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Isolation and structure elucidation of parnafungins C and D, isoxazolidinone-containing antifungal natural products

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ABSTRACT

Parnafungins, natural products containing an isoxazolidinone ring, have been isolated from *Fusarium larvarum* and have been shown to be potent inhibitors of the fungal polyadenosine polymerase. The extraction and analysis of fermentation broths of taxonomically related organisms identified as closely related *Fusarium* spp. produce not only parnafungin A and B, but also significant quantities of two related components. These members of the paranfungin family of natural products have been isolated and the structure of each has been elucidated. While structurally analogous to parnafungin A, parnafungin C is further elaborated by methylation of a phenolic hydroxyl group, and parnafungin D has both the methyl phenol ether as well as an epoxide in the xanthone ring system. Parnafungin C and D have potent, broad spectrum antifungal activity and also have been shown to target fungal mRNA cleavage and polyadenylation.

The *Candida albicans* Fitness Test (*Ca*FT) is a whole-cell screening platform used within our laboratories to discover novel classes of antifungal agents.[1–4] The *Ca*FT is comprised of over 5000 modified *C. albicans* strains, each of which is heterozygous for a unique gene of the *C. albicans* genome. By assessing which of these strains is hypersensitized to a particular chemical component, the mechanism of action of that antifungal compound can be understood. Recently, we have extended this approach to identify novel natural products with interesting mechanisms of action. In that discovery program, an extract of a fermentation of *Fusarium larvarum* demonstrated broad spectrum antifungal activity against clinically relevant fungi, with a mode of action identified by the *Ca*FT as inhibiting the cleavage and polyadenylation of mRNA. Isolation of the active components from this extract led to the elucidation of the structures of parnafungin A and B (**1** and **2**, Fig. 1), an interconverting mixture of isoxazolidinone-containing natural products.[5] Further biochemical analysis of the parnafungins led to the identification of these natural products as inhibitors of the fungal polyadenylate polymerase, the enzyme responsible for the extension of the poly(A) tail of an mRNA transcript.[1] Further, a mixture of parnafungin A and B had in vivo efficacy in a murine model of disseminated candidiasis.

Parnafungin A and B are the first examples of natural products containing the isoxazolidinone ring system. In addition to the

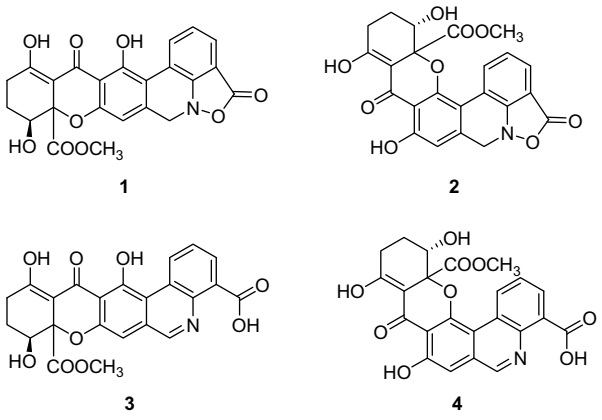

Figure 1. Parnafungin A (**1**) and B (**2**) and the corresponding benzoquinoline analogs **3** and **4**.

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isoxazolidinone, these compounds also contain a xanthone ring system, closely related to the ergochrome subunit that dimerizes to form various secalonic acids.[6,7] Ring-opening of the xanthone by a retro-Michael reaction leads to the interconversion of **1** and **2**, along with epimerization of the quaternary carbon bearing the methyl carboxylate. Further, the chemistry of these natural products is complicated by the instability of the isoxazolidinone ring. Hydrolysis of the isoxazolidinone ring, which is accompanied by the elimination of a molecule of water, generates the inactive benzoquinoline analogs **3** and **4**.

