# Cyclic Colisporifungin and Linear Cavinafungins, Antifungal Lipopeptides Isolated from Colispora cavincola

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# Cyclic Colisporifungin and Linear Cavinafungins, Antifungal Lipopeptides Isolated from *Colispora cavincola*

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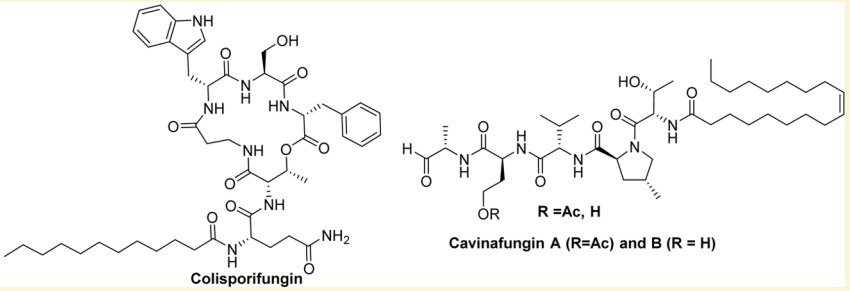
Supporting Information




**ABSTRACT:** Colisporifungin (**1**), a cyclic depsilipopeptide structurally related to the aselacins, and cavinafungins A and B, two linear peptides, were isolated from liquid culture broths of the hitherto unstudied fungus *Colispora cavincola* using a *Candida albicans* whole-cell assay as well as a bioassay to detect compounds potentiating the antifungal activity of caspofungin. The structural elucidation, including the absolute configuration of the new molecules, was accomplished using a combination of spectroscopic and chemical techniques, including 1D and 2D NMR, HRMS, and Marfey's analysis. The cyclic peptide colisporifungin displayed a strong potentiation of the growth inhibitory effect of caspofungin against *Aspergillus fumigatus* and, to a lesser extent, against *Candida albicans*. The linear peptides displayed broad-spectrum antifungal activities inhibiting growth of *Candida* species (MIC values 0.5−4 μg/mL) as well as *A. fumigatus* with a prominent inhibition of 8 μg/mL.

Invasive fungal infections (IFI), particularly in the immunocompromised patient population, are characterized by diagnostic difficulties, leading to extreme mortality with fatality rates ranging from 30% to 80% specifically in neutropenic patients.[1] *Candida* and *Aspergillus* species are the most common causes of invasive fungal infections, but other yeasts and filamentous fungi are also emerging as pathogens.[2] The most frequently used antifungal agents to treat this life-threatening infection are polyenes (amphotericin B) and triazole drugs, targeting the cell membrane, a structure common to all eukaryotic cells.[3] The echinocandins are the newest class of antifungal agents approved for treatment of IFI. They inhibit the synthesis of β-(1,3)-D-glucan in fungal cell walls, a fungal selective target. Caspofungin was the first echinocandin approved by the FDA, in 2001,[4] followed by micafungin in 2005 and anidulafungin in 2006.[5] However, in spite of significant efforts spent over many years, IFI continue to be major cause of morbidity and mortality in immunocompromised patients.[6] The alarming numbers of cases and the lack of effective treatment options have driven the search for new, broad-spectrum fungicidal agents including improving existing antifungals by reformulations as well as the search for synergistic compounds or compounds that could potentiate the effect of known antifungal drugs.[7]

As part of our program focused on the discovery of new natural product antifungal compounds and compounds potentiating the antifungal effect of caspofungin, we observed bioactivity in acetone extracts of culture broths of the fungus *Colispora cavincola* (Ascomycota, Pleosporales), isolated from plant litter collected from steppe vegetation in Argentina. These extracts were able to inhibit the growth of *A. fumigatus* and *C. albicans* particularly when combined with a sublethal dose of the antifungal agent caspofungin acetate. Bioassay-guided fractionation of these extracts using SP207ss resin column chromatography and semipreparative reversed-phase HPLC led to the isolation of colisporifungin (**1**), cavinafungin A (**2**), and cavinafungin B (**3**) from two independent fermentations harvested at different time points to maximize the production of the two sets of compounds. Colisporifungin, a depsipeptide structurally related to the aselacins, was the molecule

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Table 1. NMR Data of Colisporifungin (1) in DMSO-$d_6$

| amino acid | position | $\delta_C$, mult | $\delta_H$, mult, ($J$ in Hz) |
|---|---|---|---|
| D-Phe | 1 | 169.7, C | |
| | 2 | 54.9, CH | 3.74, m |
| | 3 | 34.8, $CH_2$ | 3.18, dd (13.7, 3.9) |
| | | | 3.11, dd (13.7, 10.5) |
| | 4 | 138.3, C | |
| | 5, 9 | 129.3, 2 × CH | 7.21−7.18, m |
| | 6, 8 | 128.1, 2 × CH | 7.25, br t (7.3) |
| | 7 | 126.1, CH | 7.21−7.18, m |
| | NH | | 7.71, d, 7.8 |
| L-Ser | 10 | 170.1, C | |
| | 11 | 57.0, CH | 4.04, m |
| | 12 | 60.5, $CH_2$ | 3.62, dd (10.8, 6.3) |
| | | | 3.52, dd (10.8, 2.9) |
| | NH | | 9.05, d (6.4) |
| | OH | | 4.84, br s |
| D-Trp | 13 | 173.8, C | |
| | 14 | 54.0, CH | 4.56, m |
| | 15 | 27.0, $CH_2$ | 3.03, m |
| | | | 2.88, dd (14.5, 10.0) |
| | 16 | 109.1, C | |
| | 17 | 127.0, C | |
| | 18 | 118.3, CH | 7.59, d (7.8) |
| | 19 | 118.2, CH | 6.96, br t (7.3) |
| | 20 | 120.9, CH | 7.06, br t (7.3) |
| | 21 | 111.3, CH | 7.32, d (8.3) |
| | 22 | 136.1, C | |
| | 23 | 123.5, CH | 7.08, br s |
| | NH(indol) | | 10.84, s |
| | NH | | 7.77, d (6.4) |
| $\beta$-Ala | 24 | 171.7, C | |
| | 25 | 33.9, $CH_2$ | 2.47, m |
| | | | 2.21, m |
| | 26 | 36.6, $CH_2$ | 3.31, m[a] |
| | | | 2.99, m |
| | NH | | 7.17, m |
| L-Thr | 27 | 168.5, C | |
| | 28 | 55.6, CH | 4.47, m |
| | 29 | 70.2, CH | 5.36, br dd, (6.4, 2.9) |
| | 30 | 15.8, $CH_3$ | 1.08, d (6.4) |
| | NH | | 8.50, m |
| D-Gln | 31 | 172.7, C | |
| | 32 | 53.7, CH | 4.49, m |
| | 33 | 26.8, $CH_2$ | 1.90, m |
| | 34 | 31.5, $CH_2$ | 2.14, m |
| | 35 | 173.3, C | |
| | NH | | 8.48, m |
| | $NH_2$ | | 7.34, br s |
| | | | 6.80, br s |
| DDA | 36 | 173.9, C | |
| | 37 | 34.7, $CH_2$ | 2.19, m |
| | 38 | 25.0, $CH_2$ | 1.47, m |
| | 39 | 28.5, $CH_2$ | 1.15−1.28, m |
| | 40 | 29.0, $CH_2$[b] | 1.15−1.28, m |
| | 41 | 28.9, $CH_2$[b] | 1.15−1.28, m |
| | 42 | 28.8, $CH_2$[b] | 1.15−1.28, m |
| | 43 | 28.7, $CH_2$[b] | 1.15−1.28, m |
| | 44 | 28.6, $CH_2$[b] | 1.15−1.28, m |
| | 45 | 31.2, $CH_2$ | 1.15−1.28, m |
| | 46 | 22.0, $CH_2$ | 1.24, m |
| | 47 | 13.9, $CH_3$ | 0.84, t (6.9) |

