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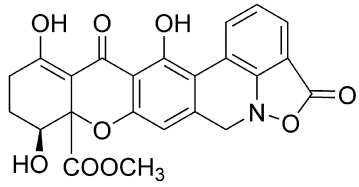
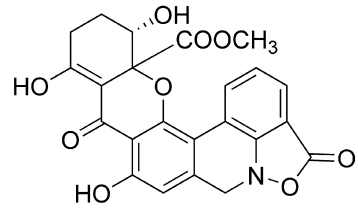


Parnafungin A



Parnafungin B

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Isolation and Structure Elucidation of Parnafungins, Antifungal Natural Products that Inhibit mRNA Polyadenylation

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Abstract: The *Candida albicans* Fitness Test, a whole-cell screening platform, was used to profile crude fermentation extracts for novel antifungal natural products with interesting mechanisms of action. An extract with intrinsic antifungal activity from the fungus *Fusarium larvarum* displayed a Fitness Test profile that strongly implicated mRNA processing as the molecular target responsible for inhibition of fungal growth. Isolation of the active components from this sample identified a novel class of isoxazolidinone-containing natural products, which we have named parnafungins. These natural products were isolated as an interconverting mixture of four structural- and stereoisomers. The isomerization of the parnafungins was due to a retro-Michael ring-opening and subsequent reformation of a xanthone ring system. This interconversion was blocked by methylation of an enol moiety. Structure elucidation of purified parnafungin derivatives was accomplished by X-ray crystallography and NMR analysis. The biochemical target of these natural products has been identified as the fungal polyadenosine polymerase. Parnafungins demonstrated broad spectrum antifungal activity with no observed activity against Gram-positive or Gram-negative bacteria. The intact isoxazolidinone ring was required for antifungal activity. In addition, the natural products were efficacious in a mouse model of disseminated candidiasis.

Introduction

In order to combat the increasing prevalence of microorganisms that are resistant to the current arsenal of antibiotics, there is a need for the identification of new structural classes of anti-infective agents. Novel screening approaches will play a critical role in their discovery. The *Candida albicans* Fitness Test (*Ca*FT) is a powerful whole-cell screening platform that can be used to understand the mechanism of action of compounds that are active against the clinically relevant fungal pathogen, *C. albicans*.[1,2] While this technology has been validated with a number of synthetic and purified compounds,[3,4] here we describe the first example of the *Ca*FT being applied to crude natural products' extracts. This integration of the *Ca*FT into our antifungal discovery platform has resulted in the identification of an interconverting family of novel natural products, which we name parnafungins based on their inhibition of polyadenosine polymerase, one component of the mRNA cleavage and polyadenylation complex.[5]

Although historically natural products screening has yielded a structurally diverse array of antifungal agents, only two classes of these have resulted in major commercial antifungal drugs: the echinocandin lipopeptides (caspofungin,[6] micafungin,[7] anidulafungin[8]) and the macrocyclic polyenes (amphotericin).[9] Members of these families of compounds target fungal cell wall biosynthesis[10] and membrane ergosterol,[9] respectively. While continued screening of natural product extracts against these established pathways could provide additional structural diversity, the inhibition of unrelated pathways of fungal metabolism may be a more productive approach to the discovery of novel antifungal agents.

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§ CIBE.

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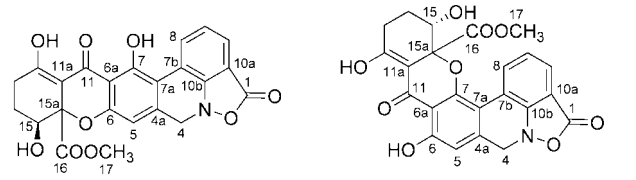
Our approach to antifungal drug discovery has utilized the *C. albicans* Fitness Test (*Ca*FT) to identify fermentation extracts that target alternative biochemical pathways distinct from those previously emphasized. The *Ca*FT employs a pool of 2868 genetically engineered *C. albicans* strains, each of which is heterozygous for a unique gene in the pathogen.[4] This strain set represents ~45% of the *C. albicans* genome. Gene selection for inclusion in the assay was based principally on documented essentiality for normal growth and/or viability in *Saccharomyces cerevisiae*[11] and/or *C. albicans*.[2,4] The individual heterozygous strains that are hypersensitive (or, at times, resistant) to sublethal concentrations of an antifungal agent typically can be correlated with the pharmacological target and/or cellular process affected by the growth inhibitory compound.[3,4] Further, the strains are bar coded so that they may be assayed *en masse* and the "fitness" of each individual strain can be quantitatively compared when subjected to the external challenge. Thus, strains displaying altered growth rates unique to the chemical treatment in this assay reflect chemical genetic profiles for the putative enzyme target and/or affected biochemical pathway.

In this approach, crude fermentation extracts with desired spectrum of antifungal activity can be evaluated using the *Ca*FT in order to identify a putative mechanism of action. This approach not only highlights possible novel targets, but also assists in the dereplication of previously described natural products. The *Ca*FT profile can be used to implicate a known antifungal that can then be confirmed by analytical techniques. When that analysis does not confirm a known structure, further isolation efforts may uncover a new component against an established target. Herein we describe a fermentation extract with intrinsic antifungal activity from lichenicolous strains of *Fusarium larvarum* (Ascomycota, Hypocreales). The *Ca*FT profile of the extract strongly implicated mRNA processing as the molecular target responsible for inhibition of fungal growth.[12,13] Isolation of the active component from the extracts provided a novel class of isoxazolidinone-containing natural products, parnafungins A and B. The presence of an isoxazolidinone ring in a natural product is unprecedented. In addition to the isoxazolidinone ring, parnafungins contain a xanthone ring system, which is most notably related to the monomeric ergochrome unit of secalonic acids.[14,15] Secalonic acids are polyketide-derived natural products[16,17] that are broad spectrum antimicrobial agents. While secalonic acids are simple dimers of the ergochrome unit linked at the 2−2′ position, more complex ergochrome-based natural products, exemplified by the beticolins, have been described.[18,19]