Parnafungin A and B were initially discovered from two fungal strains that resembled *F. larvarum* Fuckel (Ascomycota, Hypocreales) and were isolated from lichens obtained from the province of Madrid, Spain.[1,5] Subsequent to the discovery of parnafungin A and B, additional fungal species resembling *F. larvarum*, which included strains isolated from plants, plant litter and lichens, were identified as parnafungin A and B producers. From this taxonomic study,[8] it was concluded that the *F. larvarum* complex could be resolved into at least six or, possibly, seven different species. However, the examination of additional related strains is necessary before definitive taxa can be described. After fermentation of these strains, an acetone extract of each sample was analyzed by reversed phase HPLC with diode array and mass spectrometric detection (HPLC-DAD-MS) in order to confirm the production of **1** and **2** (MW 451). In all cases, the production of **1** and **2** were confirmed and, further, the *Ca*FT profiles of these extracts matched that observed for purified parnafungins A and B.

While probing the taxonomic relationship of these parnafungin producing strains, an acetone extract from strain F-155,597 was identified by HPLC-DAD-MS analysis as producing substantial quantities of two analogs along with lesser quantities of parnafungins A and B.[8] These new analogs shared similar absorbance spectra to that obtained for parnafungin A and B ($\lambda_{max} \sim 350$ nm), but differed in retention time and molecular weight (MW 465 and 479). Additional metabolites having similar absorbance spectra to that of the inactive benzoquinoline analogs ($\lambda_{max} \sim 450$ nm) were also present in this sample with corresponding molecular weights (MW 465 and 479). Following the discovery of these additional parnafungins, single ion monitoring was able to confirm that several other strains from the *F. larvarum* complex also produced these compounds, but in less pronounced quantities than that obtained from strain F-155,597. Here, we describe the isolation, structure elucidation and comparative antifungal activities of the two additional parnafungins identified from strain F-155,597, designated here as parnafungin C (**5**) and parnafungin D (**6**).

In order to determine the structure of parnafungin C and D, a large scale fermentation (1 L) of F-155,597 was prepared and extracted with one volume of EtOAc. After adsorbing this extract onto silica gel by removal of the solvent and then loading it on a silica cartridge, the column was eluted successively with 30%, 50%, and 80% EtOAc in hexanes, followed by 30% methanol in EtOAc. HPLC-DAD-MS analysis (C18) indicated that the two new parnafungin analogs were present in the 50% and 80% EtOAc in hexane fractions, with the latter cut containing predominantly **5** and **6**. The combined 80% EtOAc fractions were concentrated to dryness and further purified by preparative reversed phase C18 HPLC. This fractionation step provided purified components for full chemical characterization and structure elucidation. High resolution mass spec analysis of these samples was consistent with the molecular formulas of $C_{24}H_{19}NO_9$ (466.1131, calcd for M+H 466.1138) and $C_{24}H_{17}NO_{10}$ (480.0924, calcd for M+H 480.0931) for parnafungin C and parnafungin D, respectively.

In our earlier work,[5] the structures of parnafungins A and B were determined after methylation of the mixture of those natural products with ethereal diazomethane, thereby stabilizing the components from interconverting. The mono-methylated products

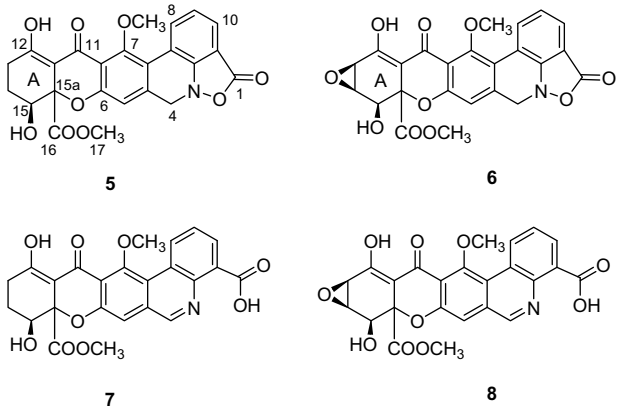

Figure 2. Parnafungin C (**5**) and D (**6**) and the corresponding benzoquinoline analogs **7** and **8**.

were purified and the structures of the methylated derivatives were resolved by NMR spectroscopy and X-ray crystallography. In that case, methylation occurred at the C12 position providing the corresponding methyl enol ethers. Methylation at the enol position prevented the retro-Michael opening of the A ring (Fig. 2), thereby blocking the equilibration between parnafungin A and parnafungin B as well as the epimerization at C15a.