[a]Water masked. [b]Interchangeable assignments.

responsible for the observed potentiation activity. The aselacins were previously isolated from two fungal species of the genus *Acremonium* using an assay to detect inhibitors of the binding of endothelin to its receptor.[8] Cavinafungins A and B are responsible for the broad-spectrum antifungal activity. These are linear lipopeptides containing a terminal aldehyde residue (alaninal). Structurally related linear peptides containing an aldehyde moiety isolated from fungi are restricted to the fellutamides A−D isolated from *Penicillium fellutanum* recovered from the gastrointestinal tract of the fish *Apogon endekataenia* (fellutamides A and B)[9] and from an undescribed species of *Metulocladosporiella* (fellutamides C and D).[10] These compounds contain in their structures a (3*R*)-hydroxydodecanoic or (3*R*)-hydroxytetradecanoic acid residue and leucinal or valinal. Fellutamide B is known to inhibit human proteasome and induce nerve growth factor synthesis[11] and is a potent inhibitor of the *Mycobacterium tuberculosis* proteasome,[12] whereas fellutamides C and D were antifungal agents active against *C. albicans* and *A. fumigatus* with MICs ranging from 4 to 16 $\mu$g/mL and against fungal proteasome ($IC_{50}$ 0.2 $\mu$g/mL).[10] Fellutamide B and some other structurally related lipopeptides have been recently isolated from AD-2-1, a strain obtained by diethyl sulfate mutagenesis of the marine-derived fungus *Penicillium purpurogenum* G59.[13] To the best of our knowledge, the isolation of colisporifungin and cavinafungins from *C. cavincola* constitutes the first report on bioactive natural products isolated from a fungus of this genus and confirms the exceptional chemical diversity and the biological functions predicted by full genomic sequences of the fungi in the Pleosporales.[14]

## RESULTS AND DISCUSSION

The fungus (CF-226670) was isolated using a dilution-to-extinction method[15] from plant litter collected from steppe vegetation near El Calafate, Santa Cruz Province, Argentina. On oatmeal agar, the fungus forms velvety to lanose, radially sulcate colonies that attained 35 to 40 mm in 21 days at 22 °C and ranged from pale to dark gray, and dark grayish-brown in reverse. On 2% malt agar, the colonies attain 25 to 35 mm in diameter in 21 days at 22 °C and ranged from pale gray to dark grayish-brown, with dark brown to brownish-black submerge mycelia, and a soluble yellowish-brown pigment in the agar (Figure S5). Only rarely, on some very nutrient-poor media, e.g., synthetic nutrient agar, was a conidial state evident (Figure S6). The conidia and conidiogenesis were very similar to that previously described for the aero-aquatic fungus *C. cavincola*.[16] The conidia arose singly from terminal cells of unbranched or sparingly branched hyphae. Conidogenesis occurred at the terminal cells by holoblastic secession from the condiogenous locus. A percurrent scar was evident at the conidiogenous locus. The conidia were cylindrical to fusoid, or narrowly clavate, with 0 to 7 transverse septa, with the basal cell being slightly inflated and bearing a secession scar, with dimensions of approximately 20 to 75 $\mu$m long and 8 to 15 $\mu$m wide, and hyaline to gray in color.



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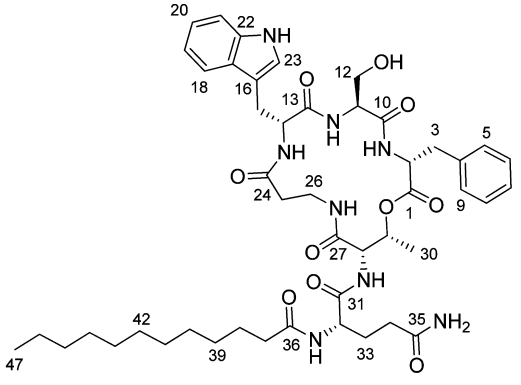
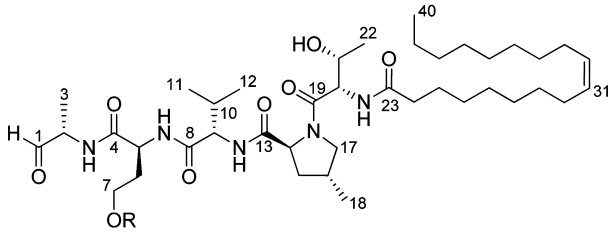
Database matching with the 28S rDNA sequence (www.fungalbarcoding.org) yielded a very high sequence similarity (99.658%) to the type strain of *C. cavincola* CBS 624.95 (Figure S7). This result was confirmed independently with amplicons sequenced with the above protocol. The ITS region was 100% identical between CF-226670 and the type strain of *C. cavincola*, thus indicating that strain CF-226670 was genetically similar to *C. cavincola* and likely conspecific. High similar scores to other authentic fungi strains, e.g., *Lophiostoma glabrotunicatum* (98.404%), indicated that CF-226670 could be classified as Ascomycota, Pleosporales, and possibly within the family Lophiostomataceae.

