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Small-Molecule Suppressors of *Candida albicans* Biofilm Formation Synergistically Enhance the Antifungal Activity of Amphotericin B against Clinical *Candida* Isolates

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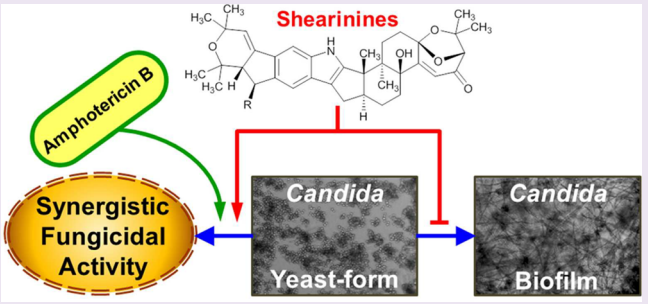
Supporting Information

ABSTRACT: A new class of fungal biofilm inhibitors represented by shearinines D (**3**) and E (**4**) were obtained from a *Penicillium* sp. isolate. The inhibitory activities of **3** and **4** were characterized using a new imaging flow-cytometer technique, which enabled the rapid phenotypic analysis of *Candida albicans* cell types (budding yeast cells, germ tube cells, pseudohyphae, and hyphae) in biofilm populations. The results were confirmed by experimental data obtained from three-dimensional confocal laser scanning microscopy and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assays. These data indicate that **3** and **4** inhibited *C. albicans* biofilm formation by blocking the outgrowth of hyphae at a relatively late stage of biofilm development (IC_{50} = 8.5 and 7.6 μM, respectively). However, **3** and **4** demonstrated comparatively weak activity at disrupting existing biofilms. Compounds **3** and **4** also exhibited synergistic activities with amphotericin B against *C. albicans* and other clinical *Candida* isolates by enhancing the potency of amphotericin B up to 8-fold against cells in both developing and established biofilms. These data suggest that the *Candida* biofilm disruption and amphotericin B potentiating effects of **3** and **4** could be mediated through multiple biological targets. The shearinines are good tools for testing the potential advantages of using adjunctive therapies in combination with antifungals.



Globally, *Candida* spp. are the most prevalent cause of mycoses[1−5] leading to an immense financial burden that exceeds ~$1 billion per year in the United States alone.[6] The majority of clinically encountered *Candida* infections involve *Candida albicans*, which possesses an assortment of disease-promoting capabilities including the ability to form biofilms.[7−9] *Candida albicans* is remarkably versatile at establishing biofilms on a variety of surfaces ranging from human tissues (e.g., mucosal membranes) to indwelling medical devices.[9−12] Biofilms shield *C. albicans* from attack by the immune system, as well as block antifungal antibiotics from reaching cells.[12−14] These defense-related attributes enable biofilms to function as infectious reservoirs,[9] which release new propagules both during and after treatment with antifungal therapeutics.

It has been observed that a subset of biofilm-embedded *C. albicans* cells exhibit a "persister" phenotype that is characterized by a state of extreme metabolic quiescence.[15−17] Persister cells are highly recalcitrant to the effects of antifungals agents,[18] and it is believed that they are a major contributing factor to infection relapse following the cessation of standard courses of antifungal antibiotics.[17] Persisters are genetically identical to drug-susceptible cells, and it is likely that the biofilm environment provides the requisite context for enabling some *C. albicans* cells to stochastically enter into this semidormant state.[15]

For these reasons, biofilms are a clinically relevant means with which *C. albicans* establishes persistent infections in humans.[12,19−22] It is anticipated that therapeutic interventions utilizing small-molecule inhibitors of biofilm formation would afford clinicians a valuable tool for reestablishing pharmacological control over recalcitrant *C. albicans* infections. However, pinpointing an appropriate chemical screening resource is a key step to identifying promising bioactive compounds that address this need.

Our research has focused on the rich chemical diversity of natural products for the discovery of bioactive small molecules that inhibit *C. albicans* biofilm formation. Although a handful of biofilm and morphological transition inhibitors have emerged in recent years,[23−26] many of these compounds suffer from mediocre potency or poor physiochemical characteristics. Natural products have long served as an unrivaled source of novel, drug-like molecules,[27,28] and it is anticipated that our

