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The discovery of moriniafungin, a novel sordarin derivative produced by *Morinia pestalozzioides*

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Abstract—A novel sordarin derivative, moriniafungin (**1**), containing a 2-hydroxysebacic acid residue linked to C-3′ of the sordarose residue of sordarin through a 1,3-dioxolan-4-one ring was isolated from the fungus *Morinia pestalozzioides*. Isolation of moriniafungin employed a highly specific bioassay consisting of a panel of *Saccharomyces cerevisiae* strains containing chimeric eEF2 for *Candida glabrata*, *Candida krusei*, *Candida lusitaniae*, *Crytpococcus neoformans*, and *Aspergillus fumigatus* as well as wild type and human eEF2. Moriniafungin exhibited an MIC of 6 μg/mL versus *Candida albicans* and IC_{50}'s ranging from 0.9 to 70 μg/mL against a panel of clinically relevant *Candida* strains. Moriniafungin was shown to inhibit in vitro translation in the chimeric *S. cerevisae* strains at levels consistent with the observed IC_{50}. Moriniafungin has the broadest antifungal spectrum and most potent activity of any natural sordarin analog identified to date.

1. Introduction

There is a significant need for broad-spectrum therapies with an improved safety profile to address the high mortality rates associated with invasive fungal infections in immuno compromised hosts, including HIV-positive patients. Despite the significant number of patients, clinicians are limited to only a few classes of antifungal drugs for treatment. Therefore, continuous efforts to design novel drugs are necessary. To meet these needs, the identification of agents with a novel mode of action would be of great benefit.

Protein synthesis has always been considered among the most attractive targets in the development of antimicrobial agents.[1] However, the application of this idea to the field of antifungal therapy is hampered by the high degree of similarity between the fungal and mammalian protein synthesis machineries.

The most important family of antifungal agents acting at the level of protein synthesis are the sordarins. Sordarin was isolated in 1969 from fermentations of the fungus *Sordaria araneosa*.[2] Sordarins are potent inhibitors of translation in fungi with an extremely high level of selectivity for fungi.[3–5] They act via a specific interaction with eEF2, by stabilizing the fungal eEF2–ribosome complex. All compounds in this class inhibited in vitro translation in *Candida albicans*, *Candida tropicalis*, *Candida kefyr*, and *Crytpococcus neoformans*, but to varying degrees. The lack of activity of the sordarins against *Candida krusei*, *Candida glabrata*, and *Candida parapsilosis*, in comparison with their extremely high levels of potency against *Candida albicans*, suggests that these compounds have a highly specific binding site, which may also be the basis for the greater selectivity of these compounds in inhibiting fungal, but not mammalian, protein synthesis.

Since the discovery of sordarin, several structurally related compounds sharing the common aglycone of

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sordarin, sordaricin, were isolated from diverse species of ascomycete fungi: zofimarin,[6] BE31405,[7] and SCH57404.[8] In a concurrent study, another group identified xylarin, which exhibited potent activity against yeasts and filamentous fungi, and was only weakly cytotoxic toward mammalian cells.[9] Another natural sordarin derivative, hypoxysordarin, was also isolated with activity against yeasts and several filamentous fungi[10] as well as GR135402, which showed to be a selective and potent inhibitor of *C. albicans* protein synthesis.[11] Synthetic derivatives of this compound (GM222712, GM237354, GM193633, and GM211676) have demonstrated good in vitro and in vivo antifungal activity against most *Candida* species, including azole-resistant isolates, *Cr. neoformans*, and *Pneumocystis carinii*.[12,13] In contrast, the new sordarin derivatives have limited activity against *Aspergillus fumigatus* and that deficiency constitutes a serious drawback.[14]

Recent efforts directed toward the synthesis and development of new sordarin antifungal agents with improved activity against pathogenic fungi and better pharmacological properties have resulted in the discovery of a new series of derivatives, the azasordarins.[15] Azasordarins displayed significant activity against *Candida* species, including fluconazole-resistant strains, with the exception of *C. krusei*. In general, the levels of cytotoxicity presented by the azasordarin derivatives were low.[16]

The potent broad spectrum in vitro activity and the fact that some sordarins have shown oral efficacy in animal models are significant advantages that justify an ongoing effort into developing the full clinical potential of the sordarins.