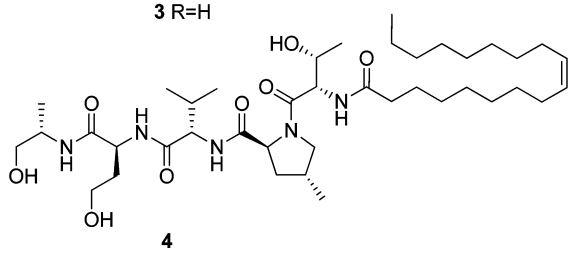
A molecular formula of $C_{47}H_{66}N_8O_{10}$ was assigned to colisporifungin (**1**) after analysis of its ESI-TOF (*m/z* 903.5006 $[M + H]^+$, calcd for $C_{47}H_{67}N_8O_{10}$, 903.4980) and $^{13}C$ NMR spectrum. The peptidic nature of the molecule was immediately inferred from the presence of a number of signals in the amide NH and $\alpha$-amino acid proton regions of its $^1H$ NMR and of carbonyl groups in the $^{13}C$ NMR spectrum (Table 1). Interpretation of the 2D NMR data revealed the presence of Phe, Ser, Trp, Thr, and Gln residues. In addition to these common amino acids, proton signals for two methylene groups at 2.47 and 2.21 ppm, and 3.31 and 2.99 ppm, coupled to carbon signals at 33.9 and 36.6, respectively, in the HSQC spectrum accounted for the presence of a $\beta$-Ala residue. Finally, signals in the aliphatic region of the $^1H$ spectrum indicated the existence of a saturated fatty acid residue that was identified as dodecanoic acid (DDA) on the basis of NMR chemical shifts and the molecular formula established by HRMS.



**1**



**2** R=OAc
**3** R=H



**4**

The planar structure of **1** was established via analysis of the correlations observed in the HMBC spectrum (Figure 1),

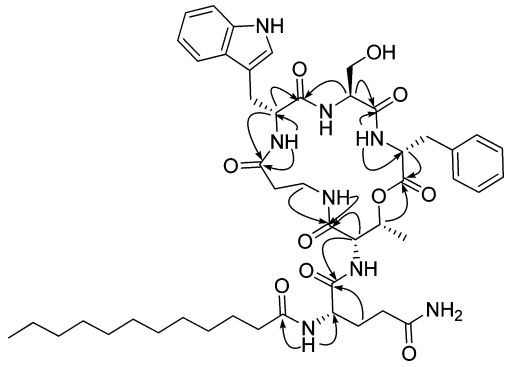


**Figure 1.** Key HMBC correlations (H to C) observed for compound **1**.

resulting in a cycle containing the sequence Phe-Ser-Trp-$\beta$-Ala-Thr linked via the amino group of the Thr to a side chain containing the Gln and DDA residues. Apart from the existence of an HMBC correlation between H-29 of Thr and C-1 of Phe, the ring closure through an ester bond between these two residues to form a cyclic depsipeptide was further supported by the low-field chemical shift of H-29 ($\delta_H$ 5.36 ppm). Finally, the absolute configuration of each amino acid residue was determined using Marfey's analysis.[17] Acid hydrolysis of the peptide followed by LC-MS analysis of the hydrolysate after derivatization with *N*-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA, Marfey's reagent) and comparison with the retention times obtained for standards revealed the presence of D-Phe, L-Ser, D-Trp, L-Thr, and D-Gln (detected as D-Glu) in the structure of colisporifungin (Figure S8).

Cavinafungin A (**2**) showed a molecular formula of $C_{42}H_{73}N_5O_9$ by ESI-TOF measurements. Similar to compound **1**, the presence of signals in the amide NH and $\alpha$-amino acid proton regions of its $^1H$ NMR and of carbonyl groups in the $^{13}C$ NMR spectrum (Table 2) revealed a peptidic nature for compound **2**. Analysis of the COSY, HMBC, and HSQC spectra established the presence of the common amino acids Thr and Val. Three additional amino acid moieties were identified as follows. An aldehyde proton resonating at 9.36 ppm coupled in the COSY spectrum to a second proton at 4.08 ppm, which was in turn coupled to a doublet methyl at 1.15 ppm, established the presence of alaninal in the molecule. A HomoSer residue was identified via COSY correlations from the $\alpha$-amino acid proton at $\delta_H$ 4.37 ppm to an aliphatic methylene (1.99/1.84 ppm), which in turn was coupled to a second oxygenated methylene at $\delta_H$ 3.97 ppm. The existence of HMBC correlations (Figure 2) from the latter protons and a singlet methyl group resonating at 1.97 ppm in the $^1H$ spectrum to a carbonyl carbon at $\delta_C$ 170.2 indicated that the hydroxy group of the HomoSer residue was acetylated. Further correlations observed in the COSY spectrum identified the third uncommon amino acid residue as 4-methylproline. Thus, a doublet methyl group resonating at 0.95 ppm was coupled to a proton at $\delta_H$ 2.32, which in turn was coupled to a downfield methylene (3.81 and 3.23 ppm) and an aliphatic (1.93 and 1.63 ppm) methylene. A correlation of the latter to an $\alpha$-amino acid proton at 4.45 ppm completed the spin system and confirmed the identity of the residue as 4-MePro, which was corroborated by correlations observed in the HMBC spectrum. Finally,



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**Table 2. NMR Data of Cavinafungins 2 and 3 in DMSO-$d_6$**