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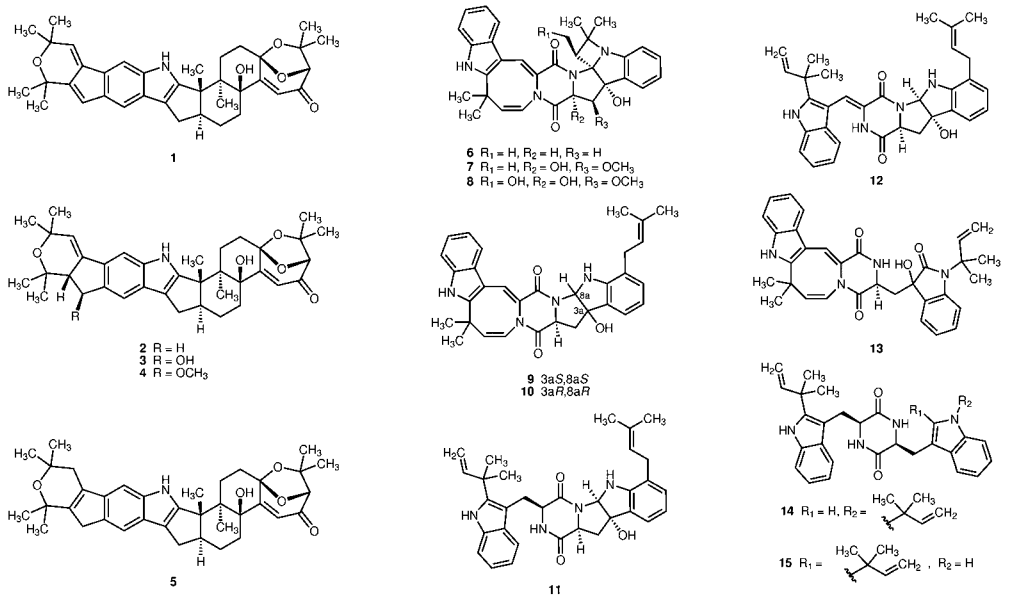

Figure 1. Structures of alkaloid metabolites produced by an Alaskan-soil-derived *Penicillium* sp. The purified new compounds 22,23-dehydro-shearinine A (**1**) , 2-dehydroxy-3-demethoxy-okaramine B (**6**), and the 3a*R*,8a*S* diastereomer of okaramine H (**9**), together with the known metabolites shearinines A (**2**), D (**3**), E (**4**), F (**5**), and eight additional alkaloids (**7**, **8**, and **10**−**15**) were part of a complex of metabolites present in the bioactive fraction (inhibition of *C. albicans* biofilm formation). Details of the isolation and structure determination of these compounds are provided in Supplementary Figure S1 and Table S1.

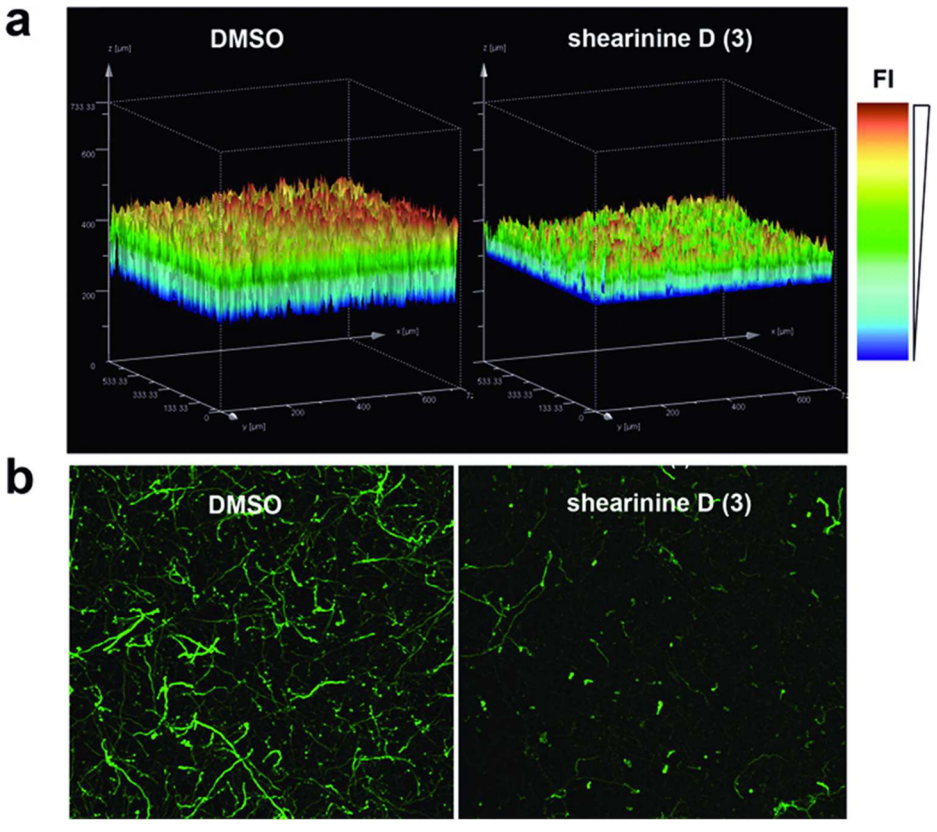

Figure 2. Three-dimensional confocal laser scanning microscopy images of *C. albicans* biofilms treated with DMSO or 10 μM shearinine D (**3**). (a) Reconstructed image detailing the thickness and fluorescence intensity of treated and untreated biofilms (note that the colors represent varying fluorescent intensity (FI) throughout the biofilm). (b) A compilation of *Z*-stack photos taken of the biofilm surface reveals a large number of long hyphae in the control group forming an interwoven network of cells, whereas samples treated with compound **3** exhibit very few hyphae and cells that did not assemble into a biofilms. All experiments were performed in triplicate on three separate occasions.

efforts will yield new inhibitors of *C. albicans* biofilm formation. Our earlier studies lend support to this approach, with novel compounds including waikialoid A,[29] mutanobactin D,[30] and others[31] having been reported.

