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Isolation, Structure, and Biological Activity of Phaeofungin, a Cyclic Lipodepsipeptide from a *Phaeosphaeria* sp. Using the Genome-Wide *Candida albicans* Fitness Test

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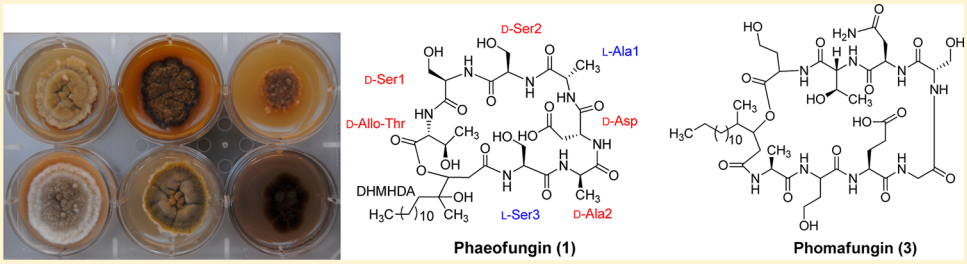
Supporting Information



ABSTRACT: Phaeofungin (**1**), a new cyclic depsipeptide isolated from *Phaeosphaeria* sp., was discovered by application of reverse genetics technology, using the *Candida albicans* fitness test (*Ca*FT). Phaeofungin is comprised of seven amino acids and a *β*,*γ*-dihydroxy-*γ*-methylhexadecanoic acid arranged in a 25-membered cyclic depsipeptide. Five of the amino acids were assigned with D-configurations. The structure was elucidated by 2D-NMR and HRMS-MS analysis of the natural product and its hydrolyzed linear peptide. The absolute configuration of the amino acids was determined by Marfey's method by complete and partial hydrolysis of **1**. The *Ca*FT profile of the phaeofungin-containing extract overlapped with that of phomafungin (**3**), another structurally different cyclic lipodepsipeptide isolated from a *Phoma* sp. using the same approach. Comparative biological characterization further demonstrated that these two fungal lipodepsipeptides are functionally distinct. While phomafungin was potentiated by cyclosporin A (an inhibitor of the calcineurin pathway), phaeofungin was synergized with aureobasidin A (**2**) (an inhibitor of the sphingolipid biosynthesis) and to some extent caspofungin (an inhibitor of glucan synthase). Furthermore, phaeofungin caused ATP release in wild-type *C. albicans* strains but phomafungin did not. It showed modest antifungal activity against *C. albicans* (MIC 16−32 μg/mL) and better activity against *Aspergillus fumigatus* (MIC 8−16 μg/mL) and *Trichophyton mentagrophytes* (MIC 4 μg/mL). The linear peptide was inactive, suggesting that the macrocyclic depsipeptide ring is essential for target engagement and antifungal activity.

Candida albicans and *Aspergillus fumigatus* are two key fungal pathogens responsible for the majority of life-threatening systemic fungal infections. These infections are particularly problematic to the patient populations that are immunocompromised.[1] Currently, three classes of drugs are used in the clinic for treatment of systemic fungal infections. These are amphotericin B,[2] azoles (e.g., fluconazole),[3] and echinocandins (e.g., caspofungin, micafungin, and anidulafungin).[4] While these treatment options are generally acceptable, they are not limitless. In addition, fungal strains resistant to these treatments are emerging, though fortunately with lower frequency. Therefore, new treatment options are needed to augment or replace these agents.

Natural products continue to be one of the best sources of antifungal agents, and two of the three classes of clinically used antifungal agents are natural product derived. However, the natural product-based drug discovery process comes with

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limitations, as compared to synthetic compounds, ranging from the high demand for resources, the specialized expertise needed in building and maintaining high-quality libraries of natural products, and the selection of diverse microbial strains capable of producing natural products, to the incompatibility of natural product extract libraries with the "blitz screen" mentality of high-throughput screens. These limitations had been further exacerbated by the ever-increasing rediscovery of known compounds. Collectively, they have exhausted the conventional "grind-and-find" approach leading to abandonment of natural product efforts by most of the big pharmaceutical companies. The key to increasing the probability of success in identifying novel scaffolds from natural products is threefold: (1) The diversity of the source microbes must be improved to increase the probability of new natural product discovery; (2) prior to committing chemical fractionation, those extracts (of fermentation broths) containing known compounds must be effectively recognized and removed; and (3) extracts containing novel biological activities (and most likely novel structures) must be positively identified and prioritized.

To address these challenges, efficient and robust natural product dereplication by chemical and biological means is needed. While large-scale chemical profiling can be readily achieved, true dereplication is resource-consuming and often strategically prohibitive if a structure is to be specifically associated with a specific biological activity. Dereplication of compounds using conventional single-target-based biochemical assays is relatively straightforward. However, parallel multitarget (protein or whole-cell based) screenings are not amenable to the challenge, since biological dereplication requires comprehensive insights of mechanisms of action of active compounds that may have not been characterized. Chemical genomic strategies could provide a solution to biological dereplication. In fact, our effort in applying the chemogenomic approach to natural products (see Roemer et al.[5] for a review) was first and foremost augmented by replenishing the natural product library with biodiversity. We have expanded our efforts to isolate microorganisms from different geography and habitats. This geographic diversity was coupled with improved high-throughput fermentation methods aimed to increase functional chemical diversity.[6]

The chemogenomic approach, known as the "fitness test", was first developed using baker's yeast and adapted in the primary human fungal pathogen, *C. albicans*.[7,8] The assay relies on a phenomenon known as chemically induced haploinsufficiency, where deletion of one allele of a given gene in a diploid fungus renders the mutant hypersensitive to a compound that inhibits the corresponding protein target at sublethal concentrations. In fact, when a collection of heterozygous deletion strains covering the entire or a significant portion of a fungal genome was tested against a panel of antifungal compounds with known modes of action (MOAs), hypervariable growth (hypersensitivity or resistance, hence the term fitness test) was restricted to only a small set of strains that correspond to aspects of their respective MOAs, including the targets. In almost all cases, the concise fitness test profiles are reflective of the MOAs of compounds tested.[7,8] As all the heterozygotes were barcoded at the deleted allele, only a small volume of pooled strains for each assay is needed to determine the relative abundance (i.e., fitness) of each strain in response to chemical perturbation, by DNA microarray methodology.

By applying the *C. albicans* fitness test (*Ca*FT), it was possible to mechanistically annotate natural products in the form of crude extracts. With a compendium of known natural products (pure), the *Ca*FT profile of any extract in question could be readily compared. If it matched the profile of a known compound, the extract was immediately analyzed by LC-MS to confirm the presence/absence of such a compound. In other cases, a *Ca*FT profile might reflect the biological MOA of a known natural product that is not present in the compendium. This could be readily remedied in the *Ca*FT and the LC-MS analyses using the compound in question. The *Ca*FT profiling of natural products had been a self-reiterating process that also grouped extracts by their biological activities. Once a novel *Ca*FT profile was identified, chemical resources could then be allocated for mechanistically guided fractionation of the representative extract.[5] This approach has been proven productive in identifying novel compounds from natural products with distinctive biological activities.[5,9−13]

In this report, we present the identification, isolation, structural elucidation, and characterization of a cyclic lipodepsipeptide, phaeofungin (**1**, Figure 1), from an extract of a fungus, *Phaeosphaeria* sp. (F-167,953). The original extract was identified for its whole-cell activity against wild-type *C. albicans* and *A. fumigatus*, two medically important human fungal

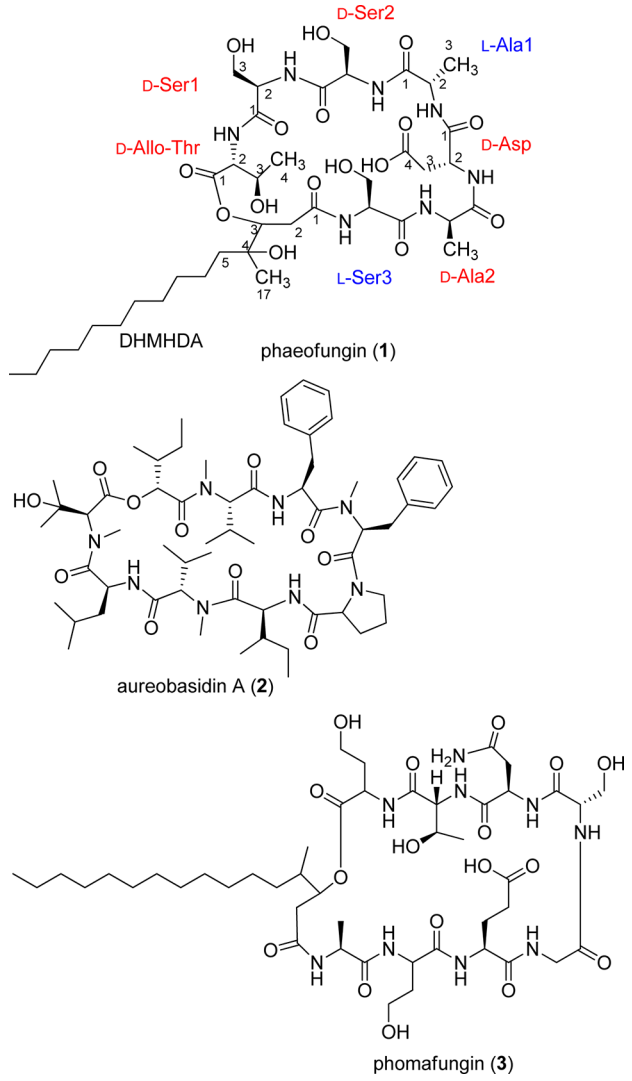

Figure 1. Structures of phaeofungin (**1**) and two other cyclic depsipeptides, aureobasidin A (**2**) and phomafungin (**3**).