| | 2 | | | 3 | | |
|---|---|---|---|---|---|---|
| amino acid | pos | $\delta_C$, mult | $\delta_H$, m, $J$ (Hz) | pos | $\delta_C$, mult | $\delta_H$, m, $J$ (Hz) |
| alaninal | 1 | 201.1, C | 9.36, br s | 1 | 201.1, C | 9.35, br s |
| | 2 | 53.8, CH | 4.08, m | 2 | 53.7, CH | 4.06, m |
| | 3 | 13.9, $CH_3$ | 1.15, d (7.2) | 3 | 13.9, $CH_3$ | 1.16, d (6.6) |
| | NH | | 8.34, d (6.5) | NH | | 8.24, d (6.3) |
| HomoSer | 4 | 171.2, C | | 4 | 171.8, C | |
| | 5 | 49.4, CH | 4.37, m | 5 | 49.8, CH | 4.34, m |
| | 6 | 31.2, $CH_2$ | 1.99, m | 6 | 35.0, $CH_2$ | 1.82, m |
| | | | 1.84, m | | | 1.71, m |
| | 7 | 60.6, $CH_2$ | 3.97, m | 7 | 57.5, $CH_2$ | 3.41, m |
| | NH | | 7.99, d (7.7) | NH | | 7.90, d (7.6) |
| | $\underline{C}OCH_3$ | 170.2, C | | | | |
| | $CO\underline{C}H_3$ | 20.6, $CH_3$ | 1.97, s | | | |
| Val | 8 | 170.8, C | | 8 | 170.7, C | |
| | 9 | 57.8, CH | 4.07, m | 9 | 57.8, CH | 4.09, m |
| | 10 | 30.3, CH | 1.94, m | 10 | 30.3, CH | 1.98, m |
| | 11 | 19.1, $CH_3$ | 0.83, d (6.6) | 11 | 19.1, $CH_3$ | 0.84, d (6.6) |
| | 12 | 18.1, $CH_3$ | 0.81, d (6.7) | 12 | 18.0, $CH_3$ | 0.81, d (6.6) |
| | NH | | 7.77, m | NH | | 7.78, m |
| 4-MePro | 13 | 171.5, C | | 13 | 171.4, C | |
| | 14 | 59.2, CH | 4.45, dd (8.4, 3.1) | 14 | 59.2, CH | 4.45, m |
| | 15 | 36.5, $CH_2$ | 1.93, m | 15 | 36.5, $CH_2$ | 1.96, m |
| | | | 1.63, m | | | 1.65, m |
| | 16 | 31.9, CH | 2.32, m | 16 | 32.0, CH | 2.33, m |
| | 17 | 53.9, $CH_2$ | 3.81, m | 17 | 53.9, $CH_2$ | 3.82, m |
| | | | 3.23, m | | | 3.26, m |
| | 18 | 17.1, $CH_3$ | 0.95, d (6.5) | 18 | 17.1, $CH_3$ | 0.94, d (6.0) |
| Thr | 19 | 169.4, C | | 19 | 169.4, C | |
| | 20 | 56.2, CH | 4.40, m | 20 | 56.1, CH | 4.42, m |
| | 21 | 66.8, CH | 3.79, m | 21 | 66.8, CH | 3.81, m |
| | 22 | 19.3, $CH_3$ | 1.07, d (6.3) | 22 | 19.3, $CH_3$ | 1.08, d (6.1) |
| | NH | | 7.84, m | NH | | 7.84, m |
| Ole | 23 | 172.2, C | | 23 | 172.2, C | |
| | 24 | 34.8, $CH_2$ | 2.10, m | 24 | 34.8, $CH_2$ | 2.12, m |
| | 25 | 25.2, $CH_2$ | 1.44, m | 25 | 25.2, $CH_2$ | 1.45, m |
| | 26 | 28.6, $CH_2$ | 1.20, m | 26 | 28.7, $CH_2$ | 1.20, m |
| | 27 | 29.1, $CH_2$ | 1.26, m | 27 | 29.1, $CH_2$ | 1.26, m |
| | 28 | 29.1, $CH_2$ | 1.26, m | 28 | 29.1, $CH_2$ | 1.26, m |
| | 29 | 29.1, $CH_2$ | 1.26, m | 29 | 29.1, $CH_2$ | 1.26, m |
| | 30 | 26.6, $CH_2$ | 1.96, m | 30 | 26.6, $CH_2$ | 1.97, m |
| | 31 | 129.6, CH | 5.30, m | 31 | 129.6, CH | 5.32, m |
| | 32 | 129.6, CH | 5.30, m | 32 | 129.6, CH | 5.32, m |
| | 33 | 26.6, $CH_2$ | 1.96, m | 33 | 26.6, $CH_2$ | 1.97, m |
| | 34 | 20.1, $CH_2$ | 1.26, m | 34 | 20.1, $CH_3$ | 1.26, m |
| | 35 | 29.1, $CH_2$ | 1.26, m | 35 | 29.1, $CH_2$ | 1.26, m |
| | 36 | 29.1, $CH_2$ | 1.26, m | 36 | 29.1, $CH_2$ | 1.26, m |
| | 37 | 29.1, $CH_2$ | 1.26, m | 37 | 29.1, $CH_2$ | 1.26, m |
| | 38 | 31.2, $CH_2$ | 1.26, m | 38 | 31.2, $CH_2$ | 1.26, m |
| | 39 | 22.1, $CH_2$ | 1.23, m | 39 | 22.0, $CH_2$ | 1.26, m |
| | 40 | 13.5, $CH_3$ | 0.84, t (6.6) | 40 | 13.5, $CH_3$ | 0.84, t (6.6) |

signals in the aliphatic and olefinic regions of the $^1$H spectrum indicated the existence of a monounsaturated fatty acid residue that was identified as oleic acid on the basis of NMR chemical shifts and the molecular formula established for **2** by HRMS. HMBC correlations (Figure 2) established the sequence alaninal-O-Ac-HomoSer-Val-4-MePro-Thr-Ole for the compound.

A molecular formula of $C_{40}H_{71}N_5O_8$ was established for compound **3** after analysis of its HRESIFTMS and $^{13}$C NMR spectra. The NMR spectra of this compound (Table 2) were very similar to those of compound **2**, with the major differences being the absence of a singlet methyl signal and the shielding of the signal corresponding to the oxygenated methylene protons of the homoserine residue from 3.97 ppm in **2** to 3.41 ppm in **3**. These findings are in agreement with the removal of the



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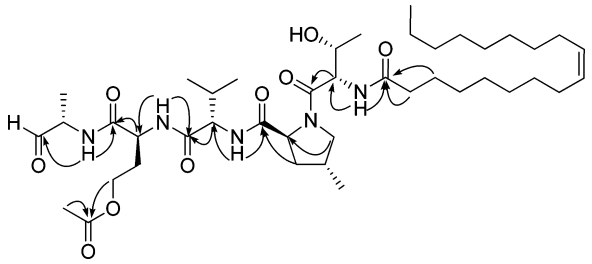

**Figure 2.** Key HMBC correlations (H to C) observed for compound **2**.

acetyl group in the homoserine group residue of **3** and were corroborated by the disappearance of signals corresponding to a methyl and a carbonyl group in the $^{13}C$ spectrum of **3** with respect to that of **2** and a slight upfield shifting of the carbon signal corresponding to the oxygenated $sp^3$ carbon of homoserine from 60.6 ppm in **2** to 57.5 ppm in **3**.

The absolute configuration of the amino acid residues present in the structures of compounds **2** and **3** was determined using the advanced Mosher's method applied to a peptide in which the alaninal residue in **2** was previously reduced to alaninol by treatment with $NaBH_4$. Hydrolysis of this peptide at 110 °C followed by derivatization of the hydrolysate with L-FDVA and comparison with the retention times obtained for standards revealed the presence of L-alaninal, L-homoserine, L-Val, and L-Thr in the structures of **2** and **3**. Double derivatization of mixtures of (2*S*,4*S*)- and (2*R*,4*S*)-4-methyl proline and (2*S*,4*S*)- and (2*S*,4*R*)-4-methyl proline with L- and D-FDVA provided standards for determining the retention time of all four of the stereoisomers of this residue and confirmed the presence of (2*S*,4*S*)-4-methyl proline (*trans* 4-Me-L-Pro) in the structure.