The *Ca*FT profile identified a number of heterozygous *C. albicans* strains to be specifically and reproducibly hypersensitive to parnafungins. These strains include multiple members of the mRNA cleavage and polyadenylation complex. Further



Parnafungin A1 (**1a**, *syn* -$COOCH_3$) Parnafungin B1 (**2a**, *syn* -$COOCH_3$)
Parnafungin A2 (**1b**, *anti* -$COOCH_3$) Parnafungin B2 (**2b**, *anti* -$COOCH_3$)

Figure 1. Structures of parnafungins. The major diastereomers, parnafungins A1 and B1, have the 15-hydroxyl and the adjacent methyl carboxylate in a *syn* relative configuration. The minor diastereomers, parnafungins A2 and B2, have these groups *anti*.

biochemical and genetic studies have confirmed that parnafungins are specific inhibitors of polyadenosine polymerase,[5] an enzyme responsible for the template-independent processive addition of adenosine to the 3′-end of mRNA to form a poly(A) tail.[20] Cordycepin (3′-deoxyadenosine), a known inhibitor of polyadenosine polymerase,[21] provided a related profile in the *Ca*FT.[5] Parnafungins have demonstrated broad spectrum antifungal activity and *in vivo* efficacy against *C. albicans* in a mouse model of infection with no observable toxicity.[5] The isolation, structure elucidation, and chemistry of parnafungins are described.

Results and Discussion

A novel class of isoxazolidinone-containing natural products, parnafungins A and B (**1** and **2**, Figure 1), were isolated from fermentations of *F. larvarum*. An acetone extract from a fermentation of this fungus was prioritized for its unique profile when assayed in the *C. albicans* Fitness Test.[5] There are two stereogenic centers in the parnafungin natural products. The absolute configuration of the 15-hydroxyl group was established as (*S*), as described below. The second stereogenic center is the adjacent 15a-quaternary carbon bearing a methyl carboxylate. Epimerization of C15a occurs readily in these natural products, leading to a mixture of parnafungin diastereomers. The major diastereomers have the hydroxyl group and methyl carboxylate in a *syn* relative configuration (as in **1a** and **2a**), while the minor diastereomers have these two substituents *anti* (**1b** and **2b**). A solution of these components in DMSO existed as an equilibrium mixture containing approximately 4:1:5:1 **1a/1b/2a/2b**.

The discovery strategy for identifying natural products for evaluation in the *Ca*FT was first to screen a library of nearly 120,000 extracts derived from microbial organisms for those with significant *in vitro* microbiological activity against *C. albicans*, *Aspergillus fumigatus*, and other clinically important fungal pathogens. Once extracts that exhibited significant anti-*C. albicans* activity had been identified, the respective producing microogranisms were refermented to provide samples for *Ca*FT profiling. Acetone extracts of these small-scale fermentations of *F. larvarum* strains MF7022 and MF7023 had significant antifungal activity: spotting extracts to agar plates seeded with *C. albicans* or *A. fumigatus* resulted in 16−20 mm and 8−20 mm zones of growth inhibition, respectively. Extracts from a liquid V8 juice−maltose medium and a solid-state brown rice medium both exhibited antifungal activity. Although *C. albicans* zones of inhibition were comparable on both media, stronger

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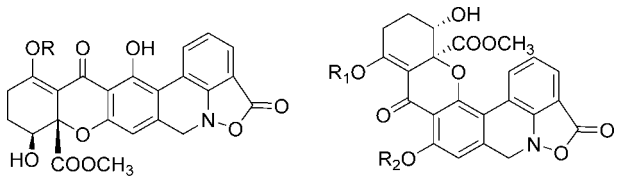

Figure 2. Parnafungin derivatives used to block interconversion among the four isomeric stereo- and regioisomers.

A. fumigatus activity was observed from extracts of the V8 juice−maltose medium. The fermentation was repeated on larger scale in this medium, and the original *Ca*FT profile was confirmed with an acetone extract. The profile obtained[5] indicated that this extract affected mRNA 3′-cleavage and polyadenylation; thus, this sample was prioritized for further isolation.

The acetone extract of the *F. larvarum* fermentation was processed through a polymeric styrene−divinyl benzene resin (CHP20P, MCI), followed by elution−extrusion countercurrent chromatography (EECCC).[22,23] The isolation of the active components was tracked through each chromatography with an agar-based assay against wild-type *C. albicans*. After these two isolation steps, analytical reversed-phase HPLC indicated the presence of four components in the active fractions. Attempts were made to purify the individual components by preparative HPLC, but each individual component rapidly equilibrated back to a mixture of components that was similar to the original. The equilibrium mixture comprised two major components and two minor components. All had related UV spectra ($UV_{max} \approx$ 345 nm), and mass spectrometric analysis of the mixture indicated that each compound had an identical molecular formula of $C_{23}H_{17}NO_9$. Isolation of the individual components at acidic pH (phosphate) slowed the rate of interconversion, but equilibration was still observed over a few hours. ^1H and ^{13}C NMR spectra confirmed the presence of four components with closely related chemical shifts.

In order to clarify the structure of these natural products, the mixture of components was treated with ethereal diazomethane. Changes in the chromatographic behavior on an analytical C18 reversed-phase column at different pHs had suggested that there was an acidic functionality in these natural products. If the acidic functional group was involved in the ability of the natural products to isomerize, then methylation could block this process. Indeed, this reaction provided a similar mixture of monomethylated components based on HPLC and mass spectrometric analysis. In addition to the monomethylated components, some dimethylated derivatives of the natural products also were generated. The reaction mixture was separable using reversed-phase (C18) HPLC and provided stable monomethylated derivatives **3** and **4** from the two major isomeric forms of parnafungin (Figure 2).