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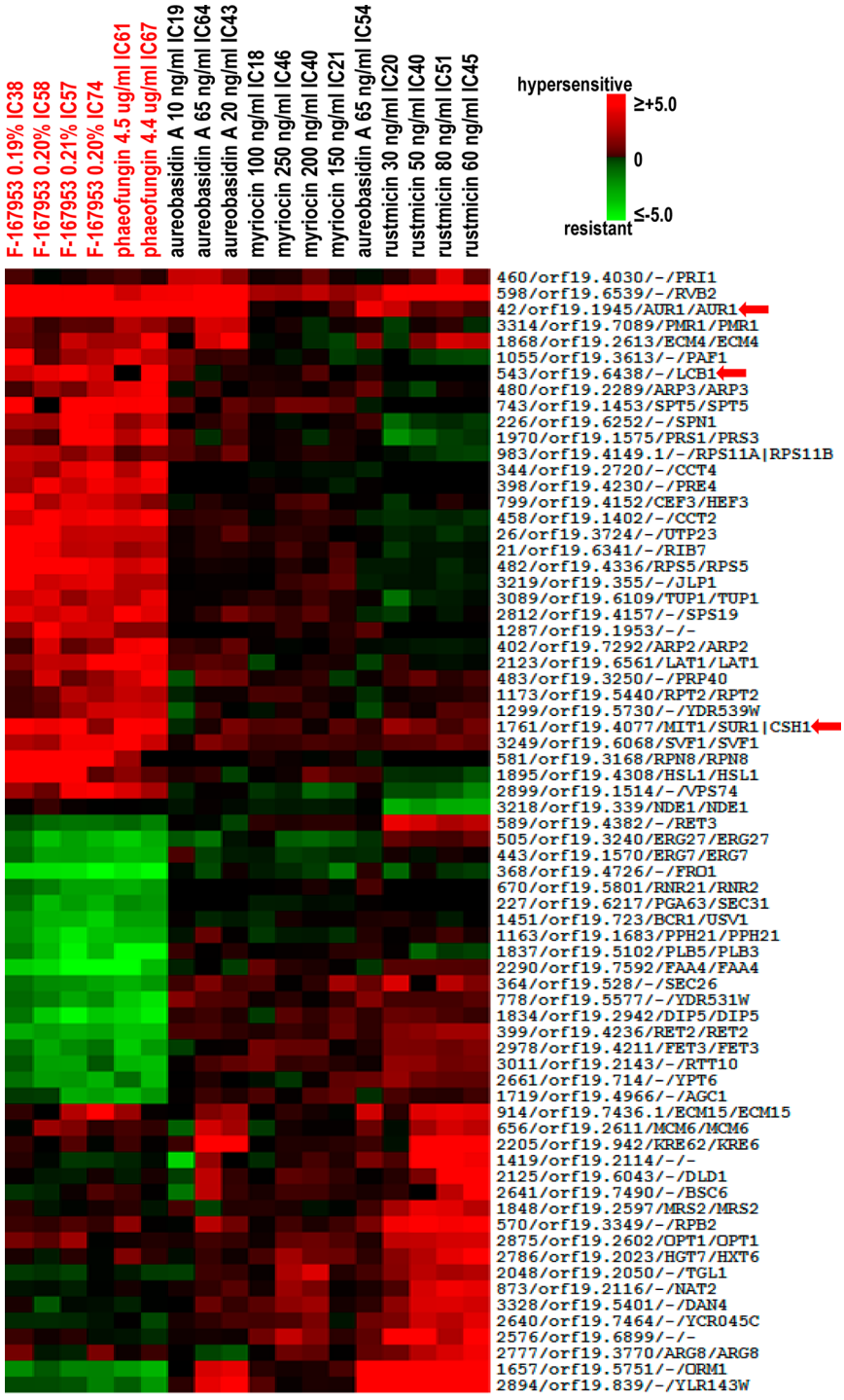

Figure 2. *Ca*FT profiles of aureobasidin A, myriocin, rustmicin, phaeofungin, and the fermentation extracts of F-167,953. The relative behavior of each heterozygous deletion strain elicited by the antifungal compounds is expressed by the normalized *z*-scores, with positive values indicating hypersensitivity, and negative values resistance. To compare the *Ca*FT profiles, strains with absolute values of *z*-scores no less than 3 in at least three independent experiments were selected. Their *z*-scores were grouped by hierarchical clustering using the centroid linkage method, the results of which are displayed in TreeView, with the scale of the heat map shown on the top-right corner, together with experimental numbers and experimental conditions. The heterozygous deletion strains are indicated by the orf19 designations of the corresponding genes, with *C. albicans* gene names and *S. cerevisiae* orthologs/homologues (if any) (as appeared in the Candida Genome Database, www.candidagenome.org). Highlighted by red arrows are those involved in the sphingolipid biosynthesis.

pathogens. When tested in the *Ca*FT, it generated a profile that contained hypersensitivities of fungal strains corresponding to the sphingolipid biosynthesis pathway. However, the *Ca*FT and LC-MS analyses failed to detect the presence of known inhibitors of the pathway (e.g., myriocin,[14] rustmicin,[15−17] minimoidin,[16] aureobasidins (e.g., aureobasidin A, **2**),[18] australifungin,[16] and syringomycins[19]). Nonetheless, the *Ca*FT profile overlapped with that of another set of extracts, which were subsequently identified to contain a cyclic lipodepsipeptide, phomafungin (**3**),[11] indicating certain struc-

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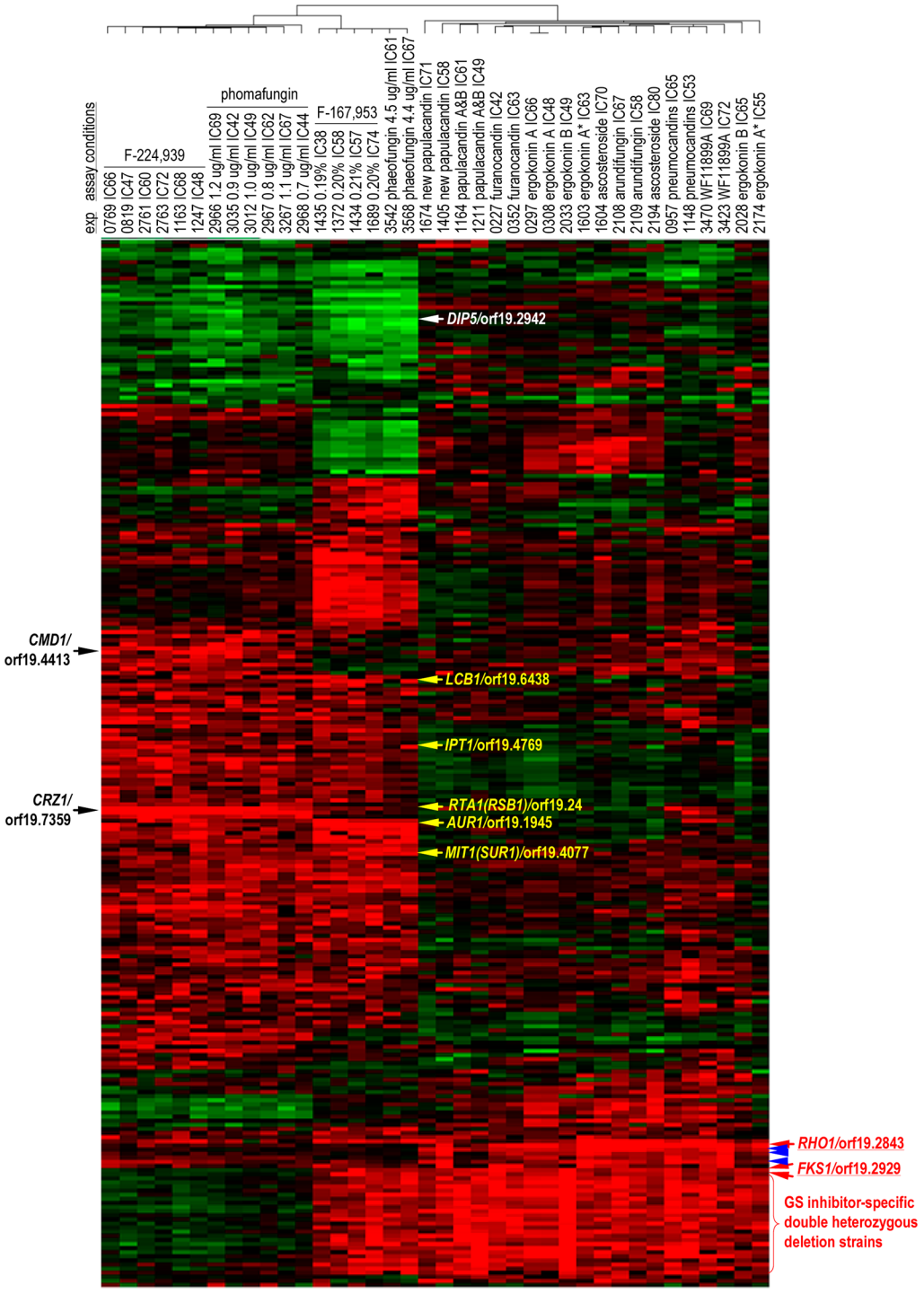