In this work, we report the discovery of a new antifungal compound, moriniafungin, isolated from extracts derived from a fungal culture identified as *Morinia pestalozzioides*. The antifungal activity in vitro and in vivo, some details of its mode of action, and the isolation and structural elucidation of the compound are described.

2. Results and discussion

2.1. Discovery of moriniafungin

During a screening program of microbial natural products searching for novel inhibitors of fungal protein synthesis, we detected antifungal activity in an extract derived from an endophytic fungus. Morphological data of the producing strain corresponded to *M. pestalozzioides* and analysis of the small subunit rDNA indicated that the strain belonged to the ascomycete family Amphisphaeriaceae (data not shown). Survey of the literature, fungal herbaria, and public culture collections suggested that *M. pestalozzioides* is a rare species previously known from a few specimens from Italy.

The growth of the wild type *Saccharomyces cerevisiae* as well as the strains expressing the *S. cerevisiae–Cr. neoformans* eEF2 and *S. cerevisiae–C. krusei* eEF2 chimeras was inhibited, while the growth of the *S. cerevisae*–human eEF2 chimeric strain was unaffected. The activity was detected in two fermentation media, AD2M2 vermiculite and MV8, at an extract concentration of 2.7 mg/mL. The positive control sordarin was active only against the wild type *S. cerevisiae* strain, because the strains harboring the chimeric eEF2s exhibited a sordarin-insensitive phenotype paralleling that of the wild-type organisms, as described by Shastry et al., (Table 1).[17]

The active component from the extract was purified and identified as a novel sordarin derivative (Fig. 1), which was named moriniafungin (**1**). Details on the isolation and structure elucidation of the compound are described below.

One of our initial objectives in order to support isolation of the active component was to increase the fermentation titers of moriniafungin. Modifications of several parameters in the fermentation conditions such as time, temperature, and light regime in medium MV8 resulted in different titers of the compound.

In general, darkness gave lower titers of moriniafungin, but both, increasing the temperature from 22 to 28 °C or increasing the fermentation time from 21 to 28 d resulted in increased titers of the active compound up to 4-fold. Temperature seemed to be a more critical factor for increasing the titers as its effect was always positive and more efficient than increasing the production time.

On the other hand, growth on other liquid media different from the original MV8 randomly affected titers of moriniafungin (at 21 d and 22 °C), but without significant improvements. The medium OP26NLW proved to be the best medium for the production of moriniafungin with an increase of 32-fold over the original medium.

Table 1. Antifungal activity of the MEK extract from isolate MF6856, expressed as the diameter of inhibition zones (in mm) in agar diffusion assay (see Materials and methods)

	S. cerevisiae expressing chimeric eEF2				
	S. cerevisiae	*C. lusitaniae*	*C. krusei*	*Cr. neoformans*	Human
MF6856 (2.7 mg/mL, AD2M2 verm)	**34c**	35v	**23h**	**28h**	32v
(2.7 mg/mL, MV8)	**28c**	19v	**21f**	**24f**	19v
Sordarin (10 μg/mL)	**15f**	0	0	0	0

All of the target organisms are from the Merck Culture Collection.
c.—clear inhibition zone; h.—hazy inhibition zone; f.—fuzzy inhibition zone; v.—very hazy inhibition zone.

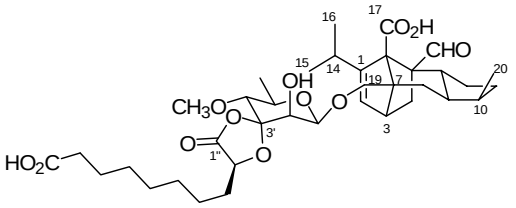

Figure 1. Structure of moriniafungin (**1**).

