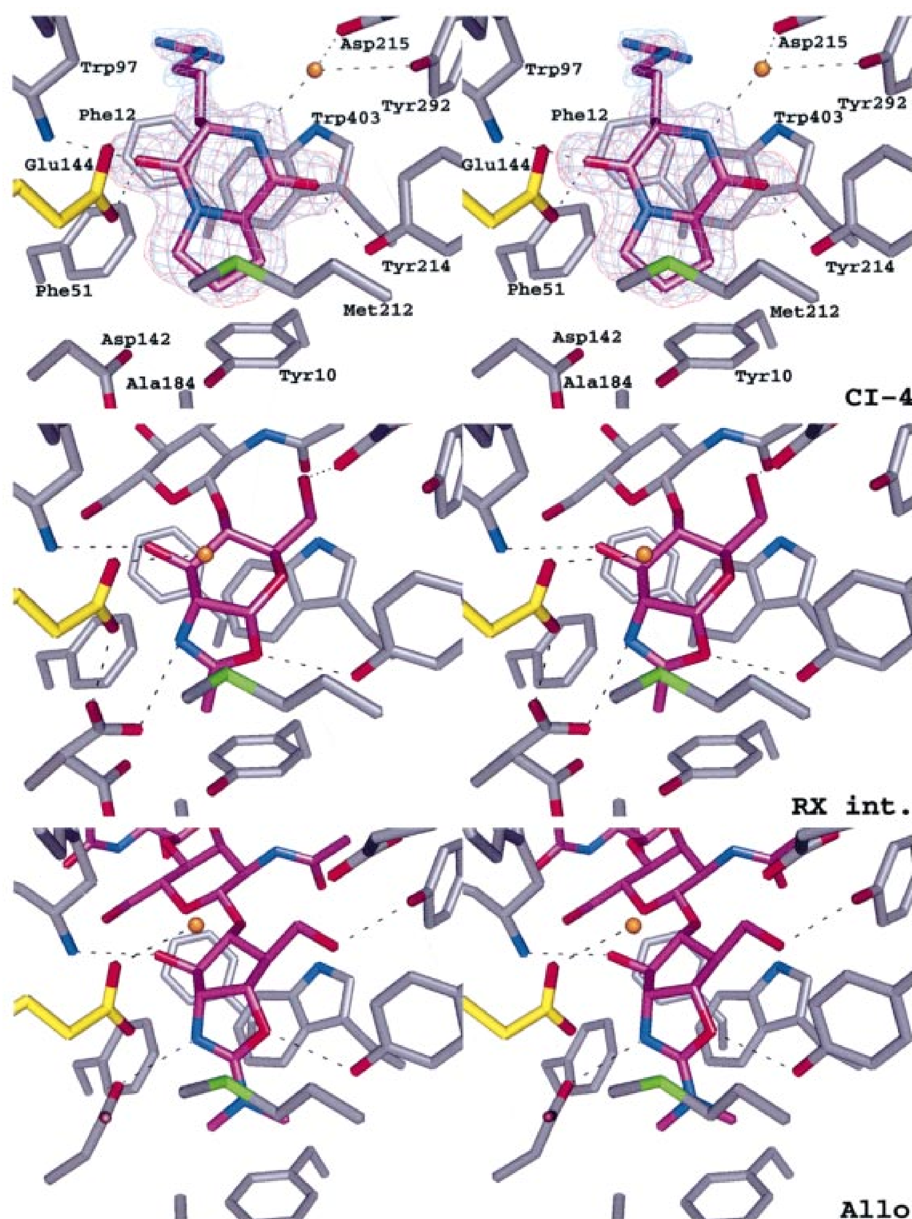

Figure 2 Comparison of the ChiB–CI-4, ChiB–allosamidin and ChiB catalytic intermediate crystal structures