A single crystal of the less polar of the two major monomethylated derivatives was suitable for X-ray diffraction and yielded the structure of 12-*O*-methyl-parnafungin A (**3**, see Supporting Information). In order to establish the absolute configuration of the C15-hydroxyl group, the 4-chlorobenzyl enol ether **5** was prepared via Mitsunobu reaction of the natural product mixture and purified to a single component that was derived from the major diastereomer of parnafungin A. Suitable single crystals of **5** were prepared by recrystallization in 1:1 ethyl acetete/chloroform. X-ray analysis provided the structure of this single component (Figure 3). The X-ray data indicated that the major diastereomer parnafungin A1 (**1a**) presented relative *syn* configuration between the 15-hydroxyl and the neighboring 15a-methyl carboxylate. The C15-hydroxyl was placed equatorially and the methyl carboxylate at quaternary center C15a was placed axially by these data. From the X-ray structure of **5**, both stereogenic centers were unambiguously established as *S*.

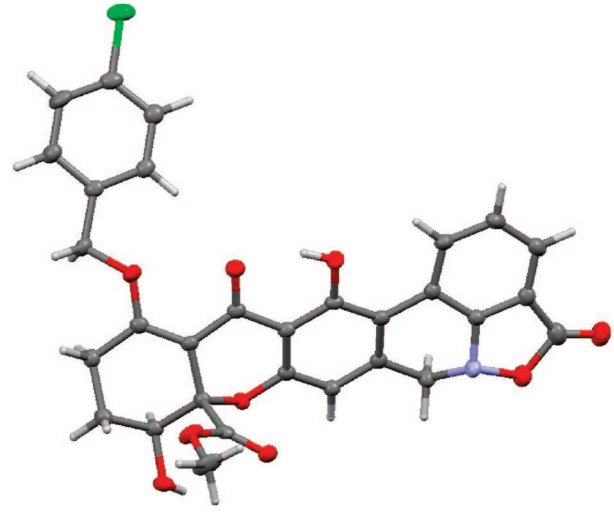

Figure 3. Crystal structure of the 12-*O*-4-chlorobenzyl ether of parnafungin A1 (**5**). From these data, the stereochemistry of the 15-hydroxyl was determined to be in the *S* absolute configuration.

The structure of parnafungin B and its enol ether derivatives were investigated both by X-ray crystallography and NMR. However, attempts to obtain suitable crystals of parnafungin B derivatives for X-ray diffraction were not successful. Full NMR characterization of **4** indicated a close relationship between the structures of parnafungin A and B. While certain analogous regions of the two structures presented distinctly different proton and carbon chemical shifts, there were no differences in the observed spin systems or heteronuclear correlations. It was apparent that a subtle structural or conformational change was responsible for the differences in the two isomers. Evidence in support of the "bent" topology of parnafungin B and its derivatives was obtained from 1D-ROESY NMR analysis of dimethylated derivative **6**. Selective excitation of the anisole methyl group at C6 produced clear ROE enhancement at H5. The reverse measurement was also positive. These correlations were only possible for parnafungin B since the C6-oxygen is part of the xanthone ring system in parnafungin A and cannot be methylated.

The interconversion of parnafungin A and parnafungin B can be explained by a mechanism similar to that described for the chlorinated xanthones, beticolin 1 (cebetin A) and beticolin 2.[24,25] In this case, a retro-Michael opening of the B ring of the xanthone (see Figure 4) of **1** exposes a second phenolic hydroxyl

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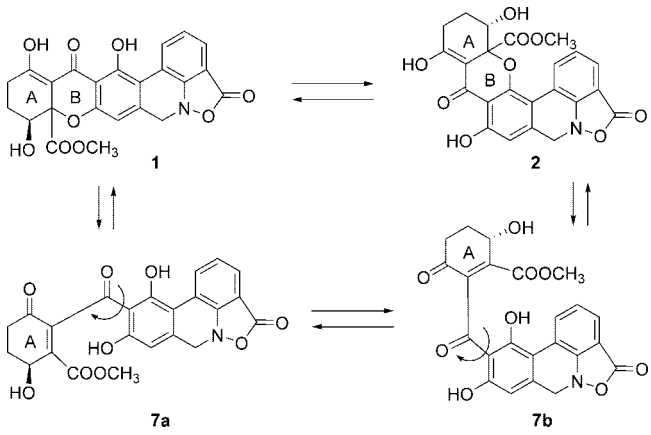

Figure 4. Pathway of interconversion of parnafungin A (**1**) and parnafungin B (**2**).

group. Subsequent ring closure then can take place with Michael addition of either the C6 or C7 phenol to the enone of the A ring. Reaction of the C6-hydroxyl in the conformation shown in **7a** regenerates parnafungin A (**1**). However, the rotation of the phenylketone bond to the conformation shown in **7b** allows for the attack of the C7-hydroxyl and the formation of the isomeric natural product parnafungin B (**2**). Further, facial selectivity of this ring closure, with the phenolic hydroxyl group reacting at either face of the enone, generates the observed thermodynamic mixture of C15a diastereomers. The major diastereomers were those in which the *S*-hydroxyl group adopted an equatorial orientation in the half-chair conformer of the A ring. All isomers have the quaternary methyl carboxylate at the A-B ring junction in an axial orientation.

Assignment of the NMR spectra to the respective structural isomers and stereoisomers in the natural product mixture (Table 1) was accomplished by extensive analysis of 1D and 2D spectra of purified derivatives **3**−**5**. The proton NMR signals of H15, H8, and H4 were particularly diagnostic of the change in geometry between parnafungin A and B. The most dramatic change in the proton chemical shifts of these compounds was that of aromatic proton H8. It presents a δ 0.35 ppm downfield shift going from parnafungin A to B. It was noted, however, that the chemical shift difference between the two diastereomers (**a**/**b**) for H8 was less than 0.05 ppm. The "bent" parnafungin B structure has more steric congestion near H8 that translated into a deshielding of the aromatic proton, whereas the diastereotopic change in the A ring had little effect on steric congestion of the remote biphenyl conformation. The H4 methylene protons also presented a significant difference in chemical shift between the structural isomers. In parnafungin A, the two H4 protons presented near magnetic equivalence and gave rise to a second-order multiplet that had nearly collapsed to a singlet at δ 4.70 ppm. In parnafungin B, these protons were clearly nonequivalent and presented as a pair of doublets at δ 4.55 and δ 4.76 ppm. The changes in the local environments of these protons were presumably due to a change in the twist of the biphenyl (C7a−C7b) bond resulting in an alteration of the magnetic environment of the C4-methylene protons.