OP26NLW liquid medium also offered a clear improvement over other vermiculite-based media and/or variations of the original MV8 liquid medium with potential for further production scale up.

2.2. Purification and structural elucidation of moriniafungin

Moriniafungin was isolated from either solid or liquid fermentations of MF6856 in three steps each of which was guided by bioactivity in an agar based susceptibility assay described herein. A second antifungal component, separable from moriniafungin, was noted in several fermentations, but the activity was non-selective versus the chimeric *S. cerevisiae* strains tested and was not identified. The dicarboxylic acid character of **1** was exploited for its purification through ion exchange on the weak anion exchange resin AG4x4 or using pH-zone-refining countercurrent chromatography.[18] Liquid fermentations, which were extracted with a water miscible solvent required a solid-phase adsorption/elution desalting step prior to ion exchange. This was accomplished through adsorption/elution on Mitsubishi SP207. The desalting step was not necessary for solid fermentations extracted with a water immiscible solvent. Final purification of **1** was straightforward using preparative RP HPLC. Initial, unoptimized, titers of moriniafungin were approximately 11 mg/L.

The structure of moriniafungin was determined based upon analysis of spectroscopic data. A molecular formula of $C_{37}H_{54}O_{12}$ (U.N. = 11) was established by correlation of FTMS exact mass measurements and the ^{13}C NMR carbon count (Table 2). Initial inspection of the NMR dataset (^{1}H, ^{13}C, dqCOSY, HSQC, and gHMBC) suggested that **1** was a sordarin derivative. The ^{13}C NMR resonances corresponding to the intact sordaricin aglycone were easily identified in the spectra of **1** and deviated less than 0.6 ppm from those for sordarin.

Novel to moriniafungin was a $C_{17}H_{26}O_8$ fragment represented by two distinct spin systems assembled from ^{1}H–^{1}H and ^{1}H–^{13}C correlations. The first of these was a modified sordarose carbohydrate. Positions 1′, 4′, 5′, 6′, and the C-4′ *O*-methyl of sordarose were clearly observed but notably absent were the 3′ secondary hydroxyl carbon and protons. Consistent with a 3′ modification was the observation of H-2′ as a doublet with a $J_{H1'–H2'}$ of <1 Hz instead of a doublet of doublets as in sordarin. Interestingly, this sordarose-like partial structure contained a ketal carbon at 108.2 ppm. This ketal carbon exhibited ^{1}H–^{13}C correlations from H-1′, H-2′, and H-4′ (Fig. 2), which allowed its assignment as C-3′. The

Table 2. NMR assignments for moriniafungin (**1**) in CD_2Cl_2 at 150/600 MHz

Position	^{13}C, δ	Mult	^{1}H, δ (m, J in Hz)
17	205.1	s	9.681 (s)
10″	178.9	s	*
18	176.2	s	*
1″	172.9	s	*
1	148.8	s	*
2	131.1	d	6.089 (dd, 1.1, 3.2)
3′	108.2	s	*
1′	98.7	d	4.562 (d, 0.9)
4′	83.0	d	3.321 (d, 9.6)
2″	76.2	d	4.594 (dd, 4.4, 7.3)
19	74.9	t	3.996 (d, 9.3); 3.716 (d, 8.7)
2′	74.1	d	3.709 (s)
6	72.9	s	*
5′	70.1	d	3.540 (dq, 6.2, 9.5)
7	65.9	s	*
7′	62.1	q	3.473 (s)
5	59.2	s	*
3	46.9	d	2.698 (dd, 3.9, 3.9)
13	42.1	d	1.994 (ddd, 6.2, 11.8, 18.2)
9	41.6	d	1.77
9″	34.2	t	2.353 (t, 7.4)
3″	32.5	t	1.83;1.65
11	32.4	t	2.05;1.22
10	31.4	d	2.07
4	29.8	t	1.25; 1.95
8	29.5	t	1.94; 1.80
*C-5″	29.3	t	1.26
*C-6″	29.2	t	1.26
*C-7″	29.1	t	1.26
14	28.0	d	2.318 (hept., 6.9)
12	26.6	t	1.86; 0.99
**C-4″	25.1	t	1.4
**C-8″	25.0	t	1.6
16	22.8	q	0.970 (d, 6.7)
15	21.3	q	1.032 (d, 6.8)
6′	17.7	q	1.316 (d, 6.2)
20	17.5	q	0.808 (d, 6.9)