Colisporifungin (**1**) and cavinafungins A (**2**) and B (**3**) were evaluated for their antifungal activity and spectrum. The linear lipopeptides **2** and **3** showed broad-spectrum antifungal activity (Table 3), inhibiting growth of *Candida* species with MIC values of 0.5−4 μg/mL. They also inhibited growth of filamentous fungi *A. fumigatus*, showing prominent inhibition at 8 μg/mL. The homoserine acetate group of **3** had no effect on antifungal activity. The antifungal activity was significantly negatively affected when they were tested in the presence of 50% mouse serum (Table 3). The reduction of **2** with sodium borohydride led to deacetylated alcohol **4**, which showed no inhibition of fungal growth at 32 μg/mL, suggesting that the aldehyde is critical for the antifungal activity. Colisporifungin (**1**) did not display any antifungal activity when tested at concentrations of 8 μg/mL against *A. fumigatus* ATCC 46645, *C. albicans* MY1055, or *C. glabrata* MY992. When colisporifungin was tested in the presence of sublethal concentrations of caspofungin against these pathogens, MICs of 4 (*A. fumigatus*) and 2 μg/mL (*C. albicans*) were observed.

**Table 3. Antifungal Activity and Spectrum of Cavinafungins (Minimum Inhibitory Concentration (MIC) in μg/mL)**

| fungal strains[a] | strain number | cavinafungin A (**2**) | cavinafungin B (**3**) | alcohol **4** |
|---|---|---|---|---|
| *C. albicans* | MY1055 | 2 | 2 | >32 |
| *C. albicans* + 50% mouse serum | MY1055 | >32 | >32 | NT[c] |
| *C. tropicalis* | ATCC750 | 0.5 | 0.5 | >32 |
| *C. glabrata* | ATCC90030 | 2 | 2 | >32 |
| *C. lusitaniae* | ATCC34449 | 2 | 2 | >32 |
| *C. krusei* | ATCC6258 | 4 | 2 | >32 |
| *C. parapsilosis* | ATCC22019 | 2 | 2 | >32 |
| *A. fumigatus* | MF5668 | >32 (8)[b] | >32 (8)[b] | NT |

[a]MICs of *Candida* species were recorded after 24h incubation, whereas the MIC of *A. fumigatus* was recorded after 48 h incubation. [b]The datum in parentheses is $MIC_{80}$ (80% inhibition reported as prominent inhibition). [c]NT (not tested).

Evaluation of the potentiation by colisporifungin of the antifungal effect of caspofungin was performed using the checkerboard methodology.[18] Dose−response curves were performed using the starting concentrations of 8 μg/mL of compound **1** and 0.24 μg/mL of caspofungin and tested against *A. fumigatus* and *C. albicans*. The results obtained are represented in Figure 3. These showed that a dose of 2 μg/mL of **1** potentiated the caspofungin antifungal effect against the pathogenic fungus *A. fumigatus*, dropping the $IC_{50}$ of this drug from ∼33 nM to 6.2 nM, representing a 5.3-fold increase in potency. Additionally, a dose of 1 μg/mL compound **1** caused a halving in the $IC_{50}$ of caspofungin when tested against *C. albicans*. As mentioned above, when colisporifungin was tested alone at doses up to 8 μg/mL against these two pathogens, no growth inhibition was observed, indicating that colisporifungin itself had no antifungal activity.

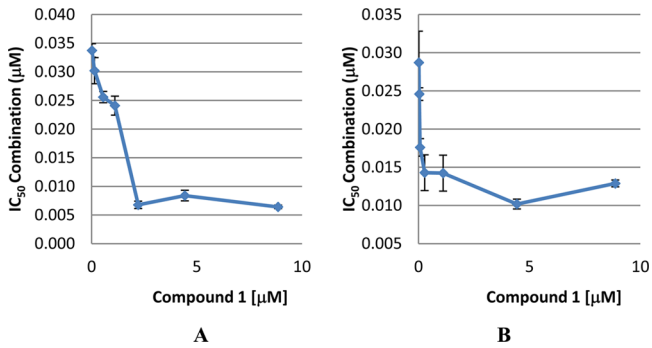


**Figure 3.** $IC_{50}$ of caspofungin vs (A) *A. fumigatus* ATCC46645 and (B) *C. albicans* MY1055 in the presence of different concentrations of **1**.

In conclusion, our systematic use of dilution-to-extinction has captured a rare fungus from plant litter. *C. cavinicola*, to date, has been reported only from Hungary.[19] Its observation in Argentina indicates such aero-aquatic fungi may be more widespread than previously recognized and that dilution-to-extinction may be an effective method for detection of these fungi. Without prior information on growth conditions for such poorly known organisms, application of a miniaturized nutrient array efficiently discerned growth conditions[20] that increased the chance of antibiotic expression on two media of a 12-media array and enabled the detection of compounds **1**−**3**.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were determined using a Jasco P-2000 polarimeter. UV spectra were obtained with an Agilent 1100 DAD. NMR spectra were recorded on a Varian "INOVA 500" spectrometer at 500/125 MHz ($^1H/^{13}C$). Chemical shifts were reported in ppm using residual DMSO-$d_6$ ($\delta$ 2.51 for $^1H$ and 39.0 for $^{13}C$) as internal reference. HMBC experiments were optimized for a $^3J_{CH}$ of 8 Hz. (+)-ESI-TOFMS was performed on a Bruker maXis spectrometer.



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**Producing Fungus and Its Characterization.** The producer microorganism (CF-226670; CBS 133614) was isolated using a dilution-to-extinction method.[15]

To estimate the approximate phylogenetic position of strain CF-226670, genomic DNA was extracted from mycelia grown on malt-yeast extract agar. The rDNA region containing the partial sequence of 28S rDNA containing D1 and D2 variable domains was amplified with primers NL1 and NL4,[21] and a DNA sequence was generated. About 0.1 μg/mL of the double-stranded amplification products was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Norwalk, CT, USA) following the procedures recommended by the manufacturer. Purified PCR products were directly sequenced using the same primer pairs as in the PCR reactions. Partial sequences obtained in sequencing reactions were assembled with Genestudio 2.1.1.5 (Genestudio, Inc., Suwanee, GA, USA).