NMR spectroscopy was used to determine the structure of parnafungin C and parnafungin D. It was readily apparent from an analysis of the ^1H and ^{13}C 1D NMR spectra that there were two methyl groups present in parnafungin C as compared with only one in parnafungins A and B (Table 1). One methyl singlet (δ 3.57 ppm) corresponded to the methyl carboxylate at the quaternary chiral center (C15a). The additional methyl singlet resonance in paranfungin C (**5**) at δ 3.86 ppm was distinct from that in the

Table 1
^1H and ^{13}C NMR data for parnafungin C (**5**)[a]

Position	δ C	mult	δ H (J in Hz)
1	167.3	qC	
4	54.9	CH2	4.73 (m)
4a	140.3	qC	
5	110.3	CH	7.06 (s)
6	160.7	qC	
6a	113.1	qC	
7	158.9	qC	
7a	115.6	qC	
7b	119.0	qC	
8	131.3	CH	8.33 (d, 8.0)
9	126.3	CH	7.43 (t, 8.0)
10	123.5	CH	7.75 (d, 8.0)
10a	112.8	qC	
10b	156.2	qC	
11	184.8	qC	
11a	102.8	qC	
12	171.4	qC	
13	25.7	CH_2	2.75 (m)
			2.54 (m)
14	23.9	CH_2	2.14 (m)
			1.90 (m)
15	70.2	CH	4.24 (d, 4.0)
15a	85.6	qC	
16	169.8	qC	
17	52.7	CH_3	3.57 (s)
7-OCH_3	61.9	CH_3	3.86 (s)
12-OH			5.9 (b)

[a] ^1H spectra were accumulated at 500 MHz and ^{13}C spectra were accumulated at 125 MHz in DMSO-d_6. Proton NMR spectra were referenced to the residual ^1H solvent peak for DMSO-d_5 at δ 2.49. Carbon spectra were referenced to the DMSO-d_6 septet at δ 39.51.

methyl enol ether derivatives prepared from parnafungin A (δ 3.82 ppm) and parnafungin B (δ 3.90 ppm). Further, for the synthetic methyl enol ethers, the methyl group protons presented HMBC correlations to C12 at δ 173 ppm, while the methyl group in parnafungin C provided an HMBC correlation to a phenolic carbon at δ 159 ppm. It remained to be determined whether the methyl ether in parnafungin C was at C7, which would be the case if the structure was analogous to parnafungin A, or on the phenolic hydroxyl group C6, which is available in parnafungin B. ROESY correlations between the methyl group and the H8 proton indicated that the methyl group was located on the C7 phenol.

Additional spectroscopic differences between the 'straight' parnafungin A and the more 'bent' parnafungin B have been described.[5] In those studies, the ^{1}H NMR signals that correspond to H4 and H8 provided further insight into the structure of **5**. The differences in the ^{1}H resonances were attributed to small changes in the twist of the biphenyl ring system. The two diastereotopic protons at C4 have near magnetic equivalence and have almost completely collapsed to a singlet at δ 4.68 ppm for **1**. This is not the case for **2**, where the two protons at C4 are well separated into a pair of doublets at δ 4.55 and δ 4.76 ppm. In the case of H8, this aromatic proton presented at δ 8.30 ppm for **1** and at δ 8.65 ppm for **2**. For parnafungin C, the signal for the C4 methylene group had almost collapsed to a singlet at δ 4.73 ppm and H8 was observed at δ 8.33 ppm. These data were clearly consistent with an assignment of the structure of parnafungin C (**5**) in the straight geometry analogous with the structure of parnafungin A (**1**). Since parnafungin C does not contain a moiety blocking the retro-Michael ring-opening of the A-ring, epimerization of C15a is still possible and an equilibrium mixture of two diastereomers was observed in the ^{1}H NMR spectra in DMSO-d_6.