Figure 3. *Ca*FT profiles of phaeofungin (**1**) and its source extract from F-167,953, and phomafungin (**3**) and its source extract from F-224,939, and other extracts identified to contain known inhibitors of the fungal glucan synthase (GS) inhibitors. The *Ca*FT results were analyzed and displayed using the same parameters described in Figure 2. Highlighted are genes that are involved in the calcineurin pathway (black), sphingolipid (yellow), potential efflux (white), and glucan synthesis (red). Strains of double heterozygous deletion of *FKS1* together with another gene implicated in the glucan synthesis/cell wall are indicated by a red bracket, with blue arrows indicating three other deletion strains ($RHO1^{+/-}$ $SMI1^{+/-}$, $RHO1^{+/-}$ $FKS3^{+/-}$, and $RHO1^{+/-}$ $CHS3^{+/-}$). These three strains, together with $RHO1^{+/-}$ and two independently constructed $GSK1^{+/-}$ strains, were not significantly hypersensitive to F-167,953 or phaeofungin. The scale of the heat map is the same as in Figure 2.

tural similarity. We proceeded to isolate and characterize the active compound produced by F-167,953. Consistent with the prediction of the *Ca*FT results, this compound turned out to be another cyclic lipodepsipeptide, termed phaeofungin (**1**). Despite biological signature overlap of the two cyclic depsipeptides, our results showed that phaeofungin (**1**) and phomafungin (**3**) are structurally and biologically distinct. This discovery demonstrates the power of the *Ca*FT, which detected and differentiated natural products in crude extracts with distinct or overlapping (often partially) activities with distinct structural and mechanistic attributes.

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments of Phaeofungin (1) in C_5D_5N and CD_3OD

AA	no.	type	δ_C C_5D_5N	δ_H, mult, J in Hz, C_5D_5N	HMBC (H→C)	δ_C CD_3OD	δ_H, mult, J in Hz, CD_3OD
Thr	1	CO	169.65			169.6	
	2	CH	61.3	4.63, m	C-1, 3, Ser1 (C-1)	61.6	4.24, d, 4
	3	CH	67.9	4.16, dq, 3, 6	C-1	68.3	4.10, m
	4	CH_3	21.6	1.41, d, 6.0	C-2, 3	20.7	1.3, d, 6.5
		α-NH		9.82, d, 6.0	C-2, 3, Ser1 (C-1)		
Ser1	1	CO	173.85			173.5	
	2	CH	57.2	5.04, dt, 5.5, 3	C-1	56.7	4.39, t, 3
	3	CH_2	62.7	4.55, dd, 12, 3	C-1	63.0	4.09, dd, 10, 2.5
				4.75, dd, 12, 3			4.25, m
		α-NH		10.15, d, 5	C-2, Ser2 (C-1)		
Ser2	1	CO	172.91			172.7	
	2	CH	58.8	5.39, dt, 9.5, 5.5	C-1, 2	58.3	4.55, t, 6
	3	CH_2	63.5	4.43, dd, 14, 6	C-1, 2	63.7	3.69, dd, 11.5, 6
				4.39, dd, 14, 5.5			3.67, dd, 11.5, 6
		α-NH		8.92, d, 9.5	C-2, 3, Ala1 (C-1)		
Ala1	1	CO	173.85			174.1	
	2	CH	51.0	4.90, pentet, 7.0	C-1, 3	51.0	4.40, q, 7.5
	3	CH_3	17.4	1.70, d, 7.0	C-1, 2	16.8	1.43, d, 7.5
		α-NH		8.07, d, 7.0	Asp (C-1)		
Asp	1	CO	174.19			174.1	
	2	CH	51.6	5.27, ddd, 9, 5, 2	C-1, 3, 4, Ala2 (C-1)	51.4	4.62, dd, 5, 3
	3	CH_2	41.7	2.78, dd, 14, 5.5	C-1, 2, 4	40.4	3.0, dd, 16, 3
				3.41, dd, 14, 3.0	C-2, 4		2.48, dd, 16, 5
	4	CO	177.80			178.2	
		α-NH		8.91, d, 9	C-2, 3, Ala2 (C-1)		
Ala2	1	CO	173.94			175.3	
	2	CH	53.2	4.74, dq, 4.5, 7.5	C-1, 3	52.5	4.27, q, 7.5
	3	CH_3	17.1	1.42	C-1, 2	16.4	1.40, d, 7.5
		α-NH		9.69, d, 5.0	C-2, 3, Ser3 (C-1)		
Ser3	1	CO	174.04			174.3	
	2	CH	56.3	5.25, dt, 9, 3		56.6	4.48, t, 2.5
	3	CH_2	63.3	4.25, dd, 11, 2.5	C-1, 2	63.0	3.79, dd, 11.5, 3
				4.64, m			4.11, m
		α-NH		8.60, d, 9.5	C-2, DHMHDA (C-1)		
DHMHDA	1	CO	172.65			174.3	
	2	CH_2	38.7	1.9, brd, 14	C-1, 3	39.3	2.69, dd, 14, 10
				2.71, dd, 14, 10	C-1, 3		2.51, brd, 14
	3	CH	79.5	5.90, d, 10	C-1, 2, 4, 5, 17, Thr (C-1)	79.3	5.19, d, 10
	4	C°	74.0			74.7	
	5	CH_2	39.3	1.56, m		39.1	1.50, m
	6	CH_2	23.7	1.60, m		24.0	1.42, m
				1.76, m			
	7−13	CH_2	30.1	1.25, m		30.4	1.25, m
			30.4 (5)			30.8 (5)	
			31.1			31.4	
	14	CH_2	32.5	1.25, m		33.1	1.25, m
	15	CH_2	23.5	1.25, m		23.7	1.25, m
	16	CH_3	14.9	0.88, t, 7.0	C-14, 15	14.4	0.89, t, 7
	17	CH_3	22.6	1.35, s	C-3, 4, 5	22.4	1.19, s

RESULTS AND DISCUSSION

Identification of a Phaeofungin-Containing Extract in the *Ca*FT. Extracts of fermentation broths of microbial strains with whole-cell antifungal activities against the wild-type *C. albicans* were tested in the *Ca*FT assay. A fungal extract from strain F-167,953 produced a complex profile that was distinct from any known natural products tested in the *Ca*FT. Several hypersensitive heterozygous deletion strains were identified that corresponded to genes involved in the sphingolipid biosynthesis, including *AUR1/orf19.1945* (the target of the antifungal cyclic depsipeptide aureobasidin A), *LCB1/orf19.6438*, and *MIT1/orf19.4077* (Figure 2). However, aureobasidin A (**2**) and other inhibitors of the sphingolipid biosynthesis yielded *Ca*FT profiles that shared no additional similarity (Figure 2). Nevertheless, when compared with other natural products from our collection, the *Ca*FT profile of F-167,953 overlapped with that of another class of fungal extracts that were subsequently identified to contain the lipodepsipeptide phomafungin (**3**)[11] (Figure 3). More intriguingly, F-167,953 induced specific hypersensitivity of a group of double

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heterozygous deletion strains, each of which contained heterozygous deletions of two genes that are functionally implicated in the fungal glucan synthesis and cell wall biosynthesis.[5] The hypersensitivity of these strains is highly reflective of the presence of fungal glucan synthase inhibitors in the extracts[5] (Figure 3). Although the complex profiles of these extracts did not directly suggest a discernible MOA, the difference between F-167,953 and the phomafungin-containing extracts indicated that their biological activities shared certain similarities but were not completely identical. Prior to any chemical fractionation effort, we predicted that the corresponding natural products present in these extracts were structurally and functionally different. These extracts were thus selected for detailed chemical purification and characterization of natural product(s) responsible for biological activities.

