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ARTICLE

New secondary metabolites produced by *Paraphoma radicina* FB55 as potential antifungal agents

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Abstract

Microorganisms in specific environments are rich sources of bioactive natural products as they produce compounds that can aid their survival in harsh environments. In an effort to investigate antifungal compounds produced by microorganisms, the fungal strain *Paraphoma radicia* FB55, isolated from a marine sediment of the Beaufort Sea, north of Alaska, was subjected to chemical investigation. Chromatography of the culture extracts yielded two new compounds (**1** and **2**) and eight known compounds (**3**–**10**). Their structures were determined using spectroscopic and chemical methods. Compound **1** was a new analog of the known compound (**3**) with an isobenzofuranone skeleton. The absolute configuration of the chiral center in **1** was established by comparison of its ECD and specific rotation values with those for a known analogue. Compound **2** is a polyketide-amino acid hybrid. Comprehensive Nuclear Magnetic Resonance (NMR) analysis indicated that **2** consisted of two substructures:5-methyl-6-oxo-2,4-heptadienoic acid and isoleucinol. The absolute configuration of the isoleucinol moiety in **2** was determined to be D using Marfey's method. All the isolated compounds were evaluated for antifungal activities. Although the antifungal activity of the isolated compounds was not potent, co-treatment of compounds **7** and **8** with a clinically available amphotericin B (AmB) lowered the IC_{50} values of AmB by synergism against human pathogenic yeast.

Introduction

Human health, as well as animal and plant ecosystems, have been significantly challenged by fungus infections, driving up expenses for industry, agriculture, and public health [1]. The main fungus pathogens responsible for the most serious fungal diseases include opportunistic pathogenic fungi including *Cryptococcus*, *Candida*, and *Aspergillus* species [2]. Contrary to the numerous varieties of antibacterial medications, few antifungal treatments and agricultural fungicides have been created despite the therapeutic importance of fungal infection and illnesses [3]. These restrictions are mostly caused by the difficulty in identifying specific therapeutic targets due to the common eukaryotic cellular architecture between fungi and humans, despite the fact that underestimate of mycotic diseases by the public and private sectors also plays a role. To date, only four classes of antifungal drugs have been used in clinical fields and many of them are derived from natural products [4]. For example, griseofulvin is produced by the fungus *Penicillium griseofulvum* and amphotericin B (AmB) is produced by an actinomycete *Streptomyces nodosus* [5]. Despite of the several antifungal agents, they still have various drawbacks, such as toxicity, fungistaticity, or a limited spectrum of activity, low tissue absorption, or frequent drug resistance [4]. These factors necessitate increased research efforts to create novel antifungal medications.

As an effort to discover antifungal compounds, a fungal strain, *Paraphoma radicina*, was isolated from a marine sediment of the Beaufort Sea, located north of Alaska.

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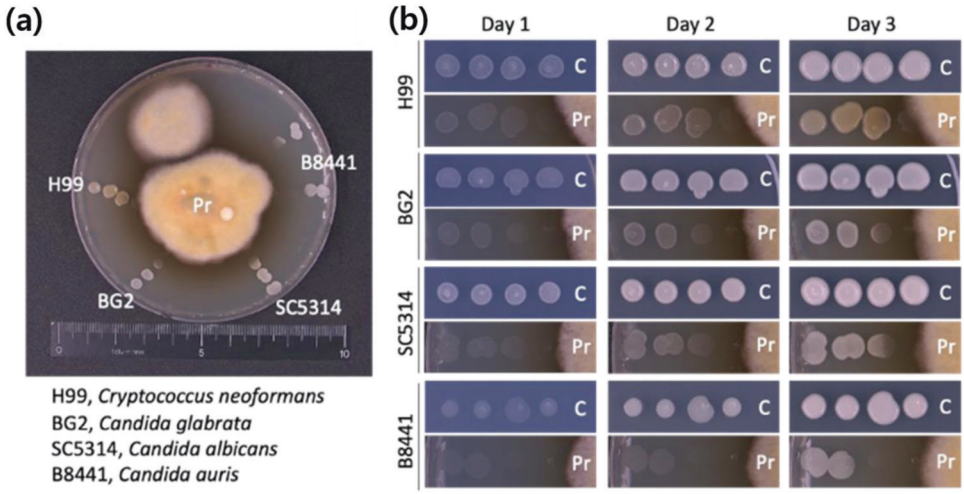