*, ** may be interchanged.

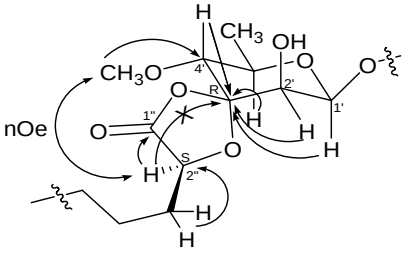

Figure 2. Critical NOE, ^{1}H–^{1}H and ^{1}H–^{13}C NMR correlations for the 1,3-dioxolan-4-one moiety of moriniafungin.

second partial structure was identified as 2-hydroxysebacic acid. Direct evidence to connect the two spin systems was lacking however. Only one 3-bond ^{1}H–^{13}C correlation between the sordarose and sebacic acid is possible that between C-3′ and H-2″, but it was not observed using various HMBC experiments optimized for different $J_{C–H}$. The sordaricin aglycone, modified sordarose, and 2-hydroxylsebacic acid accounted for 10 of the 11 unsaturations required by the proposed molecular

formula and thus connection of the two subfragments required formation of a ring. An unusual carbonyl stretch at 1803 cm^{-1} in the IR spectrum of **1** suggested the presence of a somewhat strained carbonyl and thus the two fragments were connected resulting in a 1,3-dioxolan-4-one ring.

The 1,3-dioxolan-4-one contains two chiral centers creating four diastereomeric possibilities for moriniafungin. Two of these were eliminated through the observation of a NOE between the C-4′ *O*-methyl protons and H-2″ in both NOESY and 1D difference NOE experiments (Fig. 2). This correlation is possible in only the C3′ (*R*)/C-2″ (*S*) and C3′ (*S*)/C-2″ (*R*) configurations. Unambiguous differentiation between the two remaining possible diastereomers was not possible from NMR data alone. However, co-crystallization of moriniafungin with EF2 and resolution of the complex revealed the absolute configuration to be C3′ (*R*)/C-2″ (*S*) as well as confirming the presence of the 1,3-dioxolan-4-one at C-3′.[19]

Moriniafungin is the first example of a fungal natural product containing a 1,3-dioxolan-4-one ring. The plant triterpenoid glycosides spilacleosides A and B isolated from *Ruscus aculeatus* are the only other reported natural products to contain this ring system.[20] The 1,3-dioxolan-4-one ring of spilacleosides A and B arises from condensation of a 4-oxo carbohydrate (C-4‴ of the known aculeoside A) and an α-hydroxy acid, (2*R*,3*S*)-2,3-dihydroxy-3-methylpentanoic acid. A similar route of 1,3-dioxolan-4-one ring formation is possible for moriniafungin through a 3-oxo-sordarose followed by condensation with (2*S*)-hydroxysebacic acid. The 3′-oxo analog of sordarin has not been reported as a natural product and was not observed during the isolation of moriniafungin. Interestingly, both C-2 epimers of the 1,3-dioxolan-4-one of the spilacleosides were isolated as natural products but we observed only one epimer for moriniafungin.