Producing Organisms. The fungal strain F-167,953 was isolated from a *Sedum* sp., a flowering plant, collected in the province of Albacete, Spain. Similarity searches with the 28S and ITS rDNA sequences indicated a relatively high sequence similarity with some species of *Phaeosphaeria*, *Ophiosphaerella*, and various anamorph genera associated with the family Phaeosphaeriaceae. Alignments of its 28S rDNA sequence clearly placed this fungus within the Phaeosphaeriaceae. The nearest match in the ITS region of the rDNA was an anamorph species, *Chaetosphaeronema hispidulum* (CBS 216.75), of the Phaeosphaeriaceae, with a similarity of 94.5%. A second strain, F-262,327 (from the province of Cuenca), was identified subsequently to produce phaeofungin (**1**). It yielded a nearly identical 28S rDNA sequence. The 28S rDNA sequences of these two strains were resampled from recent phylogenetic studies on fungi of the Phaeosphaeriaceae[20−22] and aligned. Neighbor joining analysis of the alignment (Figure S1, Supporting Information) placed F-167,953 and F-262,327 in the Phaeosphariaceae among a group of anamorph species including *Plenodomus fuscomaculans* and *Chaetosphaeronema coonsii*.

Prior to the *Ca*FT analysis, these extracts of fungus F-167,953 were observed to perturb the cell wall biosynthesis in a distinct cell-based assay designed to detect inhibitors of the *C. albicans* cell wall function/glucan synthesis (data not shown), a bioactivity reminiscent of what was observed in the *Ca*FT (Figure 3). The strain was revived from the freezer and grown in an eight-medium nutritional array to generate 1 mL extracts for evaluation of antifungal activity.[6] The fungus produced active *C. albicans* activity in five of the eight media tested, and the medium yielding the most potent extract was scaled up to one liter.

Isolation and Structural Elucidation of Phaeofungin (1). The fermentation broth of *Phaeosphaeria* sp. F-167,953 was extracted with an equal volume of acetone. The extract was chromatographed on an Amberchrome column and eluted with a step gradient of aqueous MeOH. The fractions from each chromatographic step were tested for their antifungal activity against the wild-type *C. albicans*. Those fractions containing the antifungal activity were finally purified by reversed-phase HPLC, and the resulting active fractions were lyophilized to afford **1** (638 mg, 638 mg/L) as an amorphous, colorless powder. The purified material was tested in the *Ca*FT, and it yielded a profile indistinguishable from the original one (Figures 2 and 3), suggesting that the purified material represents the biological activity detected in the *Ca*FT.

HRESIFTMS analysis of **1** provided a molecular formula of $C_{40}H_{69}N_7O_{16}$. It displayed end absorption in the UV spectrum and absorption bands for hydroxy and various amide/ester carbonyls in the IR spectrum. The ^{13}C NMR spectrum along with the DEPT spectrum in C_5D_5N and CD_3OD exhibited the presence of nine carboxyl carbonyls, nine methines (seven α-amino acid methines and two oxymethines), three oxymethylenes, an oxygenated quaternary, and four methyls, and the remaining carbons were aliphatic methylenes, typical of those present in a fatty chain. The ^1H NMR spectrum in C_5D_5N (Table 1) revealed seven doublets in the downfield region of the spectrum, which were exchanged in the ^1H NMR spectrum in CD_3OD (Table 1), suggesting that they are exchangeable NH groups. This indicated that compound **1** is peptidic, consisting of at least seven α-amino acids. The COSY, TOCSY, and HSQC spectroscopic analysis of **1** indicated the presence of the following moieties: three serines (Ser), a threonine (Thr), two alanines (Ala), an aspartic acid (Asp), and a β,γ-dihydroxy-γ-methylhexadecanoic acid (DHMHDA). The structure of the latter unit was confirmed by HMBC correlations of H_3-17 to C-3, C-4, and C-5 and H-3 to C-4, C-5, and C-17. Basic hydrolysis of **1** produced the acylated heptapeptide **4** (MW 921), suggesting the presence of a lactone ring in **1**. The mass spectrometric analysis of **4** showed a fragment ion at m/z 638 due to the loss of the fatty acid unit. The HRMS-MS analysis of the fragment ion at m/z 638 produced *b* series fragment ions at m/z 519, 432, 345, 274, and 159, suggesting a Ser-Ala-Asp-Ala-Ser-Ser-Thr-OH moiety (Figure 4). Whether Ser or Ala was present at the end of the

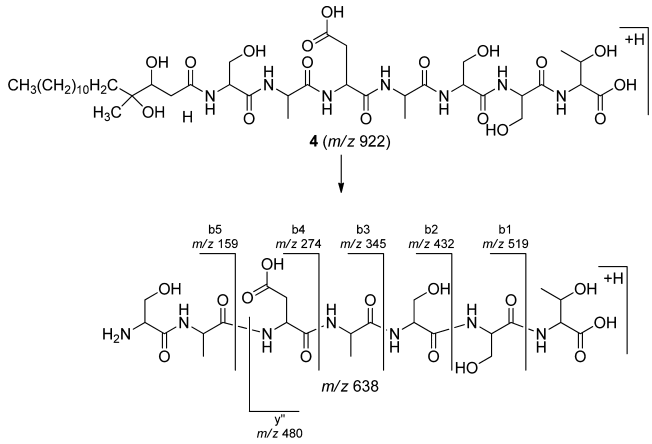

Figure 4. HRESIMS-MS fragmentation of the acyclic acylated heptapeptide **4**.

N-terminus could not be differentiated from these fragmentations due to the lack of fragmentation between these two amino acids. The amino acid sequence was corroborated by the HMBC correlations of α-CH and NH protons to the corresponding adjacent carbonyls and α-carbons in C_5D_5N (Table 1). The unambiguous assignment of the C-1 of DHMHDA unit was secured from the HMBC correlations of H-2 and H-3, which were critical for the final sequence determination. The C-1 signal of the DHMHDA unit showed the HMBC correlation to the Ser3-NH, confirming the amide bond between Ser3 and DHMHDA, thus the linear sequence. The H-3 resonance of the DHMHDA unit displayed a strong HMBC correlation to the C-1 of the threonine, confirming the macro-lactone ring in the depsipeptide structure of **1**.

The ^1H NMR chemical shifts, including shifts of carbon-bound protons in C_5D_5N, were highly concentration depend-

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ent. The same concentration-dependent phenomenon was not observed in CD_3OD. This phenomenon was likely due to the presence of a large number of hydroxylated amino acids that interacted with C_5D_5N. NOESY correlations of phaeofungin in C_5D_5N further corroborated the sequence and the assignment of the structure. The Ser2-NH showed NOESY correlations to NH groups of both the adjacent amino acids Ser1 and Ala1, suggesting that they are proximal to each other. Similar correlations were observed with the NH groups of other amino acids, confirming the amino acid sequence of the depsipeptide.

Exhaustive acid hydrolysis of phaeofungin (**1**) with 6 N HCl at 110 °C overnight followed by derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-valine, L-FDVA)[23] and comparative quantitative analysis by LC-MS with corresponding authentic standards confirmed the presence of 2 × D-Ser, a L-Ser, a D-allo-Thr, a D-Ala, an L-Ala, and a D-aspartic acid (Asp). Partial acid hydrolysis with 0.5 N HCl at 100 °C for 30 min followed by a reversed-phase HPLC purification led to the isolation of two major peptide fragments, **5** and **6** (Figure 5).