Fig. 1 Brown halo of *Paraphoma radicina* FB55 inhibited the growth of fungal pathogens. **a** Fungal competition assay between Pr (*P. radicina* FB55) and human pathogenic yeast strains. **b** Enlarged photo of the plate at the indicated culture days post yeast

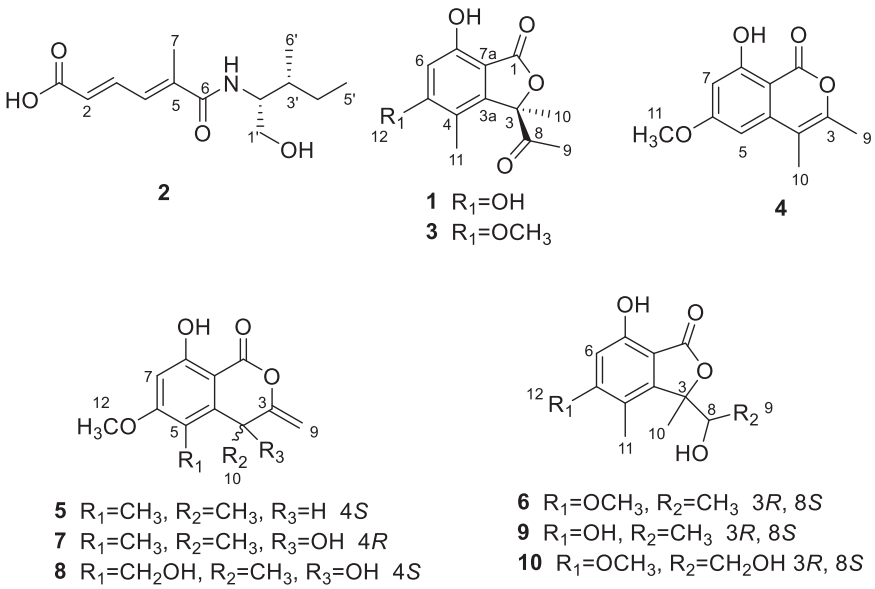

Fig. 2 Chemical structures of compounds **1**-**10** from *Paraphoma radicina*

Fungi in extreme environments are an enriched source of secondary metabolites [6, 7]. The genus *Phoma* previously included *P. radicina*; however, *P. radicina* has recently become a subcategory of the genus [8]. Polyketide-type compounds such as isochromenones, isobenzofuranone, and tetrahydronaphthalenes have been reported to be produced by this fungus [9].

In this study, we investigated the secondary metabolites produced by *P. radicina*, which afforded two new and eight known compounds. Furthermore, the inhibitory effect of these compounds on human pathogenic yeast was confirmed, and their synergistic effect with AmB was investigated. Herein, details of the isolation, structural elucidation, and determination of the absolute configuration are presented. In addition, antifungal activities of the isolated compounds against four human yeast pathogens are discussed.

Results and discussion

A competition assay was performed by inoculating *P. radicina* FB55 onto PDA medium with additional human yeast pathogens, including *Cryptococcus neoformans, Candida albicans, Candida glabrata*, and *Candida auris*. Interestingly, brown halos appeared near the colonies, and the human fungal pathogens did not grow well in the dark brown halos that were very close to the colonies (Fig. 1). Thus, we hypothesized that the substances secreted by the FB55 strain interfered with the growth of fungal pathogens, which prompted chemical investigation of this fungal strain.