Consistent with the X-ray data, the analysis of the coupling constants for H15 in methylated derivative **4** indicated that the major diastereomer of parnafungin A1 was *syn*, with the C15-hydroxyl substituent equatorial. The relatively large (~12 Hz) coupling constant was due to the diaxial orientation between H15 and one of the vicinal H14 protons, leaving the C15-OH group equatorial. In the natural product mixture, the two major structural isomers, parnafungin A and B, exist in nearly equal proportion, but the diastereomeric ratio is approximately 5:1. The two major isomers (**1a**, **2a**) presented an H15 doublet of triplets with one large 12 Hz coupling constant; both minor diastereomers (**1b**, **2b**) presented an H15 multiplet consisting of three small (<6 Hz) couplings. Further evidence of the assigned relative configuration was observed in the ^{13}C NMR spectra of these components. In both the major diastereomers, the C15 carbons of parnafungin were observed at δ ~70 ppm, while in the minor diastereomers the C15 carbons were shifted upfield to δ ~65 ppm. This difference in the carbon spectra supported the equatorial and axial orientations of the C15-hydroxyl in the major and minor diastereomers, respectively.

A unique structural aspect of the parnafungins is the presence of the isoxazolidinone five-membered ring containing the nitrogen−oxygen bond. A review of the literature did not provide a single example of this ring system in a natural product. The ^{15}N NMR chemical shift of the nitrogen was measured via indirect HMBC $^{1}H\{^{15}N\}$ spectroscopy, presenting a single resonance δ 240 ppm downfield from liquid NH_3. This signal is consistent with a nitrogen atom in a nitrogen−oxygen bond. Correlations to this nitrogen from the H4 methylene protons and the H8 aromatic proton were observed.

The isoxazolidinone was highly labile, generating the corresponding open forms, **8** and **9**, of the natural products (Figure 5). Along with ring-opening of the isooxazolidinone, there is an elimination to provide the aromatic benzoquinoline ring system. The UV spectra of the ring-opened forms had an additional absorbance at 450 nm. The mechanism of the isoxazolidinone ring-opening has not been resolved. This conversion may involve the abstraction of one of the benzylic C4 protons with elimination and concomitant cleavage of the N−O bond or, alternatively, hydrolysis of the isoxazolidinone ring followed by elimination. Since the generation of **8** and **9** formally adds and eliminates water, the molecular formulas of these degraded products, $C_{23}H_{17}NO_9$, are identical with the starting parnafungins. The rate of the ring-opening was more rapid at neutral or basic pH, but was slowed considerably when the compounds were maintained at acidic pH. Analogs **8** and **9** also were able to interconvert by a retro-Michael/Michael pathway. Opening of the isoxazolidinone ring resulted in a loss of antifungal activity. The presence of these components was a result of the chemical lability of the isoxazolidinone ring, and there was no evidence that they were generated directly by the producing organism.

The potency and spectrum of antifungal activity of the mixture of parnafungins **1** and **2** were evaluated using whole-cell *in vitro* growth assays. This natural product showed broad spectrum activity against the ascomycetous yeasts, *C. albicans* (MIC 0.014 μg/mL), *C. krusei* (0.014 μg/mL), *C. glabrata* (1.1 μg/mL), *C. tropicalis* (3.3 μg/mL), *C. lusitaniae* (1.1 μg/mL), *C. parapsilosis* (1.1 μg/mL) and *S. cerevisiae* (3.3 μg/mL). Under identical assay conditions, the MIC of caspofungin (Cancidas) against *C. albicans* was 0.01 μg/mL. The parnafungins also were active in an agar assay against *A. fumigatus*. There was no activity observed against *Staphylococcus aureus* (a Gram-positive bacterium) or *Escherichia coli* (a Gram-negative bacterium) at the concentrations tested (MIC >32 μg/mL). The purified parnafungins were analyzed in the *Ca*FT, and the original profile

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Table 1. ^{1}H and ^{13}C NMR Assignments of Parnafungins **1** and **2**[a]

		δ_C				δ_H (mult, *J* in Hz)			
no.	CH_n	1a	1b	2a	2b	1a	1b	2a	2b
11	C	185.6	185.5	184.6	185.9				
12	C	179.8	180.9	180.5	181.9				
16	C	169.7	171.1	169.5	170.9				
1	C	167.2	167.2	167.3	167.2				
6	C	160.9	158.8	161.0	158.9				
7	C	159.5	158.2	156.9	158.9				
10b	C	155.5	155.7	155.6	155.5				
4a	C	142.0	142.0	141.8	142.0				
8	CH	130.7	130.8	132.2	132.0	8.27 (d, 7.3)	8.32 (d, 7.3)	8.67 (d, 7.3)	8.63 (d, 7.3)
9	CH	125.9	125.9	126.0	126.0	7.34 (dd, 7.3, 7.8)	7.40 (dd, 7.3, 7.6)	7.32 (t, 7.3)	7.36 (dd, 7.3, 7.8)
10	CH	122.8	122.9	122.7	123.0	7.65 (d, 7.8)	7.67 (d, 7.6)	7.66 (d, 7.3)	7.70 (d, 7.8)
7b	C	118.7	118.7	118.7	118.6				
5	CH	108.5	108.5	110.4	110.9	6.72 (s)	6.74 (s)	6.67 (s)	6.72 (s)
10a	C	109.9	110.4	109.7	109.2				
7a	C	109.8	109.9	109.2	109.8				
6a	C	106.5	106.6	106.9	106.2				
11a	C	101.1	100.6	101.5	100.8				
15a	C	85.3	84.5	85.5	84.9				
15	CH	70.0	65.7	70.2	65.3	4.20 (dt, 12.0, 4.6)	4.39 (t, 4.6)	4.30 (dt, 12.0, 5.3)	4.24 (br s)
4	CH_2	54.8	54.7	55.0	54.9	4.70 (d, 11.9) 4.67 (d, 11.9)	4.70−4.68 (obscured)	4.76 (d, 11.7) 4.55 (d, 11.7)	4.73 (d, 11.9) 4.61 (d, 11.9)
17	CH_3	53.0	53.6	52.9	53.6	3.56 (s)	3.59 (s)	3.59 (s)	3.65 (s)
13	CH_2	27.7	24.7	28.0	24.9	2.82 (m) 2.60 (m)	2.70 (m) 2.40 (m)	2.82 (dd, 19.0, 8.0) 2.60 (m)	2.73 (m) 2.42 (ddd, 19.0, 13.0, 6.0)
14	CH_2	25.3	23.8	25.4	24.0	2.13 (m) 1.94 (m)	2.13 (m) 1.94 (m)	2.17 (m) 1.97 (m)	2.17 (m) 1.97 (m)
15	−OH					5.95 (d, 4.6)	5.97 (obscured)	6.01 (d, 5.3)	5.89 (d, 3.6)
6/7	−OH					12.35 (br s)	11.62 (br s)	11.47 (br s)	12.48 (br s)
12	−OH					13.85 (br)	13.85 (br)	13.85 (br)	13.85 (br)