Decoration at C-3′ of the sordarose residue of sordarin analogs generally resulted in lower MICs and increased antifungal spectrum when compared with those of sodarin. Esterification of the C-3′ hydroxyl with hexadienoic (zofimarin, GM135402) or a highly oxidized octanoic acid (hypoxysordarin) has been reported. Moriniafungin can be viewed as a C-3′ sordarin derivative but differs from other C-3′ sordarin analogs in possessing a side chain with a terminal carboxylic acid residue, which appears to be involved in binding to EF2. The terminal carboxylic acid may be partially responsible for the increased antifungal spectrum and potency of moriniafungin relative to other C-3′ analogs.

2.3. Biological activity of moriniafungin

The MICs of moriniafungin against a group of *S. cerevisiae* strains expressing the substituted *S. cerevisiae* eEF2[17] are summarized in Table 3. This compound showed a more potent activity against the *S. cerevisiae–C. glabrata* eEF2 chimeric strain (MIC_{50}, 3 μg/mL) than against the *S. cerevisiae–Cr. neoformans* and *S. cerevisiae–C. krusei* eEF2 chimeric strains (MIC_{50}, 6.25 and 12.5 μg/mL), and showed a marginal activity against *A. fumigatus* eEF2 chimeric strain (MIC_{50}, 25 μg/mL). However, it was very weakly active against *S. cerevisiae–C. lusitaniae* eEF2 strains (MIC, 100 μg/mL). The activity of moriniafungin is comparable to that of sordarin for the host *S. cerevisiae* strain (MIC_{50}, 0.2 μg/mL). In general, moriniafungin is a more potent fungal inhibitor when compared to sordarin.

Moriniafungin IC_{50}s were in the range of 0.05–1.5 μg/mL for all of the strains tested, except for the *S. cerevisiae* strain expressing the *Candida lusitaniae* eEF2 chimera, with a value of 20 μg/mL. The compound was essentially inactive against the *S. cerevisiae* human eEF2 chimeric strain (IC_{50} > 100 μg/mL).

The antifungal spectrum of moriniafungin was evaluated by comparison with that of sordarin using a panel of wild-type yeast strains (Table 4). Moriniafungin showed antifungal activity against the ascomycetous yeasts, *S. cerevisiae*, *C. albicans*, and *C. glabrata*. The compound displayed no antifungal activity against the basidiomycetous yeast *Cr. neoformans* and the ascomycetous yeasts *C. parapsilosis*, *C. krusei*, and *C. lusitaniae*, as judged by MIC values. Although in several cases, IC_{50} values were reduced for moriniafungin relative to sordarin. *C. krusei*, for example, exhibited a reduction of at least 80% in IC_{50} value. Because comparisons of intrinsic potency at the target level are difficult to make across different fungal species, with varying influx and efflux capacities, the potency of moriniafungin at its target was assessed in an in vitro translation system using the

Table 3. In vitro antifungal activity of moriniafungin and sordarin with chimera strains, evaluated in microbroth dilution assays

Chimera strains	MIC (μg/mL)		IC_{50} (μg/mL)	
	Sordarin	Moriniafungin	Sordarin	Moriniafungin
S. cerevisiae	0.2	0.2	0.15	0.05
S.c./C. glabrata	5.0	3.125	0.5	0.5
S.c./C. krusei	>100	12.5	>100	0.6
S.c./C. lusitaniae	>100	100	>100	20
S.c./Cr. neoformans	>100	6.25	60	0.1
S.c./A. fumigatus	>100	25	>100	1.5
S.c./Human	>100	>100	>100	>100

All the determinations were performed after 24 h incubation.
Results are expressed as the minimum inhibitory concentration (MIC) and at 50% inhibitory concentration (IC_{50}).