Parnafungin D (**6**) was relatively more polar than parnafungin C based on the retention times of these components on reversed phase C18 HPLC. Comparing the molecular formulas of these two components, parnafungin D had one additional oxygen atom and two fewer protons. Upon preparing a solution of **6** in DMSO-d_6 for NMR analysis, substantial decomposition of the material was observed. While parnafungin C had limited solubility in methylene chloride, parnafungin D had sufficient solubility in CD_2Cl_2 for the acquisition of a complete NMR data set (Table 2). ^{1}H and ^{13}C NMR spectra of **6** indicated that the aliphatic methylene groups of the A ring (Fig. 2) were not present in this component. While there were still two methyl groups at δ 3.67 and δ 3.90 ppm, two additional doublets were observed at δ 3.62 and δ 3.80 ppm, each corresponding to one proton by integration. The assignment of these signals as an epoxide at C13–C14 was fully consistent with these data and HMBC correlations. Since H14 does not present a ^{1}H–^{1}H coupling with H15, the epoxide is on the same face of the A ring as the hydroxyl, providing a geometry where the two protons are approximately at an angle of 90°. The remaining 1D and 2D NMR data were fully consistent with the remainder of parnafungin D being identical with that of parnafungin C. In addition, the benzoquinoline analogs **7** and **8** of parnafungins C and D, respecitively, were also identified in the HPLC chromatograms by absorbance spectra and mass spec data. ^{1}H NMR analysis of these compounds was consistent with the opening of the isoxazolidinone ring and the formation of the aromatized benzoquinoline (data not shown).

Based on our previous stereochemical analyses for parnafungin A and B,[5] the absolute configuration of the C15 hydroxyl of these natural products is (*S*) and the major diastereomer at the C15a quaternary carbon is also (*S*). The producing organism of parnafungin C and D (F-155,597) is taxonomically closely related to the original *F. larvarum* from which parnafungin A and B were isolated.[8] Further, parnafungins A and B are co-produced with parnafungins C and D by F-155,597, indicating that these new components are likely to be part of the same biosynthetic pathway. From this, the assignment of the absolute configuration of the C15 hydroxyl for parnafungin C and D is also (*S*) and the epoxide of parnafungin D is on the β face of the A ring.

The relative potencies and spectra of antifungal activity were determined for **5** and **6**, and compared to the activity of a mixture of parnafungins A and B (**1**/**2**) (Table 3). Generally, **5** and **6** were less potent against all of the *Candida* species tested, but in some cases, such as against *C. tropicalis* and *C. lusitaniae*, these analogs had comparable antifungal activity. These differences in relative potency may be indicative of slight changes in the binding of each compound against the relevant isoform of the fungal RNA polyadenosine polymerase. In order to assess whether analogs **5** and **6** had similar mechanisms of action as **1** and **2**, the activity of each compound was tested against wild-type *C. albicans* and two heterozygote strains *C. albicans* strains, each containing a single copy of CLP1 or YSH1. These two strains were previously shown to be hypersensitive to parnafungins A and B.[1] For **5** and **6** (Table 4), a 2- to 4-fold shift was observed in the MICs against the heterozygote strains, indicating that these compounds are functioning with the same mechanism of action as was determined for **1** and **2**. As was the case for benzoquinolines **3** and **4**, no antifungal activity was observed for the comparable analogs **7** and **8**.

Table 2
^{1}H and ^{13}C NMR data for parnafungin D (**6**)[a]

Position	δ C	mult	δ H (*J* in Hz)
1	168.0	qC	
4	56.3	CH_2	4.51 (m)
4a	140.6	qC	
5	113.6	CH	6.82 (s)
6	157.6	qC	
6a	112.9	qC	
7	159.0	qC	
7a	119.1	qC	
7b	119.5	qC	
8	132.0	CH	8.43 (d, 7.5)
9	126.5	CH	7.37 (t, 7.5)
10	124.4	CH	7.70 (d, 7.5
10a	111.6	qC	
10b	156.9	qC	
11	186.8	qC	
11a	101.4	qC	
12	174.8	qC	
13	53.9	CH	3.62 (d, 4.5)
14	55.7	CH	3.80 (d, 4.5)
15	74.8	CH	4.60 (d, 10.0)
15a	78.7	qC	
16	170.9	qC	
17	53.9	CH_3	3.67 (s)
7-OCH_3	62.5	CH_3	3.90 (s)
12-OH			15.9 (s)
15-OH			4.79 (d, 10.0)

[a] ^{1}H spectra were accumulated at 500 MHz and ^{13}C spectra were accumulated at 125 MHz in CD_2Cl_2. Proton NMR spectra were referenced to the residual ^{1}H solvent peak for $CDHCl_2$ at δ 5.32. Carbon spectra were referenced to the CD_2Cl_2 pentet at δ 54.0.