The structure of the ChiB–CI-4 complex is shown ('CI-4'). CNS $2|F_o|-|F_c|$, ϕ_{calc} (blue, contoured at 1.0 σ) and unbiased (i.e. before including any model of the inhibitor) $|F_0|-|F_c|,\phi_{calc}$ (red, contoured at 2.25 σ) maps are also shown. The structure of the previously proposed [16,18,19] catalytic intermediate (for which supporting crystallographic evidence has recently become available [20]; PDB entry 1E6Z) is shown ('RX int.'). The structure of the ChiB–allosamidin complex (PDB entry 1E6R [20]) is also shown ('Allo'). For all structures, amino acids bordering the active site are shown in a 'stick' representation, hydrogen bonds are shown as broken lines (- - - -). Both conformations of Asp[142], as observed in the catalytic intermediate, are shown. Two conformations of Met[212] were observed in the ChiB–CI-4 complex; only the most occupied one is shown for clarity. The catalytic residue (Glu[144]) and the ligands are shown with yellow and purple carbon atoms respectively. The *N*-acetylglucosamine in the −2 subsite, as observed in a ChiB–*N*-acetylglucosamine pentamer complex [20], is shown with grey carbons in the allosamidin complex. Key water molecules are shown as orange spheres. The Figure was made using PyMOL software (http://www.pymol.org).

CI-4 for chitinases was moderate [14], CI-4 is a useful scaffold structure for further optimization because of its straightforward chemistry. Moreover, CI-4 has been shown to possess interesting biological activities, based on its inhibition of chitinases [3,14]. *In vivo* tests showed that CI-4 inhibits separation of daughter cells in cultures of *Saccharomyces cerevisiae* and prevents the progression of the human pathogenic fungus *Candida albicans* from yeast to the infective filamentous form [14]. Chitinases have been shown to play a key a role in *S. cerevisiae* cell separation [2], and have been suggested to play a role in *C. albicans* morphological changes [14]. The notion that the biological effects of CI-4 are based on chitinase inhibition is further supported by the fact that similar effects have been observed for the well-known family 18 chitinase inhibitor allosamidin [4,14,15].

The inhibitory activity of allosamidin is attributed to its similarity to an intermediate formed during catalysis in family 18 chitinases [16]. Unlike most retaining glycoside hydrolases (e.g. hen's-egg-white lysozyme [17]), family 18 chitinases have an

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unusual reaction mechanism that involves attack of the *N*-acetyl group of the substrate itself on the anomeric carbon atom. This leads to the formation of an intermediate that consists of the glucosamine pyranose ring fused to a five-membered oxazoline ring [16,18–20] (Figures 1 and 2). Crystallographic data compatible with such an intermediate have recently been obtained {[20]; Protein Data Bank (PDB) entry 1E6Z}.

To compare the modes of inhibition of CI-4 and allosamidin and to provide a basis for further inhibitor design, we have solved the structure of CI-4 in complex with a family 18 chitinase {chitinase B (ChiB) from *Serratia marcescens* [21]} using 1.7 Å (1 Å = 0.1 nm) X-ray-diffraction data. In addition, inhibitory properties are reported, together with mutagenesis studies probing the roles of some of the active-site residues interacting with CI-4.

METHODS

ChiB from *S. marcescens* was overexpressed in *Escherichia coli*, purified and crystallized as described in [22,21], with mother liquor that included glycerol as a cryoprotectant. CI-4 was synthesized as reported previously [3]. Crystals were soaked for 16 h in mother liquor containing 70 mM CI-4 and frozen in a nitrogen gas stream. Diffraction data were collected at beamline ID29 at the European Synchrotron Radiation Facility, Grenoble, France (Table 1). Refinement was performed using CNS ('**c**rystallography and **N**MR **s**ystem') software, starting from the native ChiB structure [21], interspersed with model building in O [23]. A model for CI-4 was not included until defined by the unbiased $|F_o|-|F_c|$, ϕ_{calc} electron-density maps (Figure 2). Statistics of the final model are described in Table 1. CI-4 was observed to bind the two independent monomers in the asymmetric unit in the same position in the active site. In the interest of simplicity, the structural comparisons with other ChiB complexes are discussed using the same monomer (chain A in all the PDB files) consistently.

Mutagenesis was performed using standard methods described elsewhere [20]. Enzyme activities were determined using 4-methylumbelliferyl β-D-*N*,*N*-diacetylchitobioside as a substrate, in 50 mM citrate/phosphate buffer, pH 6.3. This substrate permits accurate determination of kinetic parameters, despite the fact that relatively low substrate concentrations have to be used ($< K_m$ [22,24]). Standard reaction mixtures contained 2.75 nM wild-type (WT) ChiB or 85–110 nM $Tyr^{214} \rightarrow Phe$ mutant (Y214F) enzyme, 0.1 mg/ml BSA and 20 μM substrate. Reaction mixtures (50 μl) were incubated for 10 min at 37 °C, after which the reaction was stopped with 1.95 ml of 0.2 M Na_2CO_3. The amount of 4-methylumbelliferone (4-MU) released was determined using a DyNA 200 fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA, U.S.A.). These conditions were used in initial inhibition tests aimed at determining IC_{50} values.