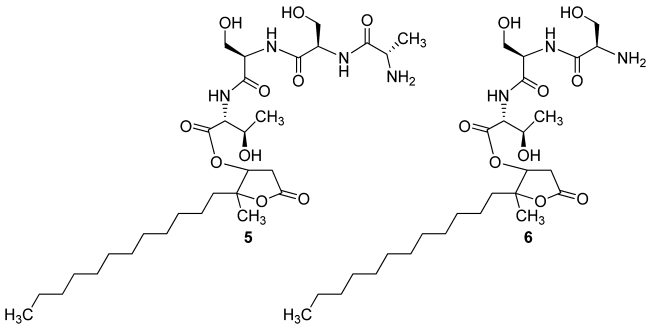

Figure 5. Fragments (**5** and **6**) from the partial acid hydrolysis of phaeofungin (**1**).

The structures of these fragments were elucidated by HRESIMS-MS analysis (Figure S2, Supporting Information). Similar exhaustive acid hydrolysis of **5** and **6** followed by analogous reaction with Marfey's reagent (L-FDVA)[23] and similar comparative quantitative analysis confirmed the presence of L-Ala, 2 × D-Ser and D-allo-Thr; and 2 × D-Ser and L-allo-Thr from **5** and **6**, respectively. On the basis of the combination of all the data presented above, a cyclo-(DHMHDA-L-Ser-D-Ala-D-Asp-L-Ala-D-Ser-D-Ser-D-allo-Thr) depsipeptide structure **1** was established for the natural product isolated from the extract F-167,953, named phaeofungin. The configurations of the two stereocenters of the oxygenated carbons of the fatty acid remain unassigned.

Characterization of Biological Activity of Phaeofungin (1). The antifungal potency and the spectrum of phaeofungin (**1**) were determined in an in vitro growth assay. The data are summarized in Table 2. Phaeofungin inhibited *C. albicans* with an MIC of 16−32 μg/mL. It showed similar activity against *C. lusitaniae* (MIC 32 μg/mL) and poorer activities against other *Candida* species (Table 2). It exhibited slightly better activities against the filamentous fungi *A. fumigatus* (MIC 8−16 μg/mL) and the dermatophytic fungi *T. mentagrophytes* (MIC 4 μg/mL). The activities of both *C. albicans* and *A. fumigatus* were negatively impacted by the presence of 50% mouse and 50% human sera. Compound **1** did not show any activity against *Staphylococcus aureus* (tested at the highest concentration of 32 μg/mL), suggesting potential for relative specificity for the fungal growth inhibition. The linear peptide **4** showed no activity against any fungal strains (MIC > 32 μg/mL), indicating the importance of the macrocyclic depsipeptide for the antifungal activity. Phaeofungin exhibited dose-dependent toxicity in DBA/2N mice when dosed (5% aqueous DMSO) intraperitoneally (ip) at over 12.5 mg/kg including lethality. The compound showed no adverse effects at 6.25 mg/kg.

When the isolated phaeofungin (**1**) was tested in the *Ca*FT, it yielded a profile that preserved characteristic features of the original extract (Figures 2 and 3), suggesting that compound **1** corresponded to the biological activity observed in the *Ca*FT of the extract. After structure elucidation, it became clear that both phaeofungin and phomafungin (**3**)[11] are cyclic lipodepsipeptides. However, as predicted by the *Ca*FT profiles, they are structurally different from each other in both the lipid and the cyclic peptide moieties: phaeofungin is cyclo-[DHMHDA-L-Ser-D-Ala-D-Asp-L-Ala-D-Ser-D-Ser-D-allo-Thr], and phomafungin is cyclo-[HMHDA-L-Ala-homoser-L-Glu-Gly-L-Ser-D-Asn-D-allo-Thr-homoser]. A close analysis of the *Ca*FT profiles revealed several functional aspects of the activity that prompted us to characterize the biological activities of both lipodepsipeptides.

It was noted that heterozygous deletions of genes involved in the sphingolipid biosynthesis are hypersensitive to phomafungin (**3**) and phaeofungin (**1**) in the *Ca*FT (Figure 3). However, a quantitative difference was noted in the relative hypersensitivity. Most notably, *AUR1*$^{+/-}$ displayed pronounced

Table 2. Antifungal Activity (MIC, μg/mL) and Spectrum of Phaeofungin (1)[a]

organism	strain	phaeofungin (**1**)	phomafungin (**3**)[b]	caspofungin
Candida albicans	MY1055	32 (16)	4	0.5
C. albicans +50% mouse serum	MY1055	>32	>32	0.25
C. glabrata	MY1381	>32	8 (4)	0.25
C. parapsilosis	ATCC22019	>32 (32)	4	0.5
C. lusitaniae	MY1396	32	4	<0.03
C. krusei	ATCC6258	>32 (32)	4	1
C. tropicalis	MY1012	>32	>32	NT
Aspergillus fumigatus	MF5668	16 (8)	4	32
A. fumigatus +50% human serum	MF5668	>32	>32	32
Trichophyton mentagrophytes	MF7004	4	2 (1)	32
Staphylococcus aureus	MB2865	>32	>32	>32

[a]The number in parentheses is MIC_{80} (prominent inhibition of growth, also referred to as MEC for *Aspergillus*). The time of incubation for all MIC measurements was 24 h except for *A. fumigatus*, which were incubated for 48 h, and *T. mentagrophytes* was incubated for 96 h. [b]MIC data of phomafungin (**3**) are taken from ref 11.

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hypersensitivity to phaeofungin, and *RTA1*$^{+/-}$ was most sensitive to phaeofungin (Figure 3). Since *AUR1* corresponds to the target of aureobasidin A (**2**), we tested how the antifungal activity of **2** was affected by either of the lipodepsipeptides. Our results demonstrated that phaeofungin and aureobasidin A are synergistic; that is, a combination of both compounds at sublethal concentrations resulted in maximal inhibition of growth of the wide-type *C. albicans* strain (Figure 6A), whereas less synergy was observed between phomafungin and aureobasidin A (Figure 6B).

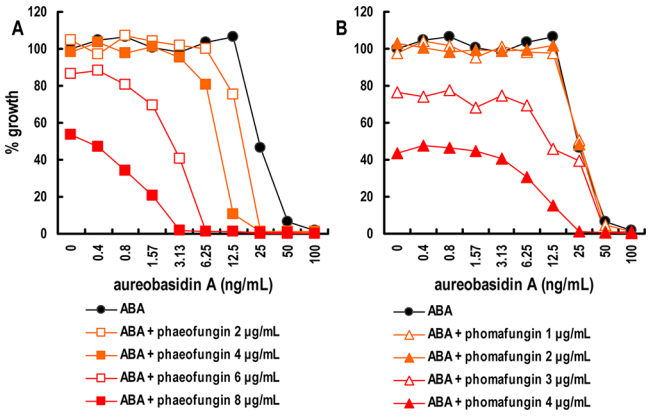

Figure 6. Interactions of the antifungal activities of aureobasidin A (ABA) with phaeofungin (A) and phomafungin (B) against the wild-type *C. albicans* strain used for the construction of heterozygous deletions strains. Sublethal concentrations of either cyclic lipodepsipeptides (as indicated) were mixed with aureobasidin A in a range of concentrations (100 to 0.4 ng/mL). The antifungal activities of mixtures were measured after 24 h incubation from the OD_{600}, which was then normalized by the growth of untreated culture.

In a cell-based screen for inhibitors of the fungal glucan synthase, the phaeofungin-containing extract from F-167,953 was shown to perturb cell wall biogenesis in *C. albicans* (data not shown). Consistent with this early observation, both the extract and purified phaeofungin induced hypersensitivity of two independently constructed *GSK1*$^{+/-}$ strains and a group of double heterozygous deletion strains that have been implicated in the action of inhibitors of the fungal glucan synthase (Figure 3). However, the rest of the profiles shared no similarity: the *RHO1*$^{+/-}$ and three additional double heterozygous deletion strains were only hypersensitive to the known glucan synthase inhibitors (Figure 3). When tested in combination, low concentrations of phaeofungin (with no inhibitory effect) potentiated the activity of caspofungin (Figure 7A). Under similar conditions, no potentiation was observed by phomafungin (Figure 7B).

Two heterozygous deletion strains for genes involved in the calcineurin pathway, *CMD1/orf19.4413* and *CRZ1/orf19.7359*, displayed specific hypersensitivity to phomafungin, but not to phaeofungin (Figure 3). We tested an inhibitor of the calcineurin pathway, cyclosporin A, in combination with either lipodepsipeptide. Cyclosporin A, not active against *C. albicans*, potentiated the potency of phomafungin (Figure 8B) but not phaeofungin (Figure 8A), consistent with the *Ca*FT results.

Despite these functional differences, the *Ca*FT profiles of both phomafungin (**3**) and phaeofungin (**1**) contained the relative resistance of a heterozygous deletion strain of *DIP5/orf19.2942* (Figure 3). The corresponding gene encodes a dicarboxylic amino acid permease. The relative resistance of the heterozygous deletion strain could indicate a potential role of the putative permease in the uptake of both lipodepsipeptides. If so, a loss-of-function mutation of *DIP5* should render the mutant strain resistant to both phomafungin and phaeofungin.

