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Antifungal Spectrum, In Vivo Efficacy, and Structure–Activity Relationship of Ilicicolin H

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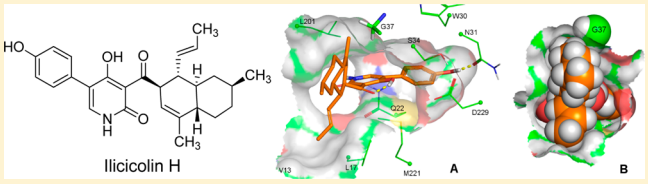
Supporting Information

ABSTRACT: Ilicicolin H is a polyketide—nonribosomal peptide synthase (NRPS)—natural product isolated from *Gliocadium roseum*, which exhibits potent and broad spectrum antifungal activity, with sub-μg/mL MICs against *Candida* spp., *Aspergillus fumigatus*, and *Cryptococcus* spp. It showed a novel mode of action, potent inhibition (IC_{50} = 2–3 ng/mL) of the mitochondrial cytochrome bc1 reductase, and over 1000-fold selectivity relative to rat liver cytochrome bc1 reductase. Ilicicolin H exhibited in vivo efficacy in murine models of *Candida albicans* and *Cryptococcus neoformans* infections, but efficacy may have been limited by high plasma protein binding. Systematic structural modification of ilicicolin H was undertaken to understand the structural requirement for the antifungal activity. The details of the biological activity of ilicicolin H and structural modification of some of the key parts of the molecule and resulting activity of the derivatives are discussed. These data suggest that the β-keto group is critical for the antifungal activity.

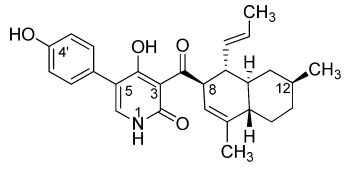


KEYWORDS: *ilicicolin H, Gliocadium roseum, antifungal activity, broad spectrum, cytochrome bc1 reductase inhibitor, in vivo efficacy*

Infections caused by pathogenic fungi (e.g., *Candida albicans* and *Aspergillus fumigatus*) are life-threatening, particularly to immunocompromised populations.[1] Three main therapeutic options exist for the treatment of such infections, including azoles (e.g., fluconazole),[2] macrocyclic polyenes (e.g., amphotericin),[3] and candins (e.g., caspofungin, micafungin, and anidulafungin).[4] Each treatment option has limitations to its utility, thus creating a need for new antifungal agents.

Screening of natural product extracts against *C. albicans* whole cell screen led to the identification of ilicicolin H (**1**) produced by *Gliocadium roseum*.[5] The structure of **1** was elucidated by 2D NMR and mass spectral studies.[6,7] This tetracyclic polyketide was originally reported in 1971 from the mycelium of an imperfect fungus, *Cylindrocladium ilicicola*, with only weak antibiotic activity against *Bacillus anthracis* (6 μg/mL).[8] The structure elucidation by chemical degradation was reported in 1976[9] followed by NMR assignment and antifungal activity against *C. albicans*.[10] The structure was confirmed by a total synthesis of racemic ilicicolin H.[11] The precursor feeding experiments helped elucidate biosynthesis as a polyketide, and the two methyl groups originate from *S*-adenosylmethionine.[12] We describe, now, the antifungal spectrum, in vivo efficacy, mechanism of action, and structure–activity relationship (SAR) from the modification of the core structure of ilicicolin H (**1**).

The antifungal spectrum of ilicicolin H is comparable to comparators including caspofungin, amphotercin B, and fluconazole (Table S1 in the Supporting Information). The activity against *C. albicans* ranged from 0.04 to 0.31 μg/mL, while in other *Candida* species, sensitivities ranged from 0.01 to 5.0 μg/mL. Fluconazole-resistant *C. albicans* strain, MY2301, was sensitive to iliciolin H. Ilicicolin H also inhibited the growth of *Cryptococcus* species with minimum inhibitory concentration (MIC) values of 0.1–1.56 μg/mL, which is generally superior to comparators. When evaluated in a limited panel of *Aspergillus* species, ilicicolin H was shown to inhibit *A. fumigatus* (MIC 0.08 μg/mL), while *A. flavus* was resistant.