The *P. radicina* FB55 was cultivated on PDA media on a large scale, which was extracted with ethyl acetate. The crude extract was subjected to a series of chromatographic methods to afford two new compounds (**1** and **2**) together with several known compounds (**3**–**10**) (Fig. 2). The known

Table 1 ^{1}H and ^{13}C NMR data for compound **1**

No.	δ_C, Type[a]	δ_H, (J in Hz)[b]
1	171.36, C	–
2	–	–
3	90.99, C	–
4	113.00, C	–
3a	150.17, C	–
5	165.74, C	–
6	104.12, CH	6.44 (s)
7	158.26, C	–
7a	103.48, C	–
8	204.46, C	–
9	23.72, CH_3	1.98 (s)
10	20.40, CH_3	1.71 (s)
11	10.04, CH_3	1.99 (s)

[a]Chemical shifts were measured at 100 MHz in CD_3OD

[b]Chemical shifts were measured at 400 MHz in CD_3OD

compounds were identified as 3-acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one (**3**) [10], polygonolide (**4**) [11], (4 *S*)-clearanol C (**5**) [12], 7-hydroxy-3-(1-hydroxyethyl)-5-methoxy-3,4-dimethyl-1(3*H*)-isobenzofuranone (**6**) [13], (4 *R*)-3,4-dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methylene-1*H*-2-benzopyran-1-one (**7**) [13], clearanol G (**8**) [5], 5,7-dihydroxy-3-(1-hydroxyethyl)-3,4-dimethylisobenzofuran-1(3*H*)-one (**9**) [14], and clearanol E (**10**) [15] by comparing their spectral data with those reported in the reference.

The molecular formula of compound **1**, $C_{12}H_{12}O_5$, (3.1 mg), was determined by QTOF-MS (*m/z* 237. 0752, $[M + H]^+$, calculated for $C_{12}H_{13}O_5$, 237.0757). The ^{1}H NMR spectral data (Table 1) of **1** show three methyl groups (at δ_H 1.71, 1.98, and 1.99) and one aromatic methine proton (at δ_H 6.44). ^{13}C (Table 1) and HSQC spectra (Fig. S4) showed presence of 12 carbons, including three methyl groups, one aromatic methine, and eight non-hydrogen-bearing sp^2 carbons (one oxygenated, two carbonyl, and five aromatics). The ^{1}H and ^{13}C NMR spectra of compound **1** were similar to those of a known compound, 3-acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one (**3**), which was also isolated in this chemical investigation. Compound **1** differed from **3** in the presence of a hydroxyl group instead of a methoxy group at C-5. The complete chemical structure of **1** was determined by interpreting its HMBC spectrum. The HMBC correlations between the aromatic methine proton (H-6) and C-4, C-5, C-7, and C-7a, and the carbonyl carbons C-1 and H_3-11 with C-4, C-3a, and C-5, suggested the presence of a 1,5-dioxygenated-4-methyl benzene moiety (Fig. 3). Further HMBC correlations between H_3-10 and the oxygenated quaternary carbon (C-3), C-3a, C-8, and H_3-9 with C-3 and C-8 indicated that the

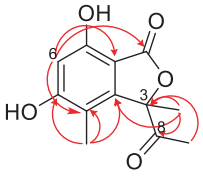

Fig. 3 Key HMBC correlations observed in compound **1**

oxygenated quaternary carbon, which bears both acetyl and methyl groups, is connected to C-3a of the benzene ring. Based on these spectral data, compound **1** has an isobenzofuranone skeleton and a hydroxyl group at C-5 instead of a methoxy group, compared to compound **3**. A similar ultraviolet (UV) spectrum (Fig. S8) to that of **3** and the molecular formula obtained by (+) HR-ESI-MS also supported the structure of **1**. Therefore, the structure of compound **1** was established to be 3-acetyl-5,7-dihydroxy-3,4-dimethylisobenzofuran-1(3*H*)-one. Compound **1** had a chiral center at C-3. The absolute configuration of **1** was established by comparing its optical rotation value and ECD spectrum with those of **3** (Fig. S7). Compound **1** had a positive specific rotation and further showed a positive Cotton effect at 291 nm and 241 nm, and a negative Cotton effect at 220 nm, which were quite similar to those of compound **3**. Therefore, the absolute configuration of C-3 was established as *R* by comparing the values in the literature [13, 16].