Table 3
Whole-cell inhibition of parnafungins A–D

Species	Strain	**1/2**	**5**	**6**
		MIC in μg/mL[a]		
C. albicans	MY2323	0.008	2	0.016
C. glabrata	ATCC90030	1.25	>10	5
C. parapsilosis	ATCC22019	0.6	>10	2.5
C. lusitaniae	ATCC34449	0.3	0.16	0.16
C. krusei	ATCC6258	0.008	0.08	0.016
C. tropicalis	ATCC750	2.5	2.5	0.6

[a] MIC was determined in Sabarose Dextrose medium.

Table 4
Heterozygote strain inhibition of parnafungins A–D

Species	Strain	1/2	5	6
		MIC in μg/mL[a]		
C. albicans	MY2323 (WT)	0.008	0.06	0.016
C. albicans	MY2323 CLP1 +/−	0.004	0.03	0.004
C. albicans	MY2323 YSH1 +/−	0.004	0.03	0.004

[a] MIC was determined in Sabarose Dextrose medium.

The biosynthesis of parnafungins is likely related to that of ergochrome-derived secondary metabolites, which are based on a series of polyketide condensations and cyclizations.[6,9] For the parnafungin family of natural products, the extended ring system beyond the typical xanthone unit as well as the incorporation of a nitrogen atom and the closure of the isoxazolidinone ring make the biosynthetic pathway an intriguing, but unresolved question. Previously, we have identified by affinity selection/mass spec techniques that the active form of the equilibrating mixture of isomers is the straight parnafungin A.[10] The fact that parnafungins C and D are direct analogs of parnafungin A and not parnafungin B supports the hypothesis that it is parnafungin A that is directly synthesized by these *Fusarium* spp. Subsequent methylation and oxidation of parnafungin A would generate **5** and then **6**. Practically, the methylation of the C7 hydroxyl simplifies the chemical properties of both of these compounds, preventing the formation of the corresponding bent geometric isomers.

Species of the *F. larvarum* complex are assumed to be mycoparisites associated with scale insects, aphids, lichens and the basidiomycete fungus *Septobasidium clelandii*.[8,11–14] Additional to the *F. larvarum* complex, the parnafungins have also been reported from two other Hypocrealean mycoparasitic fungi, *Trichonectria rectipila* and *Cladobotryum pinarense*.[8] With regards to the ecological role that the parnafungins may convey to their producing organism, it has been hypothesized that the compounds may act as potent antifungals, providing a competitive advantage for growth and, moreover, as a virulence factor mediating inter-organism interactions while colonizing hosts, for example, lichenized and non-lichenized fungi, insects, or plants.[8] The facile degradation of the parnafungins to the inactive benzoquinoline forms has been correlated with a change in pH, where ring opening was promoted in neutral or alkaline conditions, while a stabilization of the molecule is favored in acidic conditions.[5] Many fungi lower the pH of their growth medium through the secretion of organic acids during their initial phase of growth, and later, as the fungus reaches stationary growth, the medium's pH progressively becomes more alkaline;[15] in this case, alkalization of the growth medium following colonization would, hypothetically, promote the accumulation of the benzoquinoline analogs and prevent the producing organism from accumulating toxic levels of the parnafungins.[8]

Since parnafungins A and B are efficacious in a murine model of disseminated candidiasis with no observable toxicity,[1] this family of compounds can be pursued as a novel class of antifungal agents with a unique mechanism of action. The parnafungin analogs reported here provide additional information as to the types of structural modifications that are possible to the core structure while maintaining antifungal activity. Parnafungins C and D expand the available structure–activity information on this family of natural products. Further synthetic exploration of this chemical scaffold may provide parnafungin analogs with improved potency, spectrum and chemical stability, and provide a route for the further development of an antifungal agent that targets fungal RNA processing.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.081.

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