Ilicicolin H (**1**)

The in vivo efficacy of ilicicolin H was assessed in a disseminated *C. albicans* (MY1055) mouse infection model.[13] Briefly, DBA/2 mice were infected intravenously with 5.4×10^4 colony-forming units (cfu) of *C. albicans* (MY1055). Ilicicolin H was administered orally twice daily in 10% aqueous DMSO for 2 days at doses of 50, 25, 12.5, and 6.25 mg/kg. Four days after infection, kidney *Candida* burden was determined. Significant reduction ($p < 0.05$; Excel *t* test) in kidney burden relative to vehicle-treated controls was achieved at 50 mg/kg,

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with an effective dose 90 (ED_{90}, dose leading to 90% reduction of *C. albicans* in kidney) of 15.45 mg/kg/dose. Caspofungin treatment (intraperitoneally) resulted in an ED_{90} of 0.01 mg/kg. Ilicicolin H also showed significant activity ($p < 0.05$; Excel *t* test) in an abbreviated 24 h version of the candidiasis model when dosed intravenously thrice daily at a total dose of 50 or 25 mg/kg/day, with an ED_{90} of 22.3 mg/kg/day. In a similar mouse model of disseminated *Cryptococcus neoformans* (MY2061) infection (8×10^5 cfu/mouse), ilicicolin H reduced spleen burden by over 1 log cfu/g of spleen ($p < 0.05$; Excel *t* test) when administered orally at either 100 or 50 mg/kg intraperitoneally twice daily in 5% aqueous DMSO.

The antifungal activity of ilicicolin H was dependent on the carbon source used in the test media. MICs of the compound in glucose-based media were >50 μg/mL against *Saccharomyyces cerevisiae* MY 2141 and *C. albicans* MY 1055, but in media containing a nonfermentable carbon source, such as glycerol, the MICs against these yeast strains were 0.012 and 0.025 μg/mL, respectively. The effect of carbon source on antifungal activity was accounted for by inhibition of respiration. Whole cell oxygen consumption was inhibited 100% in *S. cerevisae* and in *C. albicans* at 0.003 μg/mL. Measurements of the effect of ilicicolin H on substrate-dependent rates of oxygen consumption by coupled mitochondria from *S. cerevisiae* MY 2141 revealed that complex I–III was the most sensitive complex in the respiratory chain (Figure 1). The IC_{50} values for inhibition

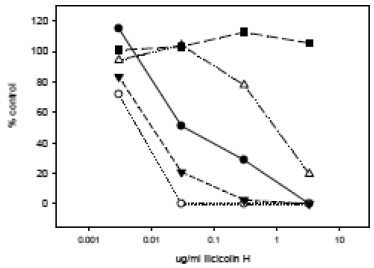

Figure 1. Effect of ilicicolin H on substrate-dependent rates of oxygen consumption by coupled mitochondria from *S. cerevisiae* MY 2141 substrates used to measure oxygen consumption. Complex I–III: NADH (●), ethanol (○), and glycerol-3-phosphate (▼). Complex II–III: succinate (△). Complex IV: ascorbate/TMPD (■).

of complex I–III by ethanol, glycerol-3-phosphate, and NADH were 0.008, 0.02, and 0.08 μg/mL, respectively. Inhibition of complex II–III and IV was orders of magnitude less sensitive with IC_{50} values of 1.0 and >10.0 μg/mL, respectively. Inhibition of complex I–III was accounted for by inhibition of NADH:cytochrome c oxidoreductase; IC_{50} values of 0.8 and 1.0 ng/mL (1.85 and 2.31 nM) were determined for *C. albicans* and *S. cerevisiae*, respectively. Ilicicolin H was shown to inhibit the center N (Q_n site, also called Q_i site) of the cytochrome bc1 complex with an IC_{50} value of 3–5 nM in the ubiquinol:cytochrome c reductase of *S. cerevisiae*.[14]