[a] ^{1}H and ^{13}C NMR spectra were recorded at 499 and 125 MHz, respectively, in DMSO-d_6. Chemical shifts are reported in ppm relative to DMSO-d_5 (2.49 ppm for ^{1}H and 39.0 for ^{13}C). Assignments for protonated carbon centers were obtained directly from COSY and HSQC data. Quarternary carbon assignments were obtained from HMBC data.

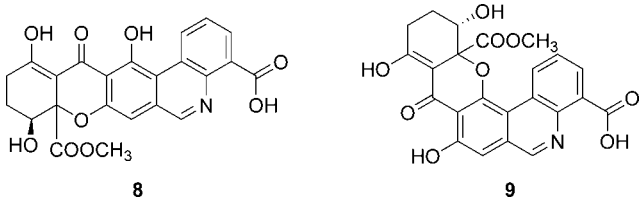

Figure 5. Ring-opened isoxazolidinones of parnafungins.

indicating an inhibitor of mRNA 3′-end processing was recovered. Hypersensitivity of heterozygote strains corresponding to additional components of the cleavage and polyadenylation complex that were not detected with the crude fermentation extract was noted in response to the purified natural product. These data further strengthen the mechanism of action hypothesis for parnafungins. A more detailed description of the mechanism of action, *Ca*FT profiles, microbiology, and *in vivo* efficacy of these natural products is described elsewhere.[5]

The possible role of parnafungins as a pathogenicity factor in the relationship with lichen hosts merits consideration. Although the usual hosts of *F. larvarum* are scale insects, herbarium specimens of *F. larvarum* and its sexual state *Cosmospora aurantiicola* have been observed on the lichens *Teloschistes chrysopthalmus* and *T. sieberianus* in New Zealand. Similar undescribed and phylogenetically related *Fusarium* species have been observed associated with parasitism of damaged and degraded lichens species.[27,28] If the lichenicolous *F. larvarum* strains described here display a natural history similar to these other lichenicolous Fusaria, then parnafungins may be involved in pathogenesis or necrosis of lichen thalli. However, it still remains to be determined if the phylogenetically and ecologically similar *F. larvarum*-like fungi are able to produce parnafungins. The only report of secondary metabolites from *F. larvarum* was that of the insecticidal dihydroisocoumarin, fusarentin.[29]

In summary, we have described the isolation and structure elucidation of parnafungins, a novel class of antifungal natural products. The *C. albicans* Fitness Test was used to probe the mechanism of these natural products, prior to refermentation, formal isolation, and structure elucidation. The targeted pathway of the parnafungins was determined to be the mRNA 3′-cleavage and polyadenylation process. Parnafungins have been found to specifically inhibit the activity of fungal polyadenosine polymerase. The biochemical characterization of this activity has identified a potential target for the screening for novel antifungal compounds and for the development of new drug candidates.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU-70 spectrophotometer. IR spectra were obtained with a Perkin-Elmer Spectrum One spectrophotometer. All NMR spectra were recorded on Varian Inova 500 MHz instruments, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. High-resolution mass spectral data were obtained with a Thermo Finnigan LTQ-FT mass spectrometer using electrospray ionization. Analytical HPLC was performed on a Hewlett-Packard 1100 HPLC system. Preparative HPLC was performed on a Waters Delta Prep 4000 preparative system. Countercurrent chromatography was performed with a Kromatron FCPC unit. CHP20P resin was purchased from MCI.

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Fungi and their Identification. Fungal strains were isolated from an unidentified lichen thallus collected in Miraflores de la Sierra, Madrid, Spain. The fungi, designated MF7022 (ATCC PAT 7894) and MF7023 (ATCC PAT 7895), are maintained in the culture collection of Merck Research Laboratories and at the American Type Culture Collection. The conidial states, observed on cornmeal agar, were indistinguishable from those of *Fusarium larvarum* var. *larvarum*, the conidial state of *Cosmospora aurantiicola*, a fungus usually associated with scale insects.[30,31]

The DNA of strains MF7022 and MF7023 was extracted and used as template for PCR reactions. Portions of the large subunit of rDNA (LSU rDNA) and the internal transcribed spacer regions 1 and 2 and the 5.8S genes (ITS) of the strains were amplified and sequenced to aid in identification and to infer phylogenetic relationships of the strains to other fungi. ITS and LSU sequences from two strains were identical, indicating they were conspecific and possibly clones from the same population. The sequences were used to query GenBank for similar ribosomal sequences. The best matches with LSU region and the percentage similarities were: *Cosmospora flammea* (U88103) 97%, *Fusarium* sp, (NRRL25126, AF228357) 96%, *Fusarium* sp. (NRRL26790, AF228359) 98%, *Fusarium* sp. (NRRL26803, AF228360) 98%, and *F. larvarum* (NRRL20473, U88107) 100%. Because authentic sequences of *F. larvarum* and other similar *Cosmospora* or *Fusarium* spp. were absent in GenBank, similarity searches with the ITS were less conclusive and retrieved a list of hypocrealean fungi of which the most similar was an unidentified fungus cultured from tropical forest litter (AF502758, 94% similarity). Sequence similarities confirmed that strains MF7022 and MF7023 belonged to the Hypocreales, and results with the LSU rDNA supported the conclusion that they were conspecific with *F. larvarum* strains associated with scale insects.