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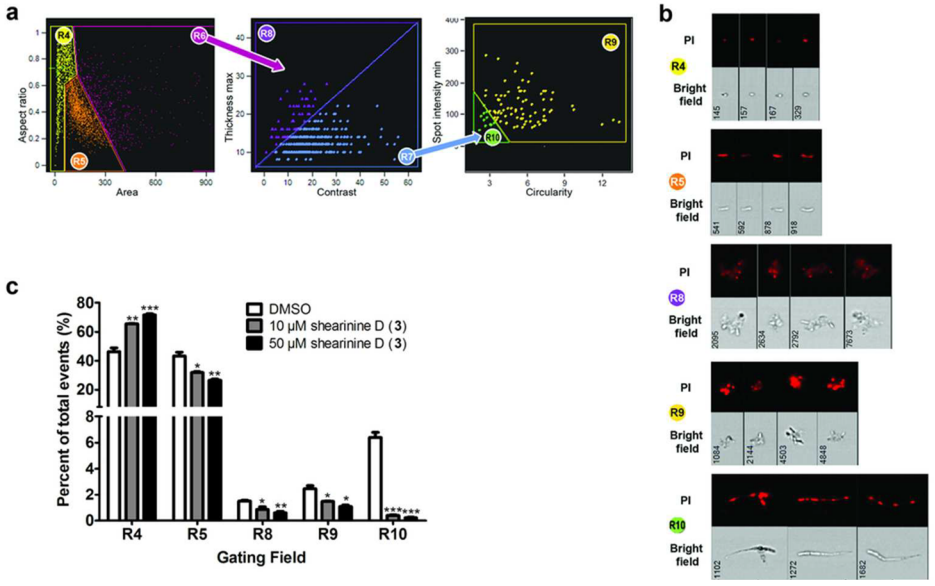

Figure 3. Morphological distribution pattern of *C. albicans* cell types as revealed by imaging flow-cytometry. (a) Distribution pattern of *C. albicans* cells and the gating parameters (R4−R10) that were used to demarcate each population. (b) Images of cells representing each of defined populations R4 (spherical cells with yeast-like morphologies), R5 (elongated cells with germ tubes/hyphal buds), R8 (biofilm matrix with cell debris), R9 (pseudohyphae), and R10 (hyphae). (c) Population distributions for each cell type. Bars labeled with asterisks were determined to be statistically different from DMSO controls (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). Data represent the mean values obtained from triplicate experiments on two separate occasions ± standard deviations.

In this study, we report on the identification of a group of fungal-derived indole-alkaloids that inhibit *Candida* biofilm formation. Our study was greatly enhanced by the application of a new imaging flow cytometer technique, which enabled us to readily quantify the morphological state distribution patterns of biofilm-associated *C. albicans* populations. We expect that this approach has the potential to provide tremendous insight into the biofilm formation process, as well as assist in the identification and classification of new biofilm inhibitors.

RESULTS AND DISCUSSION

Identification of *C. albicans* Biofilm Inhibitors. A soil sample collected in Ketchikan, Alaska, USA was received by our laboratory through an open-invitation sample-submission program. Isolate KS-017, which was determined to be a taxonomically undefined *Penicillium* sp. (based on ITS1-5.8S-ITS2 sequence homology; see Supporting Information). An ethyl acetate extract prepared from a small-scale culture of the fungus was found to inhibit *C. albicans* DAY185 biofilm formation (85% biofilm reduction at 100 μg mL^{-1}). Bioassay-guided fractionation yielded a single active fraction that was composed of several structurally related compounds (based on comparisons of ^{1}H NMR data; data not shown). All of the major components in the sample were purified and characterized *via* nuclear magnetic resonance spectroscopy and mass spectrometry dereplication, as well as *de novo* structure determination. This afforded a new indole triterpenoid, 22,23-dehydro-shearinine A (**1**); two new indole diketopiperazines, 2-dehydroxy-3-demethoxy-okaramine B (**6**) and the3a*R*,8a*S* diastereomer of okaramine H (**9**); and 12 previously reported analogues (**2**−**5**, **7**, **8**, and **10**−**15**) (Figure 1; refer to the Supporting Information for a detailed discussion of the structure characterization process for the new compounds).

To determine which of the secondary metabolites were responsible for the bioactive fraction's inhibitory properties, the purified compounds were tested individually over a 3−100 μM range in an XTT-based biofilm inhibition assay.[32] Two of the compounds, shearinines D (**3**) and E (**4**) (Figure 1), inhibited *C. albicans* DAY185 biofilm formation with IC_{50} values of 8.5 and 7.6 μM, respectively. The activities of **3** and **4** were also confirmed against a wild-type *C. albicans* SC5314, which is a virulent strain frequently used in animal infection studies and biofilm assays[33] (IC_{50} values of 2.68 and 1.99 μM, respectively). The XTT assay results for **3** were corroborated using confocal scanning laser microscopy. This revealed that *C. albicans* treated with 10 μM of **3** did not produce biofilms but instead generated irregular, sparse layers in which the majority of cells lacked characteristic hyphal phenotypes (Figure 2, panels a and b). However, neither of the compounds appeared to inhibit the proliferation and viability of *C. albicans* at concentrations of up to 100 μM. Although we are limited in our ability to compare the structure−activity relationships of these compounds with only five shearinines available, it appears that a C-22 hydroxyl/methoxyl group is essential for bioactivity, since the other shearinines lacking it were inactive at 30 μM (Figure 1).