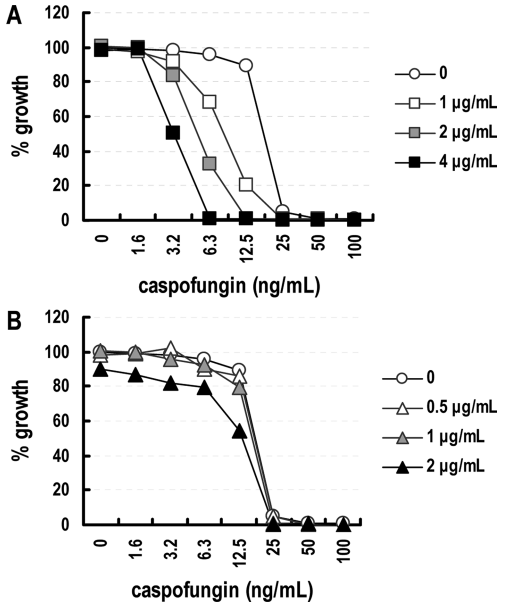

Figure 7. Interaction of the antifungal activities of caspofungin with phaeofungin (**1**) (A) and phomafungin (B) in the wild-type *C. albicans* strain. Sublethal concentrations of either cyclic lipodepsipeptide were mixed with caspofungin in a range of concentrations (100 to 1.6 ng/mL). The antifungal activities of mixtures were measured after a 24 h incubation from the OD_{600}, which was then normalized by the growth of untreated culture.

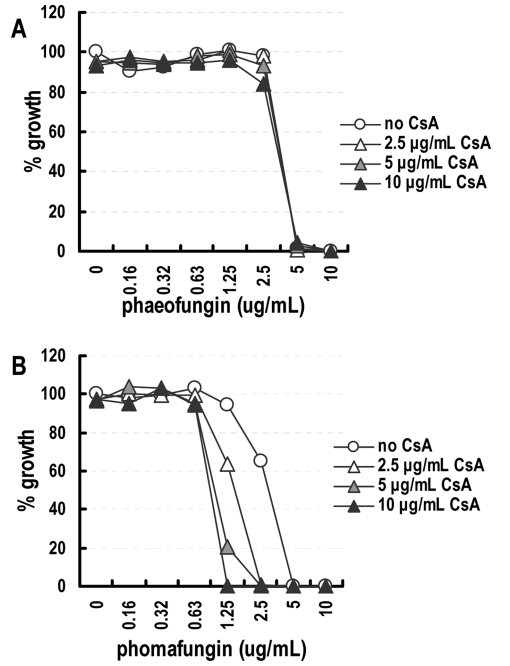

Figure 8. Interactions of cyclosporin A (CaA) with phaeofungin (**1**) (A) and phomafungin (**3**) (B) in the wild-type *C. albicans* strain. Three concentration of cyclosporine A (as indicated) were mixed with **1** and **3** in a range of concentrations (10 to 0.16 μg/mL). The antifungal activities of mixtures were measured after a 24 h incubation by OD_{600}, which was then normalized by the growth of untreated culture.

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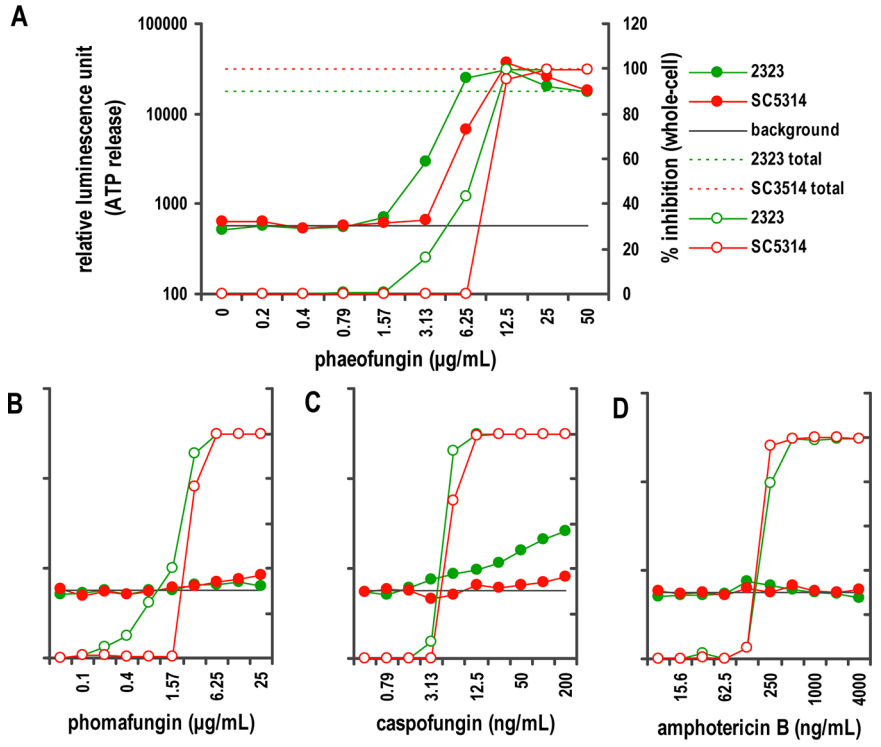

Figure 9. ATP release assay with phaeofungin (**1**) (A), phomafungin (**3**) (B), caspofungin (C), and amphotericin B (D). The ATP release (filled symbols) and the whole-cell antifungal (open symbols) activities were tested in *C. albicans* strains 2323 (green symbols) and SC5314 (red symbols). The solid blue line indicates the basal level of ATP release in the assay, the green dashed line the total of ATP release in strain 2323, and the red dashed line the total ATP release in strain SC5314.

This was indeed observed in the fungal strain in which the promoter of *DIP5* is replaced with a regulatable *tet* promoter under the repressing conditions (data not shown).

Both cyclic lipodepsipeptides (**1** and **3**) were then tested for their effects on release of ATP from the cytoplasm in two wild-type *C. albicans* strains. With the reference compound amphotericin B, no ATP release was observed even at a concentration greater than the MIC (Figure 9D). Only minimal ATP release was observed with caspofungin at concentrations greater than the MIC, and this was particularly so in strain 2323, the starting *C. albicans* strain used for strain construction (Figure 9C). While phomafungin (**3**) did not cause ATP release in either strain (Figure 9B), phaeofungin (**1**) resulted in the release of ATP in both strains. Most importantly, the maximal release correlated with the MIC (Figure 9A).

Phaeofungin (**1**) and phomafungin (**3**) are distantly related to a group of cyclic lipodepsipeptides, including syringomycin E,[24] syringostatin,[25] syringotoxin,[25] pseudomycin A,[26] and cormycin A,[27] all of which are produced by plant-associated *Pseudomonas* spp.[11] However, these cyclic lipodepsipeptides differ substantially from phaeofungin and phomafungin in both the macrocyclic ring size and composition of constituent amino acids. Both phaeofungin and phomafungin are comprised of a β-hydroxyhexadecanoic (C_{16}) fatty acid chain, which forms an integral part of the macrocycle, in contrast to the syringomycin series of peptides, which possess a shorter chain β-hydroxy fatty acid and terminate with the acylation of the amino group of a serine residue, of which the hydroxy group forms a macrocycle. In addition, phaeofungin is comprised of only seven amino acids and forms a 25-membered ring, and phomafungin is comprised of only eight amino acids, forming a 28-membered macrocyclic ring. None of the amino acids in phaeofungin and phomafungin are basic amino acids. The syringomycins family of peptides consists of at least nine amino acids (several basic), forming a 28-membered macrocyclic ring. The *Pseudomonas* lipodepsipeptides (e.g., syringomycin E) are important virulent factors for the phytopathogenic bacteria and are thought to exert their effects by forming ion channels on the plasma membrane, leading to cytolysis.[28,29] *Pseudomonas* spp. also produce large cyclic lipodepsipeptides known as the syringopeptins, comprised of 22 or 25 amino acids with an octadepsipeptide ring structure and a 3-hydroxy fatty acyl chain. They have been shown to target the yeast plasma membrane by a lipid-dependent channel-forming mechanism of action.[30] The antifungal activity of syringomycin E was functionally linked to the sphingolipid pathway in *S. cerevisiae* that is sensitive to Ca^{2+} fluctuation.[24,31] Our results also suggest a functional connection between the two fungal lipodepsipeptides and the sphingolipid and Ca^{2+} homeostasis. However, as predicted by the *Ca*FT profiles (Figure 3), phomafungin and phaeofungin are different in their biological activities: the former is potentiated by cyclosporin A (Figure 8), while the latter is potentiated by aureobasidin A (Figure 6). Furthermore, phaeofungin potentiated caspofungin at sublethal concentrations (Figure 7), consistent with the preferential hypersensitivity of the double heterozygous deletion strains to only phaeofungin in the *Ca*FT (Figure 3). Phaeofungin, but not phomafungin, was found to cause ATP release in two strains of *C. albicans* (Figure 9).