Fermentation of *F. larvarum*. Inoculum was generated by inoculating agar plugs into a 250-mL Erlenmeyer flask containing 60 mL of seed medium of the following composition in $g \cdot L^{-1}$ distilled H_2O (corn steep powder, 2.5; tomato paste, 40.0; oat flour; 10.0; glucose, 10.0; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01; $CuCl_2 \cdot 2H_2O$, 0.0025; $CaCl_2 \cdot 2H_2O$, 0.001; H_3BO_3, 0.00056; $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.00019; $ZnSO_4 \cdot 7H_2O$, 0.01). The pH was adjusted to 6.8 before autoclaving. The seed culture was incubated 5 d at 22 °C on a gyratory shaker (220 $rev \cdot min^{-1}$) prior to the inoculation of production medium. The production medium consisted of 200 mL of V8 juice (Campbell Soup Co., Camden, NJ) added to 800 mL of distilled H_2O containing the following components in g (maltose, 75; soy flour, 1; L-proline, 3; MES, 16.2). The pH was adjusted to 6.5 before autoclaving. The medium was dispensed in 100 mL aliquots in 500 mL Erlenmeyer flasks, inoculated with 1% volume of the seed culture, and agitated 20 d at 22 °C. After harvesting the fermentation (1 L), the whole broth was extracted with one volume of acetone, filtered to remove the mycelium, and concentrated to remove acetone.

Antifungal Assay. Antifungal activity was first detected with the strains *C. albicans* MY2368 and *A. nidulans* MF5668 from the Merck Culture Collection. Thawed stock of inoculum suspensions from cryovials of MY2368 were streaked on Sabouraud dextrose agar plates (SDA, 65 $g \cdot L^{-1}$) to grow new colonies. A few of the colonies were inoculated into 10 mL of Sabouraud dextrose broth (30 $g \cdot L^{-1}$ H_2O) that was incubated overnight at 37 °C. Conidia of MF5668 were produced in agar culture in Erlenmeyer flasks, filtered through glass wool, and used to prepare conidial suspensions. The *C. albicans* yeast or *A. fumigatus* conidial suspensions were adjusted to an optical density of 0.4 at 660 nm. Suspensions were added to yeast nitrogen base/dextrose agar (yeast nitrogen base, 6.75 $g \cdot L^{-1}$, dextrose 1 $g \cdot L^{-1}$, agar 17.5 $g \cdot L^{-1}$ H_2O) in the proportion of 30 $mL \cdot L^{-1}$. Twenty mL aliquots of the seeded agar media were poured into Omnitray plates (Nunc). Wells (4 mm diameter) were formed in the agar with a removable pin lid. Aqueous acetone-derived extracts (10 μL) were applied into the wells of the seeded assay plates which were incubated at 30 °C for approximately 20 h. Zones of inhibition (ZOIs) were recorded by automated image analysis.

Isolation of Parnafungins 1 and 2. The fermentation acetone extract was fractionated with CHP20P resin (50 mL), eluting with a gradient of acetonitrile in 10 mM potassium phosphate buffer at pH 3. The rich cut of activity (as measured by inhibition of growth of *C. albicans* in an agar based assay) was pooled, and a portion of this material was fractionated by EECCC with an elution−extrusion system of 1:1:1:1 hexane/ethyl acetate/methanol/water (V_c = 200 mL, 1000 rpm, 10 mL/min, ascending). The active fractions from the EECCC fractionation were analyzed by analytical HPLC (Waters Symmetry300, 300 Å, C18, 4.6 mm × 50 mm, 5 μm, 0−70% ACN in 1:9 ACN/10 mM potassium phosphate pH 3, 2 mL/min, 25 °C), and this analysis identified a mixture of related components that were responsible for antifungal activity. Typical fermentation titers were 1.2 g/L, and 650 mg of the natural product mixture was recovered by this isolation protocol. Further chromatography (C18 HPLC, pH 3) on this sample separated these components, but equilibration over 10−20 h (room temperature) returned the purified components back to the original mixture of isomeric forms. The equilibrium mixture contained two major components and two minor components as analyzed by HPLC and NMR. Analysis of these samples by mass spectrometry indicated that these components all had identical molecular weights that were consistent with a molecular formula of $C_{23}H_{17}NO_9$. ^1H and ^{13}C NMR assignments on the four individual isomeric parnafungins A1, A2, B1, and B2 are presented in Table 1. $[\alpha]_D$ +38.0 (c = 0.2, CH_2Cl_2). UV (acetonitrile): λ_{max} 343 (ϵ = 29,400 $M^{-1}cm^{-1}$), 290 (ϵ = 15,100 $M^{-1}cm^{-1}$), 265 (ϵ = 16,200 $M^{-1}cm^{-1}$), 234 (ϵ = 28,800 $M^{-1}cm^{-1}$). FTIR (ZnSe): 3475, 1750, 1611, 1579, 1500, 1455, 1431, 1364, 1348 cm^{-1}. HR-ESI-FTMS 452.0976 (calcd for $C_{23}H_{17}NO_9$ + H: 452.0982).