Table 4. In vitro antifungal activity of moriniafungin compared with that of sordarin against wild-type strains, evaluated in microbroth dilution assays

Strain	MIC (μg/mL)		IC_{50} (μg/mL)	
	Sordarin	Moriniafungin	Sordarin	Moriniafungin
S. cerevisiae (ATCC 201389)	10	10	3.9	1.2
C. albicans (MY1055)	6.25	6.25	0.4	0.9
Cr. neoformans (MY 2062)	>100	>100	>100	>100
Cr. neoformans (ATCC 66031)	>100	100	45	19
C. glabrata (MY1381)	100	25	8	1.8
C. parapsilosis (ATCC 22019)	>100	100	>100	39
C. krusei (ATCC 6258)	>100	>100	>100	21
C. lusitaniae (MY1396)	>100	>100	>100	70

All the determinations were performed after 24 h incubation. Results are expressed as the minimum inhibitory concentration (MIC) and as 50% inhibitory concentration (IC_{50}). The codes to the source of each target organism are from Merck culture collection.

hybrid *S. cerevisiae* eEF2s.[17] As summarized in Table 5, moriniafungin proved to be an inhibitor of translation in *S. cerevisiae* expressing the *C. albicans*, *C. glabrata*, and *Cr. neoformans* chimeric eEF2s, with potencies approximately the same as that of sordarin for these three chimeras. Similarly, moriniafungin has relatively weaker activity against the chimera containing the *A. fumigatus* 10 amino acid sequence of the sordarin specificity region. Most significantly, moriniafungin has acquired significant activity against the *C. krusei* chimeric construct, against which sordarin is completely inactive (Table 5).

The in vivo efficacy of moriniafungin was studied in an abbreviated 24 h version of a murine model of disseminated candidiasis with enhanced susceptibility to *C. albicans*.[21] Moriniafungin did not produce any reduction in the number of c.f.u. (colony forming units) even at high doses (data not shown).

In summary, we have described a new protein synthesis inhibitor, moriniafungin, produced by *M. pestalozzioides*, which has a broad antifungal spectrum including *C. albicans*, *C. glabrata*, and *S. cerevisiae*. The MIC distribution of moriniafungin for diverse susceptible fungal species was wider than that of sordarin. In contrast to other sordarins, this new sordarin derivative lacked in vivo activity, which constitutes a limitation. The in vitro profile of this compound warrants more studies in order to know the extent of activity of moriniafungin against other fungal species implicated in human infections.

Table 5. Evaluation of moriniafungin and sordarin in in vitro translation systems using the hybrid *Saccharomyces cerevisiae* eEF2s

Chimera Strains	In vitro translation (IC_{50}, μg/mL)	
	Sordarin	Moriniafungin
S.c./C. albicans	0.006	0.006
S.c./C. glabrata	0.05	0.03
S.c./C. lusitaniae	>100	>100
S.c./C. parapsilosis	>100	>100
S.c./C. krusei	>100	0.5
S.c./Cr. neoformans	0.035	0.03
S.c./A. fumigatus	0.22	0.16
S.c./human	>100	>100

3. Experimental

3.1. Fermentation and extraction of *M. pestalozzioides*

The producing organism MF6856, ATCC No. PTA-3862 is an endophytic fungus isolated from stems of *Sedum sediforme* (Jacq.) Pau collected in Sierra Alhamilla Almería, Spain, following a conventional method for isolation of endophytic fungi.[22] The fungus was identified as *M. pestalozzioides* Berl. & Bres. based on a combination of morphological characters and comparisons with authentic herbarium specimens. The diagnostic features of the fungus are its conidia that are multi-celled, muriform, ellipsoid, versicolored, measuring 19.5–24.7 (22.4) × 6.5 μm, with an apical cell crowned with three appendages 9–11 μm long, and a basal cell mostly lacking appendages but sometimes bearing one (rarely two) cellular appendage 3 μm long.[23,24] For production of the compound, seed flasks were prepared from fresh slants of potato dextrose agar (PDA, Difco) of the isolate MF6856 as previously described.[25] Two-milliliter portions of the resulting cultures were used to inoculate 250 mL unbaffled Erlenmeyer flasks containing 50 mL of the following media: AD2M2, MV8,[26] and OP26NLW. In the case of AD2M2, 50 cc of vermiculite was added to 20 mL AD2M2 medium plus 20 mL water, the mixture was sterilized for 20 min at 121 °C and 15 psi. The composition of OP26NLW was (g/L): glycerol, 125; glucose, 25; pectin, 20; ardamine pH, 5; ammonium sulfate, 4; glycine, 2, potassium phosphate monobasic, 4; $CoCl_2$ $6H_2O$, 0.1; pH adjustment at 7.0. Production flasks were incubated at 22–28 °C and 50% relative humidity at 220 rpm for 21–28 d for the liquid media or statically for 21–28 d for the vermiculite medium.