For the determination of kinetic parameters, standard reaction mixtures containing 0–20 μM (WT) or 0–40 μM (Y214F mutant) of the substrate were incubated at 37 °C. After 0, 2.5, 5.0 and 7.5 min, 50 μl aliquots were removed and added to 1.95 ml of Na_2CO_3 to stop the reaction. The production of 4-MU over time was linear in all cases (correlation coefficients always better than 0.98), permitting straightforward calculation of enzyme velocities. To study inhibition, reaction mixtures containing inhibitor at a concentration close to the determined IC_{50} value were used. All experiments were conducted four times (independent reactions), meaning that each $[S]/v$ pair used for further calculations represents 12 measurements (four independent experiments, with three time points per experiment). The mode of action for each inhibitor was determined by plotting the data as Lineweaver–Burk plots. The CI-4 inhibitor showed competitive inhibition, whereas inhibition by allosamidin was not purely competitive, as observed previously [20]. Kinetic parameters (K_m, V_{max}, k_{cat}) were calculated by non-linear regression using Hyper (http://www.liv.ac.uk/~jse/abouthyp.html); K_i values were calculated assuming competitive inhibition. Because of limited availability of CI-4, the (high) K_i value for inhibition of the mutant M212A ($Met^{212} \rightarrow Ala$) by this compound could not be determined accurately. The value presented is an estimate based on a limited number of experiments.

Table 1 Details of data collection and structure refinement

Values in parentheses are for the highest resolution shell. Crystals were of space group $P2_12_12_1$ ($a = 55.81$ Å, $b = 103.74$ Å, $c = 186.28$ Å), and were cryo-cooled to 100 K. All measured data were included in structure refinement. Abbreviation: RMSD, root mean square deviation.

Parameter	Value
Resolution range (Å)	30–1.7 (1.76–1.7)
Number of observed reflections	374 203 (27 598)
Number of unique reflections	114 743 (10 457)
Redundancy	3.3 (2.6)
$I/\sigma I$ (signal/noise ratio)	10.7 (3.4)
Completeness (%)	97.1 (89.7)
R_{merge}	0.047 (0.354)
R_{cryst}, R_{free}	0.168, 0.194
Number of groups	993 Residues, 1416 water, 2 CI-4 31 Glycerol, 6 SO_4^{2-} groups
RMSD from ideal geometry	
Bonds (Å)	0.013
Angles (°)	1.6
B-factor RMSD (Å^2)	
Bonded, main chain	1.4
⟨B⟩ (Å^2) (average B-factor)	23.2 (protein), 42.0 (water)

RESULTS AND DISCUSSION

CI-4 (Figure 1) was soaked in native ChiB crystals, and synchrotron diffraction data were collected to 1.7 Å resolution (Table 1). Refinement yielded a model (Figure 2) with an *R*-factor of 16.8 % ($R_{free} = 19.4$ %) and good stereochemistry (Table 1). The maps revealed an ordered CI-4 molecule occupying the active site (Figure 2), which was refined at full occupancy. The average B-factor for the CI-4 backbone and proline is 29.1 Å^2, and for the arginine side chain atoms 48.1 Å^2. This reflects the fact that the arginine side chain is more solvent-exposed and has no direct interactions with the protein (Figure 2).

Comparison of the complexes of ChiB with CI-4, allosamidin and the reaction intermediate [20] (Figure 2) shows that CI-4 not only structurally mimics the intermediate, but also makes similar interactions with the chitinase (Figures 1 and 2). The CI-4 dipeptide backbone six-membered ring overlaps with the pyranose ring of the reaction intermediate. The proline ring occupies the same hydrophobic pocket as the oxazoline ring in the intermediate. The arginine side chain occupies the same space as the *N*-acetylglucosamine in the −2 subsite [20] (Figures 1 and 2). Hydrogen bonds are formed with Glu^{144} (the catalytic acid), Trp^{97}, Tyr^{214} and Asp^{215} (water-mediated). Hydrophobic interactions are made with Ala^{184}, Met^{212} and, most notably, the completely conserved Trp^{403} (Figure 2). All of these residues are highly conserved in family 18 chitinases, including those from human pathogens [5,25,26]. This is the first structural example of a peptide-based inhibitor that mimics a glycosidase reaction intermediate.