(15*S*,15a*S*)-12-*O*-Methyl-parnafungin A1 (3) and (15*S*,15a*S*)-12-*O*-Methyl-parnafungin B1 (4). Parnafungins A and B (**1** and **2**, 100 mg) were dissolved in methanol (20 mL) and treated with freshly prepared ethereal diazomethane at 4 °C. The progress of the reaction was monitored by HPLC analysis of aliquots of the reaction mixture. The reaction was quenched by the addition of acetic acid and the solvent was removed *in vacuo*. The mixture of methylated components was purified by preparative HPLC (C18, 19 × 300 mm, 7 μm, Waters Symmetry 300Å), eluting with a gradient of acetonitrile (40−50%) in 0.1% formic acid. The major isomeric methyl enol ethers **3** (*syn* -$COOCH_3$, 29 mg, 28% based on single starting material) and **4** (*syn* -$COOCH_3$, 17 mg, 16% based on single starting material) were purified. Derivative **3** was crystallized from 1:1 methylene chloride:methanol for single crystal X-ray analysis.

(15*S*,15a*S*)-12-*O*-methyl-parnafungin A1 (**3**): ^{13}C NMR (125 MHz, DMSO-d_6): δ 24.0 (C13), 25.3 (C14), 52.7 (C17), 54.8 (C4), 56.2 (12-*O*-CH_3), 69.8 (C15), 87.0 (C15a), 103.3 (C11a), 107.4 (C6a), 107.5 (C5), 109.4 (C7a), 109.8 (C10a), 119.1 (C7b), 122.6 (C10), 125.9 (C9), 130.5 (C8), 141.1 (C4a), 155.5 (C10b), 159.3 (C6), 160.1 (C7), 167.3 (C1), 169.8 (C16), 173.2 (C12), 184.8 (C11); ^1H NMR (499 MHz, DMSO-d_6): δ 1.87 (m, 1 H, H14), 2.06 (m, 1 H, H14), 2.83 (m, 1 H, H13), 2.83 (m, 1 H, H13), 3.55 (s, 3 H, H17), 3.88 (s, 3 H, 12-*O*-CH_3), 4.15 (m, 1 H, H15), 4.70 (s, 2 H, H4), 5.92 (d, J = 3.4 Hz, 1 H, O(15)-H), 6.68 (s, 1 H, H5), 7.36 (dd, J = 7.8, 7.5 Hz, 1 H, H9), 7.68 (d, J = 7.8 Hz, 1 H, H10), 8.34 (d, J = 7.5 Hz, 1 H, H8), 14.13 (s, 1 H, O(7)-H). HR-ESI-FTMS 466.1128 (calcd for $C_{24}H_{19}NO_9$ + H: 466.1138).

(15*S*,15a*S*)-12-*O*-methyl-parnafungin B1 (**4**): ^{13}C NMR (125 MHz, DMSO-d_6): δ 24.6 (C13), 25.5 (C14), 53.0 (C17), 55.0 (C4), 56.0 (C18), 70.0 (C15), 87.4 (C15a), 103.0 (C11a), 107.7 (C6a), 108.3 (C7a), 109.6 (C10a), 109.9 (C5), 119.1 (C7b), 122.3 (C10), 125.8 (C9), 131.8 (C8), 141.0 (C4a), 155.6 (C10b), 156.6 (C7), 162.4 (C6), 167.4 (C1), 169.6 (C16), 173.1 (C12), 184.2 (C11);

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^{1}H NMR (499 MHz, DMSO-d_6): δ 2.00 (m, 1 H, H14), 2.17 (m, 1 H, H14), 2.88 (m, 1 H, H13), 2.88 (m, 1 H, H13), 3.55 (s, 3 H, H17), 3.90 (s, 3 H, 12-*O*-CH_3), 4.30 (dt, J = 12.5, 3.4 Hz, 1 H, H15), 4.55 (d, J = 11.7 Hz, 1 H, H4), 4.80 (d, J = 11.7 Hz, 1 H, H4), 5.97 (d, J = 3.4 Hz, 1 H, O(15)-H), 6.60 (s, 1 H, H5), 7.36 (dd, J = 7.5, 8.0 Hz, 1 H, H9), 7.70 (d, J = 8.0 Hz, 1 H, H10), 8.68 (d, J = 7.5 Hz, 1 H, H8), 13.25 (s, 1 H, O(6)-H). HR-ESI-FTMS 466.1129 (calcd for $C_{24}H_{19}NO_9$ + H: 466.1138).

12-(*O*-4-Chlorobenzyl)-parnafungin A1 (5). To a suspension of parnafungins **1** and **2** (61 mg, 0.14 mmol), triphenylphosphine (48 mg, 1.4 eq) and 4-chlorobenzyl alcohol (27 mg, 1.4 eq) in THF (1.5 mL) was added diisopropyl aza-dicarboxylate. The reaction mixture was stirred at room temperature and all suspended material was solubilized within 1 min, forming a dark orange solution. After 30 min, the reaction was concentrated *in vacuo*, dissolved in 1:1 EtOAc:hexanes, and partially purified by silica gel chromatography (step gradient of EtOAc in hexanes. The desired products began eluting from silica with 3:1 EtOAc:hexanes and were completely eluted with 100% EtOAc. The pooled fractions containing the benzylated products were further purified by reversed-phase preparative HPLC (Waters Symmetry 300, C18 300A, 19 mm × 300 mm, 7 μm, 10 mL/min, 55% ACN (30 min), 55−70% ACN (30 min) in 0.1% formic acid). The 4-chlorobenzyl derivatives of both major diastereomers of parnafungin were purified (analog **5**, 12 mg, 15% based on single starting material). A sample of **5** was crystallized from 1:1 chloroform:EtOAc and submitted to single crystal X-ray analysis. UV (acetonitrile): λ_{max} 338 (ϵ = 29,000 $M^{-1}cm^{-1}$), 293 (ϵ = 15,500 $M^{-1}cm^{-1}$), 265 (ϵ = 15,300 $M^{-1}cm^{-1}$), 233 (sh, ϵ = 33,000 $M^{-1}cm^{-1}$), 223 (ϵ = 37,900 $M^{-1}cm^{-1}$); FTIR (ZnSe) ν_{max} 3463, 1752, 1631, 1575, 1491, 1462, 1422, 1366, 1349 cm^{-1}.