Impact of Shearinine D on *C. albicans* Biofilms. Biofilms of *C. albicans* typically consist of complex consortia of cell types (i.e., yeast, hyphae, pseudohyphae, and germ tube forms) embedded within extracellular matrices. Although substantial progress has been made to define the genetic and biological processes regulating the transition of *C. albicans* among these morphological states, there are significant gaps in this knowledge. The development of a quantitative, phenotypic screening tool could be expected to provide substantial insight concerning an inhibitor's mode of action by offering an effective method for analyzing the distribution of *C. albicans* morphological types following compound treatment.

Accordingly, we established a new imaging flow cytometry technique, which enabled our group to discern the distribution

of yeast, hyphae, pseudohyphae, and germ tube cells in *C. albicans* biofilms. Our method utilized an imaging flow cytometer (Amnis FlowSight), which combined flow cytometry technology with fluorescent, darkfield, and brightfield cell imaging capabilities. The distinct advantage of using this approach was that it readily facilitated the assignment of biologically relevant gating parameters to complex cell mixtures, since images of each recorded "event" were available for inspection during postexperimental analysis. Although we had explored other (non-imaging-based) flow cytometry approaches for characterizing biofilms (data not shown), none had proven satisfactory since the accurate delineation of phenotypically distinct cell populations could not be readily resolved without extensive sample preprocessing.

After seeding 96-well plates with *C. albicans* DAY185, cells were treated with compound **3** (10 and 50 μM) or vehicle only (DMSO). All experiments were performed in triplicate on two separate occasions. After enzymatically digesting the biofilm matrix, the samples were stained with propidium iodide (PI), and 10^4 events were analyzed by imaging flow cytometry. The resulting image files were quantitatively analyzed, and the compiled data were used to generate a two-dimensional plot displaying each event as a function of its relative aspect ratio (length:width) and size (area) (Figure 3, panel a). This yielded three distinct event populations (fields R4, R5, and R6) representing different types of cell morphologies. Field R4 comprised spherical cells with yeast-like morphologies, while field R5 was populated by elongated cells with germ tubes/hyphal buds (Figure 3, panel b). In contrast, field R6 consisted of an unresolved mixture of varyingly branched hyphae along with biofilm matrix and cell debris (e.g., dead cells and cell fragments). Consequently, several hundred image files obtained for events in the R6 region were manually inspected to identify an operational set of gating parameters that enabled the further subdivision of this group. This resulted in the separation of the R6 region into two subgroups based on each particle's maximal thickness and fluorescence contrast (Figure 3, panel a). The resulting R7 cluster contained cells with one or more hyphal branch points, while the R8 cluster consisted almost entirely of biofilm matrix and cell debris (Figure 3, panel b). Further analysis of the R7 region demonstrated that this cluster contained pseudohyphae that displayed significant "circularity" and true hyphae, which exhibited less circularity and more diffuse PI staining (i.e., the stained nuclei of true hyphae are typically spread further apart along the length of the hyphal axis, whereas the nuclei of pseudohyphae are usually found in closer proximity to one another due to their irregular branching). This combination of features (i.e., spot intensity minimum and circularity) enabled the separation of the R7 region into two groups, pseudohyphae (R9) and hyphae (R10).

Using this approach, it was revealed that the morphological state distribution of *C. albicans* cells administered **3** was substantially altered compared with vehicle-only treated controls (Figure 3, panel c). Following exposure to **3**, the proportion of budding yeast cells (R4) was significantly increased, while the numbers of germ tube cells (R5), pseudohyphae (R9), and hyphae (R10) were significantly reduced. Taken together, these data indicate that compound **3** inhibits the hyphae formation process in *C. albicans*.

The process of *Candida* biofilm formation and maturation is rather complex, encompassing four distinct stages involving cell adherence, hyphae formation, production of an extracellular matrix, and dispersal.[34,35] Consequently, *Candida* biofilms contain a diverse population of cell types that are essential to their function.[36] Considering the potential clinical value of targeting *Candida* biofilms, characterizing the activity spectrum for each new bioactive agent will play an important role in appraising their functional roles. To date, many methods for studying the morphological distribution of cells in *Candida* biofilms have been proposed.[37] Both microscopy[38] and traditional flow cytometry[39,40] have been used to analyze specific features of these cell populations such as the presence of germ tubes and hyphae. To the best of our knowledge, none of these methods are altogether capable of concurrently providing a quantitative assessment of the full range of cell types contained within *Candida* biofilms. Furthermore, it has been our experience that microcopy-based assay approaches require a substantial investment of time to manually score each sample. This new method promises a fast and precise approach to sample evaluation, which is crucial for conducting a robust natural-products-based (or other chemical source) screening program.