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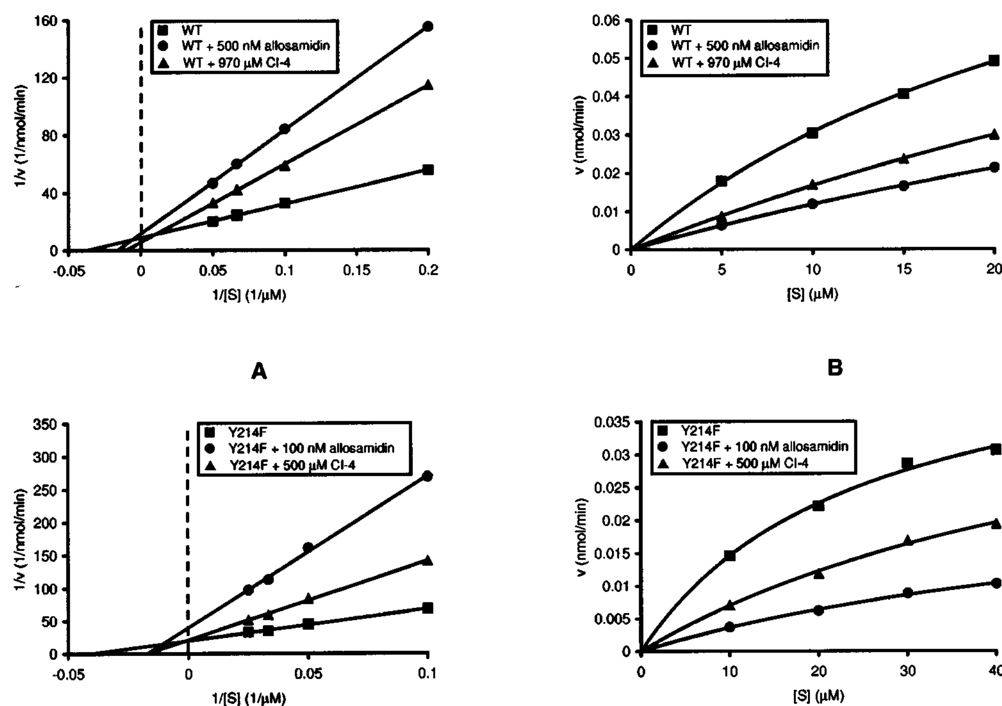

Figure 3 Determination of kinetic parameters for WT ChiB and the Y214F mutant

(**A**) Lineweaver–Burk plots; (**B**) Fit between experimental data (points) and the resulting Michaelis–Menten curves obtained by non-linear regression using Hyper software. Enzyme concentrations: WT, 2.75 nm; Y214F, 110 nM (except with allosamidin, 80 nM). Kinetic parameters obtained from the non-linear regression displayed in (**B**): WT, $K_m = 29.9 \pm 2.2$ mM, $k_{cat} = 14.9 \pm 0.7$ s^{-1}; WT, allosamidin, $K_m = 74.3 \pm 11.0$ mM, $k_{cat} = 12.1 \pm 1.5$ s^{-1}; WT, CI-4, $K_m = 74.5 \pm 8.6$ mM, $k_{cat} = 17.2 \pm 1.7$ s^{-1}; Y214F, $K_m = 24.3 \pm 4.1$ mM, $k_{cat} = 0.15 \pm 0.01$ s^{-1}; Y214F, allosamidin, $K_m = 65.2 \pm 14.4$ mM, $k_{cat} = 0.11 \pm 0.02$ s^{-1}; Y214F, CI-4, $K_m = 60.2 \pm 14.4$ mM, $k_{cat} = 0.15 \pm 0.02$ s^{-1}.

Experiments with a fluorescent hydrolysable substrate revealed that CI-4 inhibits ChiB competitively, with a K_i of 0.65 mM (Figure 3). Comparison with allosamidin, which inhibits ChiB with a K_i of 0.34 μM (Figure 3), provides an explanation for the relatively weak CI-4 inhibition (Figures 1 and 2). The nitrogen atom in allosamizoline has a pK_a of approx. 8 [27] and will thus be positively charged, similar to the oxazoline of the reaction intermediate (Figures 1 and 2). In the allosamidin structure, and in all other ChiB-inhibitor–substrate complexes [13,20,24,] (with the exception of argadin [13]), Asp^{142} is pointing towards the inhibitor to stabilize this positive charge (Figure 2). In the complex with CI-4, such conformation for Asp^{142} would be unfavourable, as this would bring it in close proximity with the aliphatic side chain of the CI-4 proline residue. In addition, experiments on allosamidins lacking the *N*-acetylallosamine sugars in the -3 and -2 subsites have revealed that the K_i of the allosamizoline unit alone is three orders of magnitude higher than that of allosamidin [28], bringing it in the same range as CI-4. Thus the two allosamidin allosamine sugars in the -2 and -3 subsites contribute the major part of the affinity of allosamidin for family 18 chitinases, and CI-4 appears to be a good mimic of the allosamizoline (Figure 1).