^{13}C NMR (125 MHz, CD_2Cl_2): δ 24.4 (C13), 25.9 (C14), 53.5 (C17), 56.3 (C4), 70.4 (C15), 71.8 (12-*O*-$\underline{C}H_2$PhCl), 87.2 (C15a), 106.2 (C6a), 107.5 (C5), 108.6 (C11a), 108.9 (C7a), 110.8 (C10a), 119.9 (C7b), 123.1 (C10), 126.1 (C9), 128.7 (2C, 12-*O*-$CH_2\underline{Ph}$Cl), 129.2 (2C, 12-*O*-$CH_2\underline{Ph}$Cl), 131.4 (C8), 134.4 (12-*O*-$CH_2\underline{Ph}$Cl), 134.7 (12-*O*-$CH_2\underline{Ph}$Cl), 141.3 (C4a), 156.2 (C10b), 159.3 (C6), 161.6 (C7), 168.2 (C1), 169.8 (C16), 170.0 (C12), 185.3 (C11); ^{1}H NMR (499 MHz, CD_2Cl_2): δ 2.0 (vb, 1 H, O(15)-H), 2.08 (m, 1 H, H14), 2.22 (m, 1 H, H14), 2.72 (ddd, J = 6.8, 11.0, 18.0 Hz, 1 H, H13), 2.88 (ddd, J = 2.0, 6.5, 18.0 Hz, 1 H, H13), 3.69 (s, 3 H, H17), 4.30 (dd, J = 4.6, 12.5 Hz, 1 H, H15), 4.54 (s, 2 H, H4), 5.20 (d, J = 12.3 Hz, 1 H, 12-*O*-$C\underline{H}_2$PhCl), 5.26 (d, J = 12.3 Hz, 1 H, 12-*O*-$C\underline{H}_2$PhCl), 6.55 (s, 1 H, H5), 7.32 (dd, J = 8.5, 7.7 Hz, 1 H, H9), 7.40 (d, J = 8.0 Hz, 2 H, 12-*O*-$CH_2\underline{Ph}$Cl), 7.46 (d, J = 8.0 Hz, 2 H, 12-*O*-$CH_2\underline{Ph}$Cl), 7.62 (d, J = 8.5 Hz, 1 H, H10), 8.47 (d, J = 7.7 Hz, 1 H, H8), 13.91 (s, 1 H, O(7)-H). HR-ESI-FTMS 576.1044 (calcd for $C_{30}H_{22}ClNO_9$ + H: 576.1061).

Ring-Opened Parnafungins 8 and 9. During the isolation of parnafungins A and B, more polar components were observed in the fermentation extracts. These components could be isolated (90 mg from a 1-L fermentation) by chromatography with CHP20P resin (MCI, eluting with 40% acetonitrile in pH 6 phosphate buffer) followed by gradient preparative C18 HPLC (Waters Symmetry 300Å C18, 19 mm × 300 mm, 7 μm, gradient 20−70% acetonitrile in 10 mM potassium phosphate, pH 3). In addition the treatment of a mixture of parnafungins A and B at neutral or basic pH generated **8** and **9** in less than 1 h. These components were also generated more slowly at acidic pH (phosphate pH 3, 10−20 h). Similar to the parent natural products, these more polar components existed as a mixture of equilibrating isomers. Isolation of the major (*syn*) diastereomer of **8** from this mixture was followed by re-equilibration by the same retro-Michael/Michael addition mechanism described for parnafungins A and B.

UV (acetonitrile): λ_{max} 432 (ϵ = 6500 $M^{-1}cm^{-1}$), 359 (ϵ = 21,600 $M^{-1}cm^{-1}$), 282 (ϵ = 30,600 $M^{-1}cm^{-1}$), 242 (ϵ = 35,700 $M^{-1}cm^{-1}$), 208 (ϵ = 46,000 $M^{-1}cm^{-1}$); FTIR (ZnSe) ν_{max} 3418, 1740, 1704, 1604, 1572, 1497, 1479, 1429, 1370, 1299, 1242 cm^{-1}. NMR assignments for the ring-opened isoxazolidinone of parnafungin A1 (**8**): ^{13}C NMR (125 MHz, DMSO-d_6/THF-d_8, 3:1): δ 25.3 (C13), 28.3 (C14), 52.6 (C17), 70.2 (C15), 84.9 (C15a), 102.1 (C11a), 106.6 (C5), 108.5 (C6a), 115.4 (C7a), 123.8 (C10a), 128.3 (C9), 130.7 (C10), 130.9 (C8), 131.8 (C7b), 140.3 (C4a), 152.8 (C4), 156.8 (C10b), 158.4 (C7), 162.0 (C6), 166.6 (C1), 169.6 (C16), 182.4 (C12), 186.2 (C11); ^{1}H NMR (499 MHz, DMSO-d_6/THF-d_8, 3:1): δ 2.02 (m, 1 H, H14), 2.23 (m, 1 H, H14), 2.65 (dd, J = 6.5, 18.5 Hz, 1 H, H13), 2.93 (ddd, J = 7.3, 11.1, 18.5 Hz, 1 H, H13), 3.56 (s, 3 H, H17), 4.33 (dt, J = 11.7, 4.5 Hz, 1 H, H15), 6.00 (d, J = 4.5 Hz, 1 H, O(15)-H), 7.48 (s, 1 H, H5), 7.87 (t, J = 8.0 Hz, 1 H, H9), 8.48 (d, J = 7.0 Hz, 1 H, H10), 9.47 (s, 1 H, H4), 9.56 (d, J = 8.0 Hz, 1 H, H8), 13.8 (b, 1 H, O(7)-H). HR-ESI-FTMS 452.0970 (calcd for $C_{23}H_{17}NO_9$ + H: 452.0976).

The supplementary crystallographic data have also been deposited as CCDC 670052 and 670053. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of parnafungins **1/2**, derivatives **3**, **4**, and **5**, the crystallographic data related to the structures **3** and **5**, and the full author lists for references 2, 3, 5, 10, and 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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