Methyl-ethyl-ketone (MEK) extracts for the primary screening were prepared by adding 1.4 volumes of MEK (Merck) to each culture. Next, the mycelia were disrupted vigorously using a homogenizer Ultra-Turrax T25 8000 1/min (IKA-Labortechnik, Germany). The mixture was extracted for 15–60 min by shaking in a Vortexer orbital shaker and was centrifuged at 3000 rpm for 15 min. Aliquots (0.8 mL) of the organic phase were removed, dried completely in a Savant Speed-Vac, and the solid residue reconstituted in 0.5 mL of dimethylsulfoxide (DMSO).

3.2. Isolation of moriniafungin

A MEK extract corresponding to 12 L of solid fermentation of MF6856 on AD2M2 medium was concentrated to dryness in vacuo. The solid residue was dissolved in methanol (MeOH) (900 mL), diluted with H_2O (300 mL), and the resulting solution adjusted to pH 6.6 with dil NaOH. The solution was clarified by filtration through Celite, and the filtrate was applied to a column of BioRad AG4x4 (formate), vol. = 200 mL. The column was then washed with 75% MeOH/H_2O (900 mL) and the moriniafungin eluted with a solution of 100 mM Na-formate, pH 4.5, in 75% MeOH/H_2O. The moriniafungin containing fractions, 200–600 mL elution volume, were combined, concd. in vacuo to approx. 260 mL, and the resulting solution was adjusted to pH 3.1 with concd. H_3PO_4. This solution was extracted three times with an equal volume of hexane/EtOAc (4:6), the organic layers combined, washed with brine, dried over Na_2SO_4, and concd. in vacuo to yield crude moriniafungin (1.13 g). This material was subjected to pH-zone-refining countercurrent chromatography (CCC), stationary phase = CH_2Cl_2 + 0.04% trifluoroacetic acid, MP = 10 mM NH_4OH, tail-to-head elution, mobile phase flow rate = 3 mL/min, coil rotation 800 rpm, and fraction vol. = 9 mL. Fractions 29–43 were combined and desalted as described above to 296 mg solid residue. Final purification of **1** was accomplished using RP HPLC (C8, 15 μm, 5 × 25 cm) using a mobile phase of CH_3CN/H_2O (6:4) containing 0.1% formic acid at a flow rate of 60 mL/min. Moriniafungin **1** eluted at 18.7 min. The resulting rich cut was concentrated to remove CH_3CN and lyophilized to yield **1**, 135 mg.

Liquid fermentations of MF6856 (28 L) were extracted with an equal portion of MeOH and clarified by filtration through a Celite pad. The filtrate (56 L) was adjusted to pH 3.1 with dilute H_2SO_4 and applied to a column of Mitsubishi SP207 Sepabead (column volume = 2.5 L) at 10 L/h. The column was then washed with 50% MeOH (8 L)/H_2O and then **1** was eluted with methanol (6.5 L). The MeOH eluate was diluted with H_2O (1850 mL) and adjusted to pH 6.5 for purification using AG4x4 (column volume = 500 mL) as described above. Final purification of the AG4x4 rich cut was accomplished using RPHPLC as described above.