Met^{212} is a residue lining the hydrophobic pocket occupied by the methyl group on the reaction intermediate and the methyl groups of the allosamizoline group in allosamidin (Figure 2). Met^{212} also makes a hydrophobic contact with CI-4, although the electron density indicates that this residue assumes two possible conformations (results not shown). To investigate the contribution of Met^{212} to CI-4 binding, we constructed the ChiB Met$^{212} \rightarrow$ Ala mutation. The mutant enzyme showed an approximate 5-fold reduction in affinity for CI-4 (results not shown), confirming the importance of the hydrophobic stacking between Met^{212} and the CI-4 proline ring (Figure 2).

Tyr^{214} is a conserved residue in family 18 chitinases. It hydrogen-bonds to the oxygen atom of the N-acetyl group on the sugar in the -1 subsite [20] and makes a similar interaction with allosamidin and the reaction intermediate (Figure 2). In the CI-4 complex, Tyr^{214} could hydrogen-bond with the backbone oxygen atom of proline. We constructed the Tyr$^{214} \rightarrow$ Phe mutation to investigate the contribution of this interaction to the affinity for CI-4. The mutant enzyme bound the inhibitor slightly better ($K_i = 0.34$ mM; Figure 3). Interestingly, the Tyr$^{214} \rightarrow$ Phe mutation has also been shown to increase substrate binding in family 18 chitinases [29,30]. In addition, the inhibition of the ChiB Tyr$^{214} \rightarrow$ Phe mutant with allosamidin showed an increased affinity with a K_i of 59 nM, compared with 340 nM for the wild-type enzyme (Figure 3). Thus it is apparent that the hydroxy group of Tyr^{214} does not contribute to the binding of either the substrate, allosamidin or CI-4. Since the Tyr$^{214} \rightarrow$ Phe mutation does significantly affect catalysis [29], it is possible that the hydrogen bond plays a role in the stabilization of the transition state rather than contributing to the binding of substrate.

Although CI-4 itself inhibits chitinases with relatively low affinity, its accessible chemical make-up could allow structure-based optimization of inhibitory and physicochemical properties. For example, whereas sugars at the -1 position hydrogen-bond directly to Asp^{215} ([20]; Figure 2), CI-4 interacts with Asp^{215} through an ordered water molecule (Figure 2). An *N*-hydroxymethyl group on the CI-4 arginine residue would allow formation of a direct hydrogen bond and may increase affinity.

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In addition, the flexible CI-4 arginine side chain does not interact with the protein directly and can be replaced with other amino acids, while leaving the structural mimicry of the reaction intermediate intact (Figures 1 and 2). This would be especially beneficial in the light of the data on the allosamidin derivatives described above, which shows that the allosamine sugars in the -2 and -3 subsites contribute considerably to the binding energy [28]. Furthermore, in complexes of family 18 chitinases with substrates, the side chain of Asp^{142} is flipped towards the substrate to interact with the *N*-acetyl side chain of the -1 sugar [20,29,30] (two Asp^{142} conformations are observed in the structure with the reaction intermediate [20]; Figure 2). With CI-4, this key interaction cannot be mimicked, because the proline side chain solely consists of methylene groups. Substitution of one of these methylene groups with an ether oxygen, the use of a hydroxyproline, or the incorporation of a group carrying a positive charge could further increase interaction with the inhibitor through stronger interaction with Asp^{142}.

CONCLUSIONS

We have shown how a cyclic dipeptide with activity against the fungal pathogen *C. albicans* inhibits family 18 chitinases through structural mimicry of a reaction intermediate. Although CI-4 shows relatively weak inhibition, this can be explained by the lack of key interactions with Asp^{142} and the -2, -3 sugar subsites. The structure of the complex leads to suggestions as to how it could be used as a template for development of more potent CI-4 inhibitors, which may have possible chemotherapeutic potential as fungicides and anti-malarials.

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