Isolation of ilicicolin H-resistant mutants in *S. cerevisiae* showed that resistance was accounted for by seven types of single amino acid changes at four sites in the cytochrome *b* gene involved in ubiquinone binding.[15,16] In an independent study, ilicicolin H-resistant mutants in *C. albicans* were isolated with a frequency of 10^{-7}. Five single amino acid changes at three sites in the cytochrome *b* gene, many of which were identical to those described in *S. cerevisiae*, were identified (Table S2 in the Supporting Information). Multiple resistant mutants were detected in both yeast strains with mutations at position G37. The MICs to ilicicolin H in the *C. albicans* resistant mutants were 250–1000-fold higher than the wild-type strain, and resistance was accounted for by resistance in both NADH:cytochrome c oxidoreductase and the ubiquinol:-cytochrome c reductase (Table S3 in the Supporting Information). Interestingly, none of the ilicicolin H-resistant mutants in *S. cerevisiae* or in *C. albicans* displayed cross-resistance with any of the known cytochrome bc1 inhibitors such as antimycin or myxothiazol.

The NADH:cytochrome c oxidoreductase from *C. albicans* MY1055 was orders of magnitude more sensitive than the enzyme from rat or rhesus liver. Ilicicolin H showed IC_{50} values of 0.8, 1500, and 500 ng/mL, respectively. In contrast, antimycin showed no selectivity in this assay with IC_{50} values of 0.5, 0.5, and 0.1 ng/mL, respectively, against *C. albicans* MY1055 and rat and rhesus NADH:cytochrome c oxidoreductase. Independent studies by Gutierrez et al.[14] showed >70-fold selectivity for the yeast ubiquinol: cytochrome c reductase (*S. cerevisiae*) as compared to the bovine reductase (IC_{50} = 87–108 ng/mL, 200–250 nM).

There are several possible explanations that may account for the selective action of ilicicolin H against NADH:cytochrome c reductase prepared from lower and higher eucaryotes. First, it is postulated that the binding interactions for the substrate, ubiquinone, differ. The yeast ubiquinol possesses a shorter length isoprene side chain (n = 4–6) as compared to mammalian ubiquinol (n = 9–10). Second, a sequence alignment of cytochrome *b*, focusing on segments containing the Q_i site amino acids, points out the differences between two lower (*S. cerevisiae* and *C. albicans*) and two higher (bovine and rat) species (Figure S1 in the Supporting Information). *S. cerevisiae* and *C. albicans* possess high overall sequence homology (76% identity and 90% similarity) with even higher homology of residues forming the Q_i site (91% identity and 95% similarity). Homology to higher species decreases (e.g., *S. cerevisiae* vs bovine: 51/72% for overall identity/similarity and 54/73% for Q_i site) and may provide a possible explanation for ilicicolin's selectivity. For example, bulky and aromatic tyrosine at position 16 in lower eukaryotes is replaced by smaller amino acid (alanine or serine) in higher eukaryotes. Other examples of dramatic differences in Q_i site amino acids between lower and higher eukaryotes occur at positions 17, 22, 27, 221, and 225 (Figure S1 in the Supporting Information).

The modest efficacy of ilicicolin H was surprising given the excellent in vitro MICs against target yeast strains and relatively good pharmacokinetics properties in mice, which showed low clearance of 16 mL/min/kg, reasonable half-life (2.5 h), and excellent oral bioavailability (F = 72%). It is postulated that low in vivo activity as compared to in vitro activity was attributed to high plasma protein binding as shown by progressive loss of activity in the presence of varying % of plasma in the cytochrome c reductase assay. A complete loss of activity was observed in the presence of 50% mouse plasma (Figure S2 in the Supporting Information). Similarly, the *C. albicans* activity (MIC) of ilicicolin H was shifted to >1000 ng/mL when tested in the presence of 10% mouse serum.

With broad antifungal spectrum, a novel mode of action, and in vivo efficacy, ilicicolin H (**1**) represented a great starting point for structural modification to define SAR and improve in vitro potency and efficacy. Because the modest in vivo efficacy is likely directly related to very high serum binding, one of the goals of the program was to explore the feasibility of reducing