3.2.1. Moriniafungin (1). IR (film on ZnSe): 1803, 1710, 1096 cm^{-1}. ESI-FTMS: obsd 691.3705 [M+H], calcd for $C_{37}H_{55}O_{12}$, 691.3694. $[\alpha]_D^{25}$ − 39.9 (*c* 0.539, MeOH). UV (MeOH): 240 nm (sh). NMR: see Table 2.

3.3. HPLC analysis of extracts

Moriniafungin titers in *M. pestalozzioides* fermentation samples in the different media were analyzed using a ZORBAX RX-C8 4.6 × 250 mm in an Agilent 1100 DAD-HPLC ChemStation. An 11–99% gradient of acetonitrile in water (0.01% TFA) with a flow rate of 0.9–1.2 mL/min was programmed at a constant temperature of 20 °C during the 22 min and monitored at 210 nm.

3.4. Yeast strains

All the wild-type fungal strains used for growth inhibition assays are from the Merck culture collection and are identified by the Merck identification number or the ATCC strain number.

The isogenic *S. cerevisiae* strains harboring eEF2 constructs containing the sordarin selectivity regions from selected fungi (*S. cerevisiae*, *C. lusitaniae*, *C. krusei*, and *Cr. neoformans*) and human eEF2 are described in Shastry et al.[17] The *S. cerevisiae–A. fumigatus* eEF2 was constructed by replacing the residues 521–523 in *S. cerevisiae* eEF2 with the corresponding residues from the *A. fumigatus* eEF2 following the protocol described in Shastry et al.[17]

3.5. Differential susceptibility test against eEF2 isogenic yeast strains

The search for potential inhibitors of eEF2 was performed using an agar-based differential susceptibility test against five different isogenic yeast strains differing only by the chimeric eEF2 constructs as described above.

The assay plates were prepared by inoculating each strain to a final optical density at 600 nm of 0.01 units/mL into separate flasks containing YPAD/KCl supplemented with 1.5% Noble agar. Aliquots of 100 mL of the seeded agar media were poured into Nunc square plates (24 × 24 cm). Twenty-microliter aliquots of the MEK fermentation extracts were applied onto the surface of the assay plates seeded with the target microorganisms. Sordarin (12.5 and 6.25 μg) was tested as a positive control, while cycloheximide (1 μg) was used as negative controls in each assay plate. The plates were incubated at 28 °C, and zones of inhibition were scored after 24 h. An extract producing zones of inhibition on any of the fungal strains and no zone or a very hazy zone on the *S. cerevisiae*–human eEF2 containing strain was considered to be a positive readout.

3.6. Broth microdilution assay for MIC and IC_{50} determination

MIC and IC_{50} values for the wild-type fungal strains and *S. cerevisiae* strains with substituted eEF2s were determined from growth inhibition assays in which cells were inoculated in YPAD medium containing sordarin or moriniafungin serially diluted 2-fold from 100 to 0.2 μg/mL, followed by incubation at 29 °C for approximately 16 h.[3] The lower limit of sensitivity was 0.005 μg/mL.

3.7. In vitro translation assays

In vitro translation assays with extracts prepared from chimeric constructs in *S. cerevisiae* were performed as described previously.[3,4,17]

3.8. Determination of in vivo efficacy

For the evaluation of the in vivo activity of the compound, a murine model of disseminated candidiasis with

enhanced susceptibility to *C. albicans* but increased sensitivity for discriminating antifungal efficacy was used (TOKA-Lite, for Target Organ Kidney Assay). Basically, immunosuppressed mice were challenged intravenously with *C. albicans* and treated with titrated dilutions of the compound administered intraperitoneally. Five mice per group were used. After 24 h, the mice were sacrificed and their kidneys were removed, homogenized, and serial dilutions were plated. Yeast colonies were enumerated for determination of colony forming units (c.f.u.) per gram of kidneys. The details of the method have been fully described by Bartizal et al.[21]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.08.046.

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