Shearinines Block Hyphae Formation. To further characterize the inhibitory activities of the shearinines, the effects of **3** and **4** were assessed against both nascent and intact biofilms. Both compounds substantially suppressed hyphal outgrowth in developing biofilms at concentrations of 30 μM (Figure 4, panel a), which led to an accumulation of metabolically active (based on XTT assay) pseudohyphae. These results were compared to the effects of 5,8,11,14-eicosatetraynoic acid (ETYA), which had been reported to function as a yeast-to-hyphae transition inhibitor.[25] As expected, *C. albicans* biofilm formation was blocked in cells treated with the reference compound, whereas cell viability and proliferation persisted at concentrations as high as 100 μM. However, the resulting pseudohyphae exposed to as little as 30 μM ETYA exhibited marked cellular swelling, which was in contrast to the normal-looking pseudohyphae that resulted from administration of either shearinine (Figure 4, panel a).

The activities of **3** and **4** were assessed in a time-of-addition assay to determine when during the biofilm development process *C. albicans* was responsive to the inhibitory effects of the shearinines (Figure 4, panel b). Unlike waikialoid A, another potent biofilm inhibitor that was recently described by our group,[29] compounds **3** and **4** showed an extended spectrum of activity that resulted in relatively similar levels of biofilm-formation disruption at time points of up to 6 h after *C. albicans* inoculation. This timeline of activity is consistent with the flow cytometry observations that shearinines have little effect on the relative proportion of *C. albicans* cells exhibiting germ tubes but instead have a more striking effect on limiting the fraction of cells exhibiting extended and/or branched hyphae. These data support the conclusion that compounds **3** and **4** impact a stage in the biofilm development process that is activated much later than the target disrupted by waikialoid A.

The disruption of preformed biofilms is considered an important attribute for the development of a therapeutically useful agent capable of combating established *C. albicans* infections. In these cases, antibiotics such as amphotericin B and caspofungin exhibit reduced efficacy due to limited drug penetrance. This results in the need for dosing patients with much higher levels of the drugs, which poses significant toxicological risks.[41] Both **3** and **4** were able to cause the partial disruption and detachment of preformed *C. albicans* biofilm in a dose-dependent manner resulting in 51 $\pm$ 2% and 34 $\pm$ 1% reductions, respectively, of 48-h-old biofilm at 100 μM (Figure

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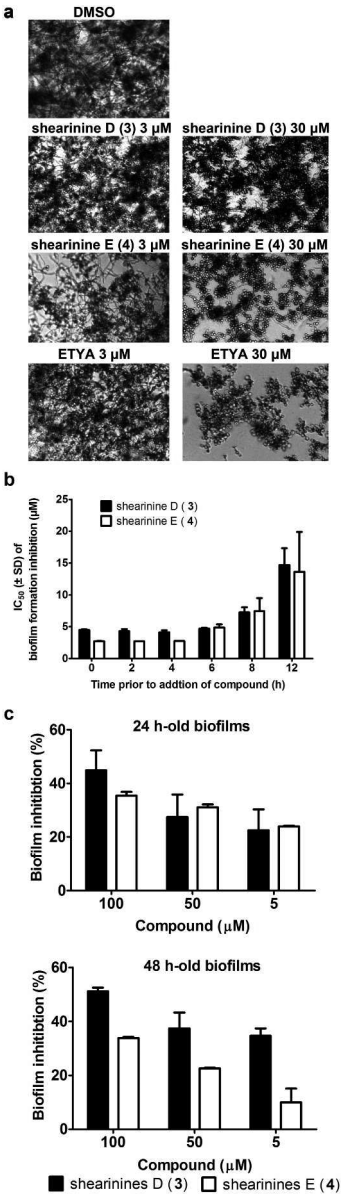

Figure 4. Effect of shearinines D (**3**) and E (**4**) on *C. albicans* DAY185 biofilm formation. (a) Both compounds **3** and **4** strongly inhibited hyphae formation at 30 μM, but only compound **4** exhibited activity at 3 μM. 5,8,11,14-Eicosatetraynoic acid (ETYA) was used as positive control. (b) Time of addition assay showing that compounds **3** and **4** are still both effective at limiting biofilm formation for up to 6 h, which is well after germ tube formation and hyphae formation is initiated under these experimental conditions. (c) Dose–response data revealing that **3** and **4** cause the detachment of preformed biofilms. Data represent the means of triplicate experiments performed on two separate occasions ± standard deviations.

4, panel c). Cells freed from the biofilms remained viable, which was consistent with our previous observation that the shearinines are not acutely toxic to *C. albicans*. Whereas an initial model rationalizing the higher concentrations of shearinines required for disrupting preformed biofilms could involve their limited permeation into the biofilm matrix, we find this explanation to be incongruous with other observations made in the course of our experiments. Namely, 10× reduced concentrations of shearinines were found to be efficacious at synergistically enhancing the antifungal activity of amphotericin B against established biofilms (*vida infra*). Therefore, it is likely that the activities of the shearinines against established biofilms involve a mechanism unrelated to the disruption of their formation.