Despite these functional differences, the exact mechanisms of action of both fungal lipodepsipeptides remains elusive. However, we speculate that phaeofungin (**1**) and phomafungin (**3**) target the plasma membrane and that their activities are differently affected (both qualitatively and quantitatively) by

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changes in sphingolipid content, Ca^{2+} concentrations, and cell wall integrity. In the case of **1**, its interaction with the plasma membrane could lead to release of ATP and cytolysis.

Cyclic lipodepsipeptides are chemically and biologically diverse secondary metabolites produced by many organisms. Their biological properties have been exploited successfully in antimicrobial therapeutics, e.g., echinocandins (caspofungin, micafungin, and anidulafungin, inhibitors of cell wall synthesis)[32] and daptomycin (targeting bacterial plasma membrane).[33] Using a chemogenomic assay, the *Ca*FT, we identified two fungal lipodepsipeptides, phaeofungin (**1**) and phomafungin (**3**). The *Ca*FT profiles of these original extracts indicated that these two active compounds are structurally and biologically related, but not identical. This prediction was confirmed upon isolation and characterization of both compounds and subsequent biological analysis. It provides an example in which the application of a chemogenomic approach could be used to discern biological activities, hence the structures of the active compounds, in a crude extract. This serves as a powerful tool for the discovery of novel natural product antifungal agents particularly when combined with interesting chemical diversity.

EXPERIMENTAL SECTION

Geneal Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. The UV spectrum was recorded on a Perkin-Elmer Lambda 35 UV/vis spectrometer. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter; IR spectral data were obtained on a Perkin-Elmer Spectrum One spectrometer. The NMR spectra were obtained on a Varian Inova 500 or 600 MHz spectrometer operating at 500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C nuclei. The chemical shifts were referenced to residual C_5D_5N (δ_H 8.74 ppm and δ_C 150.4 ppm) and CD_3OD (δ_H 3.30 ppm and δ_C 49.00 ppm). Data were collected uniformly at 25 °C in 3 mm NMR tubes. A Nalorac 3 mm H{CN} indirect Z-gradient probe was used for all samples. Varian standard pulse sequences were used for all data collection. The 2D TOCSY data were collected with a 4900 Hz spin-lock field held for 80 ms, using the flopsy16 mixing scheme. The 1D TOCSY data were collected with a 6200 Hz spin-lock field using the MLEV17 mixing scheme. Mixing times were arrayed: 20, 40, 60, 80, and 90 ms. Peak selection was achieved using an IBURP waveform with a 50 Hz bandwidth. ^{1}H homonuclear correlation data were obtained with the Varian gCOSY or gDQF-COSY pulse sequences. ROESY data were collected using a 4500 Hz spin-lock field applied for 200 ms. Single- and multiple-bond heteronuclear connectivity data were observed using the gHSQC and gHMBC pulse sequences, respectively. The gHMBC data were collected using a mixing time optimized for a 7 Hz heteronuclear coupling constant. High-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT using electrospray ionization using a Finnigan Ion Max source with source fragmentation on and equal to 18 V.

Fungal Material. The fungal strain (F-167,953, E-000504901) was isolated from living stems and leaves of a *Sedum* sp. (Crassulaceae) collected in Motilleja, Albacete, Spain (Figure S1, Supporting Information). A second strain (F-262,327, E-000531145) producing phaeofungin (**1**) (Figure S1, Supporting Information) was isolated from the stems and leaves of *Teucrium* sp. (Lamiaceae) collected at Serrania de Cuenca, Cuenca, Spain. Strains were maintained as frozen mycelia in 10% glycerol at −80 °C.

DNA Sequencing and Characterization of Fungal Strains. Total genomic DNA was extracted from mycelia grown on YM agar. The rDNA region containing the partial sequence of 28S rDNA including the D1 and D2 variable domains was amplified with the primers NL1 and NL4. Sequences of 28S rDNA were used to generate a neighbor-joining tree (Figure S1, Supporting Information) that demonstrated a close relationship between the two cyclic lipodepsipeptide-producing strains and showed that they belong to the Phaeosphaeriaceae. To achieve further phylogenetic resolution, the same genomic DNA samples were used to amplify and sequence the intertranscribed spacer regions and 5.8S gene of the rDNA (ITS) with primers 18S-3 and Nl1r.

PCR reactions were performed following standard procedures (5 min at 93 °C followed by 40 cycles of 30 s at 93 °C, 30 s at 53 °C, and 2 min at 72 °C) with Taq DNA polymerase (Q-bioGene), following the procedures recommended by the manufacturer. The amplification products (0.10 μg/mL) were sequenced using Bigdye Terminators version 1.1 (Perkin-Elmer, Norwalk, CT, USA), following the manufacturer's recommendations. For all the amplification products, each strand was sequenced with the same primers used for the initial amplification. Partial sequences were assembled using Genestudio software (Genestudio, Inc.), and consensus sequences were aligned with Clustal X. Neighbor-joining analyses (Figure S1, Supporting Information) were used to approximate the phylogenetic relationships among strains.

Fermentations in Microarrays and Scale up for Isolation. Our screening strategy relied on high-throughput generation of 1 mL scale extracts of organisms grown under varied fermentation parameters followed by assay for antibiosis caused by cell-penetrable molecules using bioassays with *C. albicans* and *S. aureus*. Organism-and-medium combinations yielding extracts with a minimum potency and activity spectrum were scaled up to provide larger fermentations suitable for profiling in the *Ca*FT and further processing for an extract library and for chemical fractionation, if needed. The strategy and protocols for fermentation of fungi on nutritional microarrays have been described previously.[6] Each week, 160 to 240 fungal strains were selected for fermentation. These fungi were grown 2 to 3 weeks in 60 mm Petri dishes containing YM agar (Fluka or Difco malt extract 10 g, Difco yeast extract 2 g, agar 20 g, distilled H_2O 1000 mL). Three to four mycelial disks were cut from each 60 mm plate. Mycelia disks were crushed in the bottom of tubes (25 × 150 mm) containing 8 mL of SMYA medium (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 mm^2). Tubes were agitated on an orbital shaker (200 rev min^{-1}, 5 cm throw), and rotation of the cover glasses continually sheared hyphae and mycelial disk fragments to produce homogeneous hyphal suspensions. Tubes were agitated 4 to 6 days at 22 °C. Hyphal suspensions from these tubes were transferred to master inoculum plates. Master plates of fungal inoculum were used to inoculate eight-media nutritional arrays in a 10-column × eight-row pattern.[6] Nutritional arrays were grown statically for 21 days at 22 °C.

The detection of antifungal activity from strain F-167,953 originated from a 1 mL fermentation in the medium GLUS [glucose 20 g; yeast extract (Becton Dickinson) 1 g; monosodium glutamate 14.5 g; KCl 0.5 g; K_2HPO_4 0.25 g; $CaCl_2{\cdot}2H_2O$ 0.20 g; $MgSO_4{\cdot}7H_2O$ 0.02 g; $ZnSO_4{\cdot}7H_2O$ 0.01 mg; $CuSO_4{\cdot}5H_2O$ 0.005 mg; distilled H_2O 10 000 mL]. This fermentation was scaled up to 1 L by growing F-167,953 in 500 mL flasks with 150 mL of liquid GLUS agitated at 220 rpm, 22 °C for 22 days. The liquid fermentations were extracted with an equal volume of acetone and pooled. A 4 mL aliquot was frozen and tested for *Ca*FT, and the remaining extract was used for the isolation of active metabolites.

***Candida albicans* Fitness Test.** The assay was reported elsewhere.[8] Its application to biological dereplication of natural products has been published.[5,8−13]

Extraction and Isolation of Phaeofungin (1). One liter of the fermentation broth was extracted with 1 L of acetone and filtered, and the filtrate was concentrated under reduced pressure to remove most of the acetone. The 500 mL of the mainly aqueous solution remaining after concentration was charged to a 60 cubic centimeter Amberchrome column. The column was eluted by a 100 min linear gradient of 0 to 100% aqueous MeOH at 10 mL/min, and 5 mL fractions were collected every 0.5 min. The antifungal activity eluted in fractions 13−19, which were concentrated under reduced pressure and lyophilized to yield 1.3 g of a partially enriched fraction. This fraction was dissolved in 20 mL of MeOH and chromatographed on a 1 L Sephadex LH20 column, eluted with 100% MeOH at 10 mL/min.