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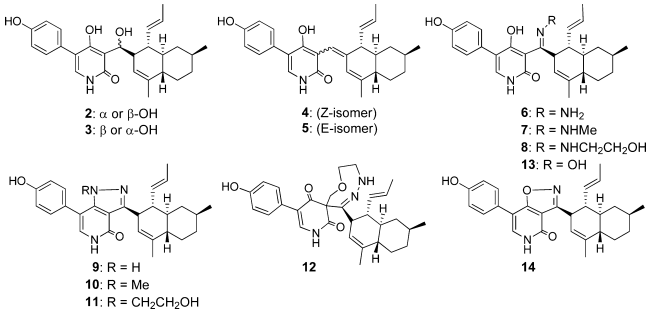
protein binding to increase in vitro and in vivo antifungal activity. To accomplish this goal, structural modifications to ilicicolin H were performed using a chemical, biotransformation, and enzymatic approaches. For initial SAR, the semisynthetic compounds were tested for whole cell MIC for *C. albicans* (MY1055), IC_{50} of *C. albicans*, and rat liver NADH:cytochrome c1 reductases. The effect of 50% serum on enzymatic activity was assessed as well.



The highly acidic and metal chelating *β*-diketo group (C4−C3−C7) is likely playing a significant role in the serum binding; therefore, this became a first focal point for chemical modification. Sodium borohydride reduction of **1** produced >70% yield of a diastereomeric mixture of two alcohols **2** and **3** (ratio 4:1), which underwent facile elimination under acidic condition to afford the exocyclic olefins **4** and **5**. To eliminate the chelating properties of the *β*-diketone while conserving the overall conformation and electronic properties of ilicicolin H, a series of oxime, hydroazone, pyrazole, isoxazole, and novel 1,4,5-oxadiazocines analogues were synthesized. The syntheses of hydrazones (**6**−**8**), pyrazole (**9**−**11**), and oxadiazocine (**12**) were recently reported.[17] The oxime (**13**) and isoxazole (**14**) were prepared by reaction of **1** with hydroxyl amine at room temperature followed by heating with acetic acid. Both geometrical isomers of oxime and hydrazones were formed but did not show any difference in their activity; therefore, the data for only one of the isomer are shown. Activities of these compounds are reported in Table S4 in the Supporting Information. In general, chemical modifications of the *β*-diketo group led to significant loss of the whole cell *C. albicans* activity with concomitant attenuation of fungal cytochrome bc1 reductase activity. Surprisingly, these compounds became less selective with respect to rat liver cytochrome c1 reductase activity. However, compounds that retained the general molecular shape of ilicicolin H, for example, hydrazones **6**−**8** and corresponding pyrazoles **9**−**11**, lost only 10−20-fold antifungal activity as compared to compounds with significant change in the molecular shape (e.g., **2**−**5**), which lost >250-fold activity (Table S4 in the Supporting Information). It appears that the retention of the shape is not sufficient for the retention of the activity, for example, the oxadiazocine (**12**), oxime (**13**), and isoxazole (**14**) did not show any activity at 10000 ng/mL. The whole cell activity SAR and the fungal reductase activity SAR did not track well. The pyrazole **9** showed about 10-fold loss in the antifungal activity (MIC 250 ng/mL) while maintaining the same fungal reductase activity (IC_{50} 3 ng/mL) and selectivity.

To facilitate the understanding of the structural aspects of ilicicolin H enzyme inhibition and SAR, a binding model of ilicicolin H in *C. albicans* cytochrome bc1 was constructed. Because no crystal structure of ilicicolin H and *C. albicans* cytochrome bc1 complex is available, we used a homology model based on the crystal structure of yeast (*S. cerevisiae*) cytochrome bc1 complex with ubiquinol in Q_i site.[18]

As mentioned before, the residues forming cytochrome *b* Q_i site in both proteins are practically identical, with binding site similarity ∼95% (Figure S1 in the Supporting Information). The homology model therefore provides a reliable representation of the *C. albicans* Q_i binding pocket that could provide insights into ilicicolin H SAR. Because antimycin is a potent Qi site inhibitor, we used antimycin's binding mode in the crystal structure of bovine bc1 complex[19] as an initial template for ilicicolin H binding (Figure S3 in the Supporting Information). A refined model of ilicicolin H binding pose was subsequently obtained by minimization of a complex with protein heavy atoms fixed.[20]

The initial overlay of ilicicolin H to antimycin presents several notable features. The hydrophobic decalin group of ilicicolin H is pointing out of the binding pocket toward the lipid bilayer. The allyl group is positioned similarly to the pentyl group of antimycin, interacting with hydrophobic residues at the exposed end of the binding pocket (V13 and L17 in Figure 2A). The hydroxy phenyl group of **1** is roughly