**Fermentation.** The antifungal activity and potentiating effect of antifungal agents by extracts of strain CF-226670 were initially detected in two of a 12-media nutritional array in 1 mL micro-fermentations in deep-well 96-well plates.[22] The most potent activity from extracts of a medium designated as supermalt (malt extract 50 g, yeast extract 10 g, $FeSO_4 \cdot 7H_2O$ 20 mg, $ZnSO_4 \cdot 7H_2O$ 20 mg, distilled $H_2O$ 1 L) was selected for further study. The initial sample was prepared by cutting three to four mycelial discs from each 60 mm plate and crushing them in the bottom of tubes (25 × 150 mm) containing 10 mL of Sabouraud maltose broth supplemented with yeast extract and dilute agar (SMYA) (Difco neopeptone 10 g, maltose 40 g, Difco yeast extract 10 g, agar 4 g, distilled $H_2O$ 1000 mL) and two cover glasses (22 × 22 mm). Tubes were agitated on an orbital shaker (200 rpm, 5 cm throw) to produce homogeneous hyphal suspensions. After growing the inoculum stage for 4 days, a 0.5 mL aliquot was used to inoculate 15 mL of supermalt medium in 10 × 50 mL sterile polystyrene tissue culture tubes (Techno Plastic Products AG, Trasadingen, Switzerland). The tubes were incubated statically for 21 days at 22 °C. Mycelium and broth from these tubes were extracted with acetone, and after removing the acetone by evaporation, the aqueous residue was used to detect the molecules that potentiate the effect of antifungal agents. To further characterize the molecule responsible for the activity, a 1 L fermentation was prepared. Ten mycelial discs were used to inoculate 50 mL of SMYA. After 4 days, 1 mL aliquots of this culture were used to inoculate supermalt medium (10 × 100 mL of medium in 500 mL Erlenmeyer flasks). The flasks were incubated statically at 22 °C, 70% relative humidity, for 22 days. Additional screening of medium formulations indicated that significant amounts of compound **1** can be produced in MV8 medium (maltose 75 g, V8 juice 200 mL, soy flour 1 g, L-proline 3 g, MES 16.2 g, $H_2O$ 800 mL) in agitated fermentations. Larger amounts of colisporigfungin were prepared by using the same supermalt medium incubated in an aerated agitated fermentation (220 rpm, 5 cm throw) for 10 to 14 days at 22 °C, 70% relative humidity.

**Extraction and Isolation.** A 1 L culture of CF-226670 (*C. cavincola*) grown for 22 days (maximal production of **1** and minimal production of **2** and **3**) in supermalt medium was extracted by addition of acetone (1 L), agitation at 220 rpm for 1 h, centrifugation at 8500 rpm, filtration, and evaporation of the organic solvent under a nitrogen stream. The aqueous residue was loaded onto an SP207ss column (65 g, 32 × 100 mm) that was eluted with a gradient of acetone in water (10% to 100% acetone in 12.5 min + 100% acetone for 15 min, 8 mL/min, 20 mL/fraction). Fractions 6–8 from this chromatography were pooled and subjected to preparative HPLC (Zorbax SB-$C_8$ PrepHT, 21.2 × 250 mm, 7 μm, gradient $H_2O$ + 0.1% TFA–$CH_3CN$ + 0.1% TFA from 5% to 100% organic in 40 min, 20 mL/min, UV detection at 210 and 280 nm). Fractions containing the compound of interest from this chromatography were further purified by semipreparative reversed-phase HPLC (Zorbax RX-$C_8$, 9.4 × 250 mm, gradient $H_2O$ + 0.1% TFA–$CH_3CN$ + 0.1% TFA from 5% to 60% $CH_3CN$–TFA in 22 min, held at 60% $CH_3CN$–TFA for 11 min, and from 60% to 100% $CH_3CN$–TFA in 2 min, UV detection at 210 and 280 nm) to yield 22 mg of colisporifungin as a white, amorphous solid eluting at 28.8 min.

A second supermalt-based liquid fermentation of F-226,670 (ca. 4 L) grown for 12 days (with maximal production of **2** and **3** and lower production of **1**) was extracted using the same procedure as above. The filtered aqueous residue (ca. 4 L) was loaded along with water (1:1) onto a reversed-phase SP207ss column (65 g, 32 × 220 mm), and the column was eluted with a linear gradient of acetone in water (8 mL/min; 10–100% acetone in 12.5 min with a final isocratic step of 100% acetone for 15 min), collecting 12 fractions of 20 mL. These fractions were evaporated to dryness in a centrifugal evaporator and analyzed by LC/MS in order to locate the target compounds. Fractions containing the lipopeptides **2** and **3** were redissolved in DMSO and purified by reversed-phase preparative HPLC (Agilent Zorbax SB-$C_8$ PrepHT, 21.2 × 250 mm) eluting with a linear gradient of $H_2O$–$CH_3CN$ (20 mL/min, 5–100% $CH_3CN$ in 80 min, UV detection at 210 nm). Finally, the enriched subfractions of this chromatography were repurified by reversed-phase semipreparative HPLC (Agilent Zorbax RX-$C_8$, 9.4 × 250 mm) with the same linear gradient at a flow of 3.6 mL/min, to yield lipopeptides **2** (16.2 mg) and **3** (2.1 mg), eluting at 78.2 and 76.1 min, respectively.

*Colisporifungin (**1**):* white, amorphous solid; $[\alpha]^{20}_D$ +8.47 (*c* 0.1, MeOH); IR (ATR) $\nu$ $cm^{-1}$ 3281, 2925, 2853, 1736, 1543, 1455, 1441, 1234, 1025; $^1H$ and $^{13}C$ NMR data see Table 1; (+)-ESI-TOFMS *m/z* 903.5006 $[M + H]^+$ (calcd for $C_{47}H_{67}N_8O_{10}$, 903.4980).

*Cavinafungin A (**2**):* pale yellow oil; $[\alpha]^{20}_D$ −73.10 (*c* 0.05, MeOH); $^1H$ and $^{13}C$ NMR data see Table 2; (+)-ESI-TOFMS *m/z* 792.5483 $[M + H]^+$ (calcd for $C_{42}H_{74}N_5O_9$, 792.5481), 814.5300 $[M + Na]^+$ (calcd for $C_{42}H_{73}N_5O_9Na$, 792.5481).

*Cavinafungin B (**3**):* pale yellow oil; $[\alpha]^{20}_D$ −66.22 (*c* 0.25, MeOH); $^1H$ and $^{13}C$ NMR data see Table 2; (+)-ESI-TOFMS *m/z* 750.5379 $[M + H]^+$ (calcd for $C_{40}H_{72}N_5O_8$, 750.5375), 772.5188 $[M + Na]^+$ (calcd for $C_{42}H_{73}N_5O_9Na$, 772.5195).