The molecular formula of compound **2** was established as $C_{13}H_{21}NO_4$ based on (+) HR-ESI-MS (*m/z* 256.1537 $[M + H]^+$, calcd. for $C_{13}H_{22}NO_4$, 256.1543). The ^{1}H and ^{13}C NMR spectra (Table 2) together with HSQC (Fig. S12) revealed the presence of three methyl groups, three olefinic methines [δ_H 7.59 (1H, dd, $J = 15.0$, 11.5 Hz), 6.84 (1H, d, $J = 11.5$ Hz), and 6.12 (1H, d, $J = 15.0$ Hz)], two sp^3 methines [δ_H 3.98 (1H, m) and 1.72 (1H, m)], two sp^3 methylenes [δ_H 3.62 (2H, m), 1.51 (1H, m), and 1.20 (1H, m)], and two carbonyl groups (δ_C 172.20 and 170.81). By interpretation of ^{1}H-^{1}H COSY, two partial structures were obtained: (1) CH=CH-CH= corresponding to C2-C3-C4 and (2) CH_2(O)-CH-CH(CH_3)-CH_2-CH_3 corresponding to C1′-C2′-C3′(C6′)-C4′-C5′. Both partial structures were connected by the interpretation of the HMBC correlations. The olefinic protons H-2 (δ_H 6.12) and H-3 (δ_H 7.59) of the first spin system showed HMBC correlations with the carboxylic carbon at δ_C 170.81, indicating the presence of a terminal carboxylic acid. HMBC correlations of the methyl protons at δ_H 2.08 with C-4, C-5, and the carbonyl carbon at δ_C 172.20 indicated that the methyl group was attached to C-5, which was further connected to the carbonyl carbon. Thus, the presence of a methyl hexadienoic acid moiety was confirmed. H-2′ (at δ_H 3.98) in the second partial structure showed a strong HMBC correlation with a carbonyl carbon next to C-5, which allowed the connection of C-2′ with the carbonyl carbon C-6 (δ_C 172.20). Considering the

Table 2 ^{1}H and ^{13}C NMR data for compound **2**

No	δ_C, Type[a]	δ_H, (J in Hz)[b]
1	170.81, C	–
2	127.82, CH	6.12 (d, 15.0)
3	139.90, CH	7.59 (dd, 15.0, 11.5)
4	130.82, CH	6.84 (d, 11.5)
5	142.04, C	–
6	172.20, C	–
7	14.05, CH_3	2.08 (3H, s)
1'	63.32, CH_2	3.62 (2H, m)
2'	56.61, CH	3.98 (m)
3'	36.91, CH	1.72 (m)
4'	27.62, CH_2	1.20 (m)
		1.51 (m)
5'	11.94, CH_3	0.95 (3H, t, 7.5)
6'	15.30, CH_3	0.92 (3H, d, 6.8)

[a]Chemical shifts were measured at 150 MHz in CD_3OD

[b]Chemical shifts were measured at 600 MHz in CD_3OD

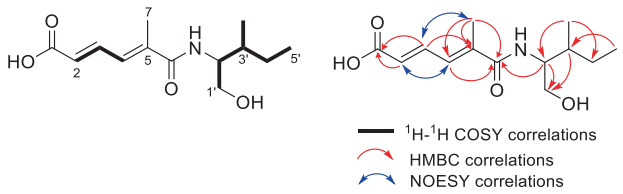

Fig. 4 The ^{1}H-^{1}H COSY, Key HMBC, and NOESY correlations of compound **2**

molecular formula obtained by HR-ESI-MS together with the downfield-shifted H-2′, the second partial structure appeared to be connected to the first by an amide bond. The HMBC correlation of H-1′ with C-2′ and C-3′, of H-2′ with C-1′, C-3′, C-4′, and C-6′, and of H_3-6′ with C-2′, C-3′, and C-4′ together with COSY correlations established the structure of isoleucinol. Thus, an isoleucinol moiety (partial structure 2) was connected to the methyl-hexadienoic acid moiety via an amide bond to form compound **2**.