Shearinines Synergize with Amphotericin B against *Candidas*. The abilities of **3** and **4** to enhance the antifungal activity of the clinically approved agent, amphotericin B, were also tested. This is important because biofilms are reported to increase the resistance of *Candida* to antifungal therapies.[42] Whereas the shearinines did not inhibit the viability or proliferation of *C. albicans* at concentrations of up to 100 μM, the addition of 2.5 μM or more of **3** and **4** to cells treated with a subtherapeutic dose (0.2 μM) of amphotericin B (MIC = 2.5 μM) substantially enhanced its potency against *C. albicans* in developing biofilms (Figure 5, panel a). Treatment of cells with 2.5 μM **3** or **4** resulted in an approximately 8-fold increase in the potency of amphotericin B (Figure 5, panel b). The presumed synergistic activities of compounds **3** and **4** were tested in a checkerboard assay,[43] which is used to delineate synergistic drug combinations. This analysis confirmed the proposed synergistic effects of **3** and **4** with both compounds affording FICI indices of <0.15 (Table 1). The synergistic activities of **3** and **4** against *C. albicans* DAY185 grown on silicone discs were also observed by fluorescence microscopy (Figure 5, panel c). Initially, concentrations of **3** (5 μM) and amphotericin B (0.3 μM) were identified that were not able to inhibit *C. albicans* hyphae formation or cell proliferation, respectively. However, when **3** and amphotericin B were coadministered to *C. albicans* at these same concentrations, the combination treatment was effective at arresting cell growth. The same combination of the two agents showed a similar synergistic effect against another isolate, *C. albicans* SC5314 (Supplementary Table S2). The synergistic effects of shearinines and amphotericin B were also tested against established *C. albicans* biofilms. However, the combination treatment provided a more marginal enhancement of amphotericin B's antifungal activity (Figure 5, panel d).

In light of the synergistic effects exhibited by shearinines with amphotericin B against *C. albicans*, we investigated if similar antagonistic effects could be achieved against a wider selection of *Candida* spp. A total of 28 clinical isolates representing six *Candida* spp. were obtained from the University of Oklahoma Medical Center (*C. albicans*: 11 isolates, *Candida glabrata*: 8 isolates, *Candida parapsilosis*: 6 isolates, *Candida tropicalis*: 1 isolate, *Candida kefyr*: 1 isolate, and *Candida krusei*: 1 isolate), and each specimen was evaluated in the checkerboard assay. We found that 16 of the strains exhibited synergistic responses to the combination treatment of amphotericin B and compound **3** (Table 2 and Supplementary Table S2). Cell proliferation was not inhibited by **3** in any of the isolates at concentrations of up to 100 μM. Interestingly, 10 of the *Candida* isolates were unable to form hyphae or pseudohyphae under the conditions tested in our lab (Supplementary Table S2), whereas only four of these isolates failed to exhibit synergistic sensitivities to the combination of **3** and amphotericin B. These data imply that the shearinines might (i) act through a mechanism that impacts a more global cellular process that affects pathways beyond those solely involved in hyphae formation and biofilm formation or (ii) disrupt multiple biological targets.

Summary and Future Directions. In light of the deleterious effects that *C. albicans* and other *Candida* spp. have on human health, new strategies must be implemented to

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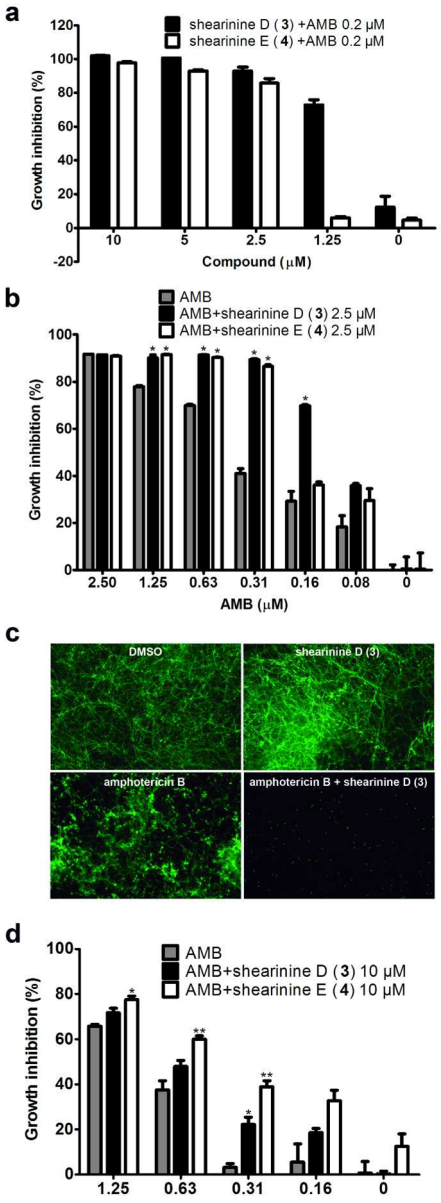

Figure 5. Synergistic activities of shearinines D (**3**) and E (**4**) with amphotericin B (AMB) against *C. albicans*. (a) The effects of 0.2 μM amphotericin B with different concentrations of compounds **3** and **4** on *C. albicans* growth. (b) The effects of 2.5 μM of compounds **3** and **4** with different concentrations of amphotericin B on *C. albicans* growth. Sets of asterisks represents that results for cells administered the combination treatment were statistically different from amphotericin B-only treated controls ($P < 0.05$). (c) Fluorescence images of *C. albicans* treated with 5 μM **3** and 0.3 μM amphotericin B. (d) Both **3** and **4** enhance the activity of amphotericin B against the 48-h-old *C. albicans* biofilms. Bars labeled with asterisks were determined to be statistically different from amphotericin B-treated controls (*$P < 0.05$, **$P < 0.01$). All experiments were performed in triplicate on three separate occasions.