**Reduction of Cavinafungin A (2).** To a 2 mg solution of **2** in MeOH (0.3 mL) was added a 0.5 M solution of $NaBH_4$ in 2-methoxyethyl ether (0.1 mL). The solution was stirred at room temperature for 48 h and was directly purified by reversed-phase HPLC using Zorbax $C_8$ (9.4 × 250 mm) eluting with a 30 min linear gradient of 20–100% aqueous acetonitrile containing 0.1% TFA. Fractions eluting at 22–23 min were pooled and lyophilized to give 1 mg of reduced deacetylated product **4**. $^1H$ NMR (500 MHz) D-alaninol: 7.45 (1H, d, *J* = 6.5 Hz, NH), 4.62 (1H, m, OH), 3.70 (1H, m, H-2), 3.30/3.16 (2H, m, $H_2$-1), 0.98 (3H, d, *J* = 7 Hz), HomoSer: 7.78 (1H, d, *J* = 7.8 Hz, NH), 4.46 (1H, m, OH), 4.22 (1H, m, H-2), 3.36 (2H, m, $H_2$-3), 1.77/1.63 (2H, m, $H_2$-2), Val: 7.79 (1H, m, NH), 4.04 (1H, m, H-2), 1.95 (1H, m, H-3), 0.82 (3H, d, J = 6.5 Hz, $CH_3$), 0.81 (3H, d, J = 6.5 Hz, $CH_3$), 4-MePro: 4.44 (1H, m, H-2), 3.80/3.23 (2H, m, $H_2$-5), 2.32 (1H, m, H-4), 1.95/1.64 (2H, m, $H_2$-3), 0.95 (3H, d, *J* = 6.5 Hz, $CH_3$), Thr: 7.84 (1H, m, NH), 4.65 (1H, m, OH), 4.39 1H, m, H-2), 3.78 (1H, m, H-3), 1.07 (3H, d, J = 6.5 Hz), Ole: 5.30 (2H, m, H-9,10), 2.10 (2H, m, $H_2$-2), 1.95 (4H, m, $H_2$-8, 11), 1.20–1.43 (22H, m), 0.83 (3H, t, *J* = 6.6 Hz); (+)-HRESIFTMS *m/z* 752.55377 $[M + H]^+$ (calcd for $C_{40}H_{74}N_5O_8$, 752.55374).

**Marfey's Analysis of Compound 1.** A sample (220 μg) of compound **1** was dissolved in 0.44 mL of 6 N HCl and heated at 110 °C for 16 h. The crude hydrolysate was evaporated to dryness under a $N_2$ stream, and the residue was dissolved in 100 μL of water. A 1% (w/v) solution (100 μL) of L-FDVA (Marfey's reagent, *N*-(2,4-dinitro-5-fluorophenyl)-L-valinamide) in acetone was added to an aliquot (50 μL) of a 50 mM solution of each amino acid (D, L, or DL mixture) and to the aqueous solution of the peptide hydrolysate. After addition of 20 μL of 1 M $NaHCO_3$ solution, each mixture was incubated at 40 °C for 60 min. The reactions were quenched by addition of 10 μL of 1 N HCl, and the crude mixtures were diluted with 700 μL of acetonitrile and analyzed by LC/MS on an Agilent 1100 single quadrupole. Separations were carried out on a Waters XBridge $C_{18}$ column (4.6 × 150 mm, 5 μm) maintained at 40 °C. A mixture of two solvents, A (10% acetronitrile, 90% water) and B (90% acetronitrile, 10% water), both containing 1.3 mM trifluoroacetic acid and 1.3 mM ammonium formate, was used as the mobile phase under a linear gradient elution mode (10–30% B in 35 min, 30–100% B in 1 min, isocratic 100% B for 4 min) at a flow rate of 1 mL/min.



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Retention times (min) for the derivatized (L-FDVA) amino acid standards under the reported conditions were as follows: L-Phe: 34.66; D-Phe: 37.87; L-Ser: 12.63; D-Ser: 14.94; L-Trp: 35.33; D-Trp: 37.72; L-Thr: 13.02; D-Thr: 22.88, L-allo-Thr: 13.83; D-allo-Thr: 16.14; L-Glu: 15.42; D-Glu: 19.59. Retention times (min) for the observed peaks in the HPLC trace of the L-FDVA-derivatized hydrolysis product of compound **1** were as follows: D-Phe: 37.85; L-Ser: 12.63; D-Trp: 37.72; L-Thr: 13.01; D-Glu: 19.62.

**Reduction and Marfey's Analysis of Compounds 2 and 3.** A 240 $\mu$g sample of each lipopeptide was treated with 12 $\mu$L of a solution 0.5 M $NaBH_4$ in 2-methoxy ethyl ether and allowed to stand at room temperature overnight. A second portion of reagent (6 $\mu$L, 0.5 M $NaBH_4$) was added to the mixture, and the reaction was monitored by LC/MS until consumption of starting material. The reaction was quenched by addition of 60 $\mu$L of 1 N HCl, and the mixture was evaporated under nitrogen to dryness.

The solid residue obtained from reduction of each lipopeptide was hydrolyzed at 110 °C for 16 h with 0.48 mL of 6 N HCl. The crude hydrolysate was evaporated to dryness, and the residue was dissolved in 100 $\mu$L of water. This solution was divided into two 50 $\mu$L portions. To each 50 $\mu$L portion of hydrolysate were added 20 $\mu$L of 1 M sodium bicarbonate and 100 $\mu$L of L-FDVA or D-FDVA (1% in acetone). The reaction mixtures were incubated at 40 °C for 60 min. After this time the reaction was quenched by addition of 20 $\mu$L of 1 N HCl, and the crude mixture was diluted with 700 $\mu$L of acetonitrile and analyzed by ESI LC/MS on an Agilent 1100 single quadrupole MS. Three different chromatographic methods (A–C) were employed to unequivocally assign the absolute configuration of all the amino acid residues present in both peptides. Method A was used for the separation of L- and D-FDVA derivatives of 4-Me-proline and valine and employed an Agilent Zorbax SB-$C_8$ (2.1 × 30 mm) column maintained at 40 °C, a mixture of two solvents, A (10% acetronitrile, 90% water) and B (90% acetronitrile, 10% water), both containing 1.3 mM trifluoroacetic acid and ammonium formate, and a linear gradient elution mode (10–30% of B in 35 min) at a flow rate of 0.3 mL/min. Method B was employed for the separation of L-FDVA derivatives of threonine and homoserine on a Waters XBridge $C_{18}$ (4.6 × 150 mm, 5 $\mu$m) column maintained at 40 °C. The same mixture of solvents used in method A was used as mobile phase under the same linear gradient (B, 10–30%, 35 min), at a flow rate of 1.0 mL/min. Method C allowed the separation of L-FDVA derivatives of alaninol and was performed on a Waters XBridge $C_{18}$ (4.6 × 150 mm, 5 um) column maintained at 40 °C. The same mixture of solvents used in methods A and B was used as mobile phase under a linear gradient elution mode (B, 10–65%, 6 min; isocratic 65% 1 min; 65–100% 1 min) at a flow rate of 1.0 mL/min.