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Fractions (20 mL each) were collected every 2 min. The activity eluted in fractions 38−58. These fractions were combined and concentrated to give 1.0 g of solid. This solid material was dissolved in 7 mL of MeOH. One milliliter of this material was purified by preparative RP HPLC (Zorbax Rx C_8 21.2 × 250 mm) using a 40 min gradient of 10−95% aqueous acetonitrile (0.1% TFA) at 12 mL/min. The activity eluted at 20−26 min. The chromatography was repeated seven times, and fractions from each run were pooled and lyophilized to afford 638 mg (638 mg/L) of phaeofungin (**1**) as a colorless powder: $[\alpha]^{23}_{D}$ +6.8 (*c* 0.45, MeOH); UV (MeOH) λ_{max} end absorption; IR (ZnSe) ν_{max} 3294, 2923, 2853, 1643, 1517, 1378, 1255, 1068 cm^{-1}; for ^1H and ^{13}C NMR see Table 1; ESIMS *m/z* 904 $[M + H]^+$, 886 $[M - H_2O + H]^+$, 868 $[M - 2H_2O + H]^+$; HRESIFTMS *m/z* 904.4865 (calcd for $C_{40}H_{69}N_7O_{16}$ + H, 904.4879), 868.4688 (calcd for $C_{40}H_{65}N_7O_{14}$ + H, 868.4668).

Basic Hydrolysis of Phaeofungin (1). Phaeofungin (**1**) (20 mg) was suspended in water (5 mL), treated with 0.1 NaOH (2 mL), and stirred at room temperature for 90 min. The reaction mixture was directly charged on a 5 mL Amberchrome column and washed thoroughly with water, and the linear peptide was eluted with methanol, which was concentrated under reduced pressure and rechromatographed by reversed-phase HPLC using similar conditions to those used for the purification of **1**. Lyophilization of fractions gave linear peptide **4** as a colorless, amorphous powder: ^1H NMR (500 MHz, C_5D_5N) δ 9.51 (1H, d, *J* = 6 Hz), 9.37 (1H, d, *J* = 7.5 Hz), 9.31 (1H, d, *J* = 8 Hz), 9.04 (1H, d, *J* = 8 Hz), 8.87 (1H, d, *J* = 6.5 Hz), 8.86 (1H, d, *J* = 6 Hz), 5.43 (1H, brd, *J* = 5.5 Hz), 5.34 (1H, brt, *J* = 6 Hz), 5.32 (1H, t, *J* = 5.5 Hz), 5.26 (1H, dd, *J* = 11, 6 Hz), 5.20 (1H, t, *J* = 6 Hz), 4.95 (1H, pentet, *J* = 7 Hz), 4.79 (1H, q, *J* = 7 Hz), 4.70 (1H, pentet, *J* = 6 Hz), 4.52 (1H, dd, *J* = 6, 5.5 Hz), 4.50 (1H, m), 4.44 (1H, dd, *J* = 11, 5.5 Hz), 4.36 (1H, dd, *J* = 11, 6 Hz), 4.33 (1H, dd, *J* = 10.5, 5 Hz), 3.49 (1H, dd, *J* = 16.5, 5.5 Hz), 3.43 (1H, dd, *J* = 16.5, 7 Hz), 3.06 (1H, brd, 14 Hz), 2.99 (1H, dd, *J* = 14, 10 Hz), 1.90 (1H, dt, *J* = 5, 13 Hz), 1.79 (1H, dt, *J* = 4.5, 13 Hz), 1.67 (3H, d, *J* = 7 Hz), 1.63 (3H, d, *J* = 7 Hz), 1.58 (2H, m), 1.42 (3H, s), 1.28−124 (18H, m), 0.87 (3H, t, *J* = 7 Hz); HRESIFTMS *m/z* 922.4979 (calcd for $C_{40}H_{71}N_7O_{17}$ + H, 922.4985); HRESIFTMS-MS (*m/z*) 638.2632 (calcd for $C_{23}H_{40}N_7O_{14}$, 638.2630), 519.2072 (calcd for $C_{19}H_{31}N_6O_{11}$, 519.2050), 480.1960 (calcd for $C_{17}H_{30}N_5O_{11}$, 480.1940), 432.1740 (calcd for $C_{16}H_{26}N_5O_9$, 432.1730), 345.1421 (calcd for $C_{13}H_{21}N_4O_7$, 345.1400), 274.1040 (calcd for $C_{10}H_{16}N_3O_6$, 274.1030), 159.0770 (calcd for $C_6H_{11}N_2O_3$, 159.0760).

Acid Hydrolysis of Phaeofungin (1). Phaeofungin (0.5 mg) was dissolved in 0.5 mL of 6 N HCl in a Reacti-Vial and heated at 110 °C overnight. The reaction was cooled to room temperature, and volatile material was removed under a stream of nitrogen. To the completely dried material was added 200 μL of a solution of 1% 1-fluoro-2,4-dinitrophenyl-5-L-valine amide (L-FDVA) in acetone. To this solution was added 40 μL of an aqueous solution of 1.0 M sodium bicarbonate. This mixture in a Reacti-Vial was heated in a Reacti-Therm heating module for 1 h. The solution was allowed to cool to room temperature, and 20 μL of 2 M HCl was added. The solution was degassed and analyzed by reversed-phase HPLC (Zorbax SB-C_8, 2.1 × 30 mm, 3.5 μm, elution with a 35 min linear gradient of 5−20% of solvent A: 90:10 H_2O/CH_3CN containing 1.3 mM TFA and 1.3 mM ammonium formate, and solvent B: 90:10 CH_3CN/H_2O containing 1.3 mM TFA and 1.3 mM ammonium formate, at a flow rate of 0.3 mL/min) and compared with the authentic samples of the Marfey's derivatives of the corresponding authentic amino acids. Retention times of authentic standards were as follows: L-Ser (7.0 min), D-Ser (9.0 min), L-Ala (12.5 min), D-Ala (23.4 min), L-Thr (7.6 min), D-Thr (17.2 min), L-allo-Thr (8.3 min), D-allo-Thr (12.2 min), L-Asp (7.9 min), D-Asp (11.0 min).

Partial Acid Hydrolysis of Phaeofungin (1). Phaeofungin (**1**) (5 mg) was dissolved in 0.2 mL of 0.5 N HCl in a Reacti-Vial and heated at 100 °C for 30 min. The reaction mixture was allowed to cool at room temperature, concentrated under a stream of nitrogen, and chromatographed by reversed-phase HPLC [Zorbax RX-C_8, 9.4 × 250 mm, 5 μm, elution with a 32 min linear gradient of 5−100% of solvent A (H_2O with 0.1% TFA) and solvent B (CH_3CN with 0.1% TFA) at a flow rate of 3.6 mL/min]. Fractions eluting at 23.5, 24.5, and 25.0 min were lyophilized to give pure phaeofungin, **5**, and **6**. The structures of compounds **5** and **6** were elucidated by LCMS-MS analysis (Figure S2, Supporting Information). **5**: HRESIMS *m/z* 631.3922 (calcd for $C_{30}H_{54}N_4O_{10}$ + H, 631.3913), 560.3538 (M − Ala), 473.3224 (M − Ala-Ser), 386.2897 (M − Ala-Ser-Ser). **6**: HRESIMS *m/z* 560.3516 (calcd for $C_{27}H_{49}N_3O_9$ + H, 560.3542), 473.3219 (M − Ser), 386.2878 (M − Ser).

Compounds **5** and **6** were separately hydrolyzed exhaustively, similar to the exhaustive hydrolysis of phaeofungin (**1**). The hydrolyzed mixture of amino acids was derivatized by Marfey's reagent in an identical manner and was analyzed in the similar manner by reversed-phase HPLC. Compound **5** yielded 2 × D-Ser (t_R 9.0 min), L-Ala (t_R 12.5 min), and D-allo-Thr (t_R 12.2 min), and **6** afforded 2 × D-Ser (t_R 9.0 min) and D-allo-Thr (t_R 12.2 min).

Antifungal Assay. The whole-cell antifungal activity assay was described elsewhere.[13] The MIC (minimum inhibitory concentration) against each of the strains was determined as previously described.[34] 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.

ATP Release Assay. A Promega BacTiter Glo assay was used according to the manufacturer's instructions to measure the release of ATP from the fungal cells in the presence of various antifungal compounds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR, neighbor joining analysis of the 28S rDNA, and photos of the phaeofungin-producing strains. 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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