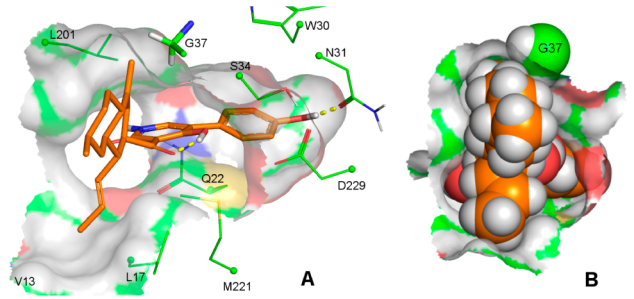


Figure 2. (A) Binding mode of ilicicolin in the *C. albicans* model. Several key residues are shown as thin sticks. G37 is shown in thicker sticks. (B) To better appreciate the steric constraints of the binding pocket, ilicicolin H is shown in CPK representation. The necessity for a perpendicular orientation of the left- and right-hand side of ilicicolin H is obvious. G37 C*α* carbon with hydrogens shown is also displayed in CPK.

aligned with antimycin's *N*-formylamino-salicyl group pointing deep into the pocket and likely being responsible for some of the binding affinity and specificity. The shape of the binding pocket necessitates a conformation where two sides of the molecule fall roughly into two perpendicular planes. While antimycin served as a good template for initial positioning of gross hydrophobic and polar features of ilicicolin H, the two inhibitors represent distinct chemical classes and differ in the details of their interactions in the binding pocket. Specifically, the key interaction of D229 with antimycin's phenolic OH and the formylamido NH is absent in ilicicolin, which instead engages hydroxyl phenyl in the interaction with N31. The amide linker in antimycin also presents very different donor/acceptor pattern than the pyridinone moiety in ilicicolin H, even though both functional groups are assumed to occupy the same space. Those and other differences in chemical nature and interaction patterns of the two inhibitors unsurprisingly lead to differences in their biophysical characterization as discussed by Trumpower.[21]

The ilicicolin H bioactive conformation (Figure 2) has left-hand side of the molecule buried deep in the binding pocket and lipophilic right-hand side aligned with the pocket opening and rotated ∼90° relative to hydroxyl-pyridinone central ring.

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The adjacent carbonyl linker is forming an intramolecular hydrogen bond with the hydroxyl group of the pyridinone ring. The presence of this internal hydrogen bond is supported by two observations. In solution, ilicicolin H forms six-center hydrogen bond as confirmed by the NMR spectrum (data not shown). More importantly, compound **9**, the pyrazole analogue, with locked conformation similar to those with intramolecular hydrogen bond, is active in the fungal cytochrome c1 reductase assay (Table S4 in the Supporting Information).

The highly twisted nature of the proposed bioactive conformation has important implications for SAR. The binding mode further suggests that the phenolic group of the hydroxy phenyl makes a hydrogen bond interaction with N31 side chain, which is likely to be responsible for the specificity of ilicicolin's binding. The crystal structure of antimycin complex suggests that D229 makes critical hydrogen bonds. As already mentioned above, we do not observe this interaction since it would only be accessible to ilicicolin H analogues with hydroxy substitution in the ortho or meta position. The model also allows us to speculate about the loss of activity of ilicicolin H in G37 mutants. Mutations of that residue will increase the bulk in that region and cause a steric clash with ilicicolin H. The tight packing of ilicicolin H relative to G37 in the pocket is illustrated in Figure 2B.

Ilicicolin H is a natural product produced by an imperfect fungus, *C. ilicicola* and *G. roseum*, which showed broad spectrum antifungal activity. It imparts its activity by selectively inhibiting fungal cytochrome c1 oxidoreductase activity and respiration. It demonstrated modest in vivo activity in a *Candida* and *Cryptococcus* infection mouse model. The in vivo activity was limited by high plasma protein binding. Preliminary medicinal chemistry efforts pointed out the criticality of the β-diketone feature of the molecule and lead to mostly less active or inactive compounds. The homology model suggests that its binding mode has some similarities but also differences relative to antimycin binding and provides valuable insight to SAR and fungal specificity. These studies open the window for future work on ilicicolin H and the development of new modes of action antifungal agents.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedure for bioassays and mutation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

†Deceased.

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