Retention times (min) for the derivatized amino acid standards under the reported conditions were as follows: L-Val-L-FDVA: 12.49 min; D-Val-L-FDVA: 23.27 min; L-Thr-L-FDVA: 13.04 min; D-Thr-L-FDVA: 23.09 min; L-*allo*-Thr-L-FDVA: 13.82 min; D-*allo*-Thr-L-FDVA: 18.20 min; L-HomoSer-L-FDVA: 13.11 min; D-HomoSer-L-FDVA: 17.04 min; *cis*-4-Me-L-Pro-L-FDVA: 11.35 min; *trans*-4-Me-L-Pro-L-FDVA: 12.34 min; *cis*-4-Me-L-Pro-D-FDVA (equivalent to *cis*-4-Me-D-Pro-L-FDVA): 17.40 min; *trans*-4-Me-L-Pro-D-FDVA (equivalent to *trans*-4-Me-D-Pro-L-FDVA): 17.03 min; L-Phe: 34.66; D-Phe: 37.87; L-Ser: 12.63; D-Ser: 14.94; L-Trp: 35.33; D-Trp: 37.72; L-Thr: 13.02; D-Thr: 22.88, L-allo-Thr: 13.83; D-allo-Thr: 16.14; L-Glu: 15.42; D-Glu: 19.59; L-alaninol-L-FDVA: 5.98 min; D-alaninol-L-FDVA: 6.58 min. Retention times (min) for the observed peaks in the HPLC trace of the derivatized hydrolysis product of compound **2** were as follows: L-Val-L-FDVA: 12.42 min; L-Val-D-FDVA (equivalent to D-Val-L-FDVA): 23.22 min; L-Thr-L-FDVA: 13.05 min; L-Thr-D-FDVA (equivalent to D-Thr-L-FDVA): 23.06 min; L-HomoSer-L-FDVA: 13.05 min; L-HomoSer-D-FDVA (equivalent to D-HomoSer-L-FDVA): 17.08 min; *trans*-4-Me-L-Pro-L-FDVA: 12.29 min; *trans*-4-Me-L-Pro-D-FDVA (equivalent to *trans*-4-Me-D-Pro-L-FDVA): 17.02 min; L-alaninol-L-FDVA: 5.98 min; L-alaninol-D-FDVA (equivalent to D-alaninol-L-FDVA): 6.59 min. Similarly, the derivatized amino acid residues present in the hydrolysate of compound **3** gave retention times of L-Val-L-FDVA: 12.50 min; L-Val-D-FDVA (equivalent to D-Val-L-FDVA): 23.29 min; L-Thr-L-FDVA: 13.09 min; L-Thr-D-FDVA (equivalent to D-Thr-L-FDVA): 23.09 min; L-HomoSer-L-FDVA: 13.09 min; L-HomoSer-D-FDVA (equivalent to D-HomoSer-L-FDVA): 17.10 min; *trans*-4-Me-L-Pro-L-FDVA: 12.32 min; *trans*-4-Me-L-Pro-D-FDVA (equivalent to *trans*-4-Me-D-Pro-L-FDVA): 17.07 min; L-alaninol-L-FDVA: 5.99 min; L-alaninol-D-FDVA (equivalent to D-alaninol-L-FDVA): 6.60 min.

**Antifungal Assay.** The whole-cell antifungal activity assay was described elsewhere.[10] The MIC (minimum inhibitory concentration) against each of the strains was determined as previously described.[23] Cells were inoculated at $10^5$ colony-forming units/mL followed by incubation at 37 °C with a 2-fold serial dilution of compound in the growth medium for 37 °C for 20 h. MIC is defined as the lowest concentration of an antifungal inhibiting visible growth.

**Strains and Media for Potentiation Assays.** Two fungal strains were used in this study: *A. fumigatus* ATCC 46645 and *C. albicans* MY1055. All manipulations were carried out in a laminar flow hood using aseptic techniques. Cell viability of *A. fumigatus* was measured using resazurin as previously described.[24] *C. albicans* suspensions from cryovials were streaked on Sabouraud dextrose agar (SDA, 65 g/L) plates for confluent growth. *C. albicans* plates were incubated for 18 h at 37 °C, and then colonies were harvested from the SDA plates and inoculated in 10 mL of SDB (Sabouraud dextrose broth). The suspension was incubated overnight with agitation. To prepare the inocula, a suspension with $OD_{600}$ = 0.25 was prepared in RPMI-modified medium (10.4 g/L of RPMI-1640 medium, 6.7 g/L of yeast nitrogen base, 1.8% (w/v) glucose, and 40 mM HEPES (pH 7.1)), and subsequently the suspension was diluted 1:10 ((~2–5) × $10^5$ cells/mL) and kept on ice until used to inoculate 96-well microtiter plates.

**Caspofungin Potentiation Assay.** The potentiation assay was performed in 96-well plates, and samples were tested with and without a sublethal dose of caspofungin (0.015 $\mu$g/mL *for A. fumigatus* and 0.03 $\mu$g/mL for *C. albicans*). Culture plates were incubated at 37 °C for 24 h. Resazurin 0.002% was added to the microtiter plates 3–4 h before reading. The change of the color of resazurin was visually observed, and the growth inhibition was quantified by measuring fluorescence (excitation 570 nm, emission 615 nm) using an EnVision Multilabel (PerkinElmer). All the experiments were performed in triplicate. The Z′ factor obtained in all the experiments was between 0.85 and 0.95.

**Checkerboard Tests for Antifungal Interactions *in Vitro*.** Optimal interactions between caspofungin and compound **1** against *A. fumigatus* and *C. albicans* were determined by checkerboard layouts of double dilutions.[18] Final concentrations ranging from 0.24 to 0.00018 $\mu$g/mL of caspofungin (columns) and from 8 to 0.015 $\mu$g/mL of compound **1** (rows) were tested in the 80 central wells of the same microdilution plate. Growth inhibition was determined using the above-described resazurin test.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

$^1H$ and $^{13}C$ NMR spectra (Figures S1–S4) for compounds **1**–**3**, photos of the fungus and its conidia and a phylogenetic tree (Figures S5–S7), and HPLC chromatograms of Marfey's analysis of compounds **1** and **2** (Figures S8, S9) are available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ DEDICATION

Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California−San Diego, for his pioneering work on bioactive natural products.

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