combat these infectious pathogens. Targeting *Candida* biofilms has been identified as one such approach.[9] However, concerns over the spectrum of protection afforded by this tactic, as well as the potential to reliably enhance the efficacy of existing antifungal therapeutics, has tempered the biomedical community's willingness to enthusiastically embrace this methodology. This study offers support for the concept that small molecules that target biofilm-related processes are potentially useful tools for improving clinicians' abilities to exert pharmacological control over *Candida* infections. The synergistic effects of the shearinines present a promising opportunity for exploring how the currently limited range of antifungal antibiotic options could be strengthened to more effectively combat *Candida* infections. Moreover, the synergistic activities of the shearinines point to a potentially useful strategy for helping to limit the significant toxic side effects associated with amphotericin B use. By lowering the amount of amphotericin B that is administered to patients, there is the potential to alleviate many of the drug's self-limiting properties such as nausea and vomiting, fever, breathing difficulties, and nephrotoxicity.[44] The shearinines are a good tool for testing the potential advantages of using adjunctive therapies in combination with antifungals. Despite these intriguing activities, further research will be needed to determine whether the biofilm disruption effects and synergism with amphotericin B are related through a single cellular target or result from the disruption of multiple targets. Nevertheless, given the wide spectrum of unique *Candida* biofilm disrupting compounds that have been detected from our initial natural products studies,[29−31] we are optimistic that new and more potent bioactive compounds will emerge as prospective candidates for preclinical exploration.

METHODS

Strains and Medium. The reference strain *C. albicans* DAY185[45] was a gift from C. A. Kumamoto (Tufts University) and A. Mitchell (Carnegie Mellon University), and *C. albicans* SC5314[33] was a gift from A. Dongari-Bagtzoglou (University of Connecticut Health Center). Clinical isolates were obtained from the University of Oklahoma Medical Center (*C. albicans*: 11 isolates, *Candida glabrata*: 8 isolates, *Candida parapsilosis*: 6 isolates, *Candida tropicalis*: 1 isolate, *Candida kefyr*: 1 isolate, and *Candida krusei*: 1 isolate). These strains were cultured in brain heart infusion medium (BHI medium, Becton Dickinson) or RPMI-1640 plus MOPS medium [RPMI-1640 medium (Sigma) buffered to pH 7.0 with 0.17 M MOPS (3-(*N*-morpholino)-propanesulfonic acid, Sigma)] as required.

Assays for Growth Inhibition and Biofilm Formation. The effects of compounds on the growth of *C. albicans* were tested using the method described in the NCCLS 2002 CLSI M27-A2 guidelines.[29] The biofilm assay was performed as described with the following modifications. Cells of *C. albicans* DAY185 or SC5314 were cultured in BHI medium (Becton Dickinson) at 37 °C overnight. The cells were pelleted by centrifugation, washed with sterile PBS (phosphate-buffered saline, pH 7.4), and resuspended in RPMI-1640 plus MOPS medium. Test compounds were prepared in DMSO at stock concentrations of 20 mM before being serially diluted in RPMI-1640 plus MOPS medium for testing. 5,8,11,14-Eicosatetraynoic acid (ETYA, Santa Cruz Biotechnology) was used as a positive control. Aliquots of yeast suspension (100 μL containing 2.5×10^3 cells mL^{-1}) were added to the medium containing the diluted compounds or DMSO [final concentrations did not exceed 1% (v/v)] before being transferred to 96-well plates (Corning). After 48 h of incubation at 37 °C, the viability of the yeast was measured using the XTT assay.[32] In brief, yeast cells were treated with 0.1 mg mL^{-1} XTT at 37 °C for 1 h. Absorbance measurements were taken at 492 nm using a microplate reader (Infinite M200). The minimum inhibitory concentrations (MIC) for growth were defined as the lowest antifungal concentrations that caused ≥85% reduction in metabolic activity.

For measuring biofilm formation, the medium was aspirated and the wells were washed twice with sterile PBS to remove nonadherent cells. Fresh medium (100 μL RPMI-1640 plus MOPS) was then added back to each well. The formation of biofilms was measured using the XTT assay. All experiments were performed in triplicate on three separate occasions. The 50% inhibitory concentration values (IC_{50}) for biofilm inhibition were calculated using GraphPad Prism 5.

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Table 1. Summary of the Synergistic Interactions of Amphotericin B (AMB) with Shearinines D (3) and E (4) against *C. albicans* as Determined by the Checkerboard Assay[a]

compound	AMB (μM)			compound (μM)			
	MIC_{single}[b]	$MIC_{combination}$	FIC	MIC_{single}	$MIC_{combination}$	FIC	FICI
shearinine D (3)	2.5	0.3	0.12	>100	2.5	<0.025	<0.15 S[c]
shearinine E (4)	2.5	0.3	0.12	>100	2.5	<0.025	<0.15 S

[a]All experiments were performed in triplicate on three separate occasions. [b]The MIC was defined as the lowest concentration causing ≥85% reduction in metabolic activity. [c]Synergistic effect.

Table 2. Summary of the Effects of Amphotericin B and Shearinine D (3) Combinations against a Panel of 28 *Candida* Clinical Isolates[a]

species	isolates tested	isolates exhibiting the indicated interaction		
		synergistic effect	indifference	antagonistic effect
C. albicans	11	7	4	0
C. glabrata	8	5	3	0
C. parapsilosis	6	2	4	0
C. tropicalis	1	1	0	0
C. kefyr	1	1	0	0
C. krusei	1	0	1	0

[a]All experiments were performed in triplicate on three separate occasions.

Biofilm Formation on Silicone Discs. Biofilms were formed on silicone discs (Bentec Medical).[37] In brief, silicone elastomer discs were pretreated with fetal bovine serum and inoculated with *C. albicans* DAY185, which were recovered in BHI medium and washed with sterile PBS. After incubating at 60 rpm agitation at 37 °C for 90 min, the silicone discs were washed with sterile PBS to remove adhered cells and transferred to 6-well plates with 3 mL of RPMI-1640 plus MOPS medium. After adding DMSO (vehicle control), amphotericin B, test compound, or amphotericin B plus test compound, the plates were incubated on a shaker incubator (60 rpm agitation at 37 °C for 48 h). The silicone discs were stained with Alexa Fluor 488 conjugated concanavalin A (Invitrogen) in the dark at 37 °C for 30 min, and biofilms were observed by confocal laser scanning microscopy (Leica) or fluorescence microscope (Molecular Devices).

Morphological Analysis by Imaging Flow Cytometry. Cells of *C. albicans* DAY185 were seeded in 96-well plates at 5×10^4 cells $well^{-1}$ and treated with DMSO or test compound in RPMI-1640 plus MOPS medium at 37 °C for 24 h. After the medium was discarded, the bioflims were digested with lyticase (Sigma) at 37 °C for 30 min.[46] The digested cells were washed with 1 M sorbitol (Sigma) and fixed with 70% ethanol/30% sorbitol[47] at 4 °C for 36 h. After washing with PBS, the cells were treated with ribonuclease A (Amresco) and stained by propidium iodide (PI; Sigma) at 37 °C for 30 min.[48] The cell suspensions were analyzed using an ImageStream imaging flow cytometer (Amnis Corporation).

Hyphae Formation Assay.[29] Cells of *C. albicans* was grown in BHI medium at 37 °C overnight. The cells were pelleted, washed, and suspended in sterile PBS (pH 7.4). Cells were seeded in 96-well plates at 1×10^6 cells $well^{-1}$ and incubated at 37 °C for 1 h. Wells were washed twice with sterile PBS to remove nonadherent cells. RPMI-1640 containing 2% (w/v) glucose and compound in DMSO [final concentration did not exceed 1% (v/v)] were added to each well, and the plates were incubated at 37 °C for 24 h. Hyphae formation was observed with a phase contrast microscope.

Biofilm Time-of-Addition Assay. Using the techniques described above for the biofilm formation inhibition assay, compounds (from 3 to 100 μM) were added at 0, 2, 4, 6, 8, 12 h after seeding *C. albicans* DAY185 cells in a 96-well microplate. At 48 h after inoculation, the wells were washed twice with PBS, and the amount of cells and biofilm were determined by XTT assay. The IC_{50} values for biofilm reduction were calculated using GraphPad Prism 5. All experiments were performed in triplicate on three separate occasions.

Pre-Formed Biofilm Assay. The methods used to assess established biofilms were the same as those described above except that *C. albicans* DAY185 cells were cultured in RPMI-1640 plus MOPS medium in a 96-well microplate at 37 °C for 24 h ("24-h-old biofilm") or 48 h ("48-h-old biofilm") prior to treatment with test compounds.

Checkerboard Assay for Synergistic Effects. To evaluate the synergistic effects of shearinines and amphotericin B, a checkerboard assay was used.[43] *Candida* cells were seeded in 96-well plates and treated with different concentrations of test compounds and amphotericin B, alone or in combination, in RPMI-1640 plus MOPS medium at 37 °C for 48 h. The viability of the yeast was measured using the XTT assay, and the MIC for growth was defined as the lowest antifungal concentrations that caused ≥85% reduction in the metabolic activity. The interactions of test compounds and amphotericin B were based on the fractional inhibitory concentration index (FICI).[43] FICI values are calculated as follows: $(MIC_{\text{drug A in combination}}/MIC_{\text{drug A alone}}) + (MIC_{\text{drug B in combination}}/MIC_{\text{drug B alone}})$. The interpretation of the FICI is determined as follows: ≤0.5, synergistic effect; >0.5 but <4, indifference; and ≥4, antagonistic effect.

Statistical Analysis. Results were expressed as the means ± standard deviations. Analyses were performed using two-tail Student's *t* tests. *P* values <0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Additional methods, natural products characterization, tables of NMR data, additional assay data for clinical isolate, and ITS sequence information for the fungal isolate (KS-017). This material is 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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