The geometry of the two olefinic groups was established using coupling constants and NOESY correlations (Fig. S18). The configuration between C-2 and C-3 was classed as *E* based on the coupling constants ($J = 15.0$ Hz), and the strong NOESY correlations between H-2 and H-4 and between H-3 and H_3-7 suggested *trans* configuration at C-4 and C-5 (Fig. 4). The absolute configurations of the chiral centers in **2** were determined using Marfey's method because of the isoleucinol moiety with two chiral centers. Compound **2** was hydrolyzed to obtain an amino alcohol moiety, which was further added to 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (FDLA) for derivatization via HPLC-MS analysis. A commercially available standard of

Table 3 Antifungal activity of the compounds against human fungal pathogens

Compound	*C. albicans*		*C. glabrata*		*C. auris*		*C. neoformans*	
	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}
1	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	64	>128
4	–	–	–	–	–	–	128	>128
5	–	–	–	–	–	–	128	>128
6	–	–	–	–	–	–	–	–
7	128	>128	128	>128	64	>128	64	>128
8	–	–	–	–	–	–	>128	>128
9	–	–	–	–	–	–	64	>128
10	–	–	–	–	–	–	–	–

IC_{20} and IC_{50} refer to 20% and 50% inhibitory concentrations (μg/mL) by MIC assay

L-isoleucinol was derivatized with FDLA to enable direct comparison of their retention times [17, 18]. When analyzed by LC-MS using selective ion monitoring, the molecular ion at *m/z* 412 $[M + H]^+$ for isoleucinol-FDLA was detected in the positive ion mode. L-Isoleucinol-L-FDLA was eluted at 21 min by HPLC, while L-FDLA derivative of the hydrolysate of **2** was eluted at 26 min. Therefore, racemization of the isoleucinol standard was conducted to obtain both L-isoleucinol-L-FDLA and D-isoleucinol-L-FDLA. The retention times for the FDLA derivatives of the racemized product were 21 and 26 min, respectively. Consequently, the isoleucinol in **2** was established as D form [19].

Fungal growth inhibition of the compounds isolated from cultures of *P. radicina* FB55 was evaluated using MIC assay (Table 3). The activity of compound **2** could not be evaluated due to the limited amount of sample. Compound **7** inhibited the growth of human pathogenic yeasts *C. albicans*, *C. glabrata*, *C. auris*, and *C. neoformans*. The IC_{20} was 128 μg ml^{-1} for *C. albicans* and *C. glabrata*, and the IC_{20} was 64 μg ml^{-1} for *C. auris* and *C. neoformans*. Interestingly, compounds **3, 4, 5, 8**, and **9** showed growth inhibition activity only in *C. neoformans*. Thus a checkerboard assay was conducted against *C. neoformans*, an obviously pathogenic yeast. By comparing its interaction with amphotericin B (AmB), one of the most effective fungicides against *C. neoformans*, the synergistic effects of each compound were investigated as a potential strategy for combination therapy. Interestingly, compound **7** lowered the IC_{50} value of AmB from 0.5 μg ml^{-1} to 0.125 μg ml^{-1}, and the compound **8** lowered it to 0.25 μg ml^{-1} (Fig. 5a). In contrast, compounds **5** and **9** did not interact with AmB, despite their moderate growth inhibition capacity (Fig. 5b). Furthermore, compounds **3, 4**, and **6** exhibited antagonism with increased IC_{50} values of AmB from 0.5 μg ml^{-1} to 1.0 μg ml^{-1} (Fig. 5c). Compounds **1** and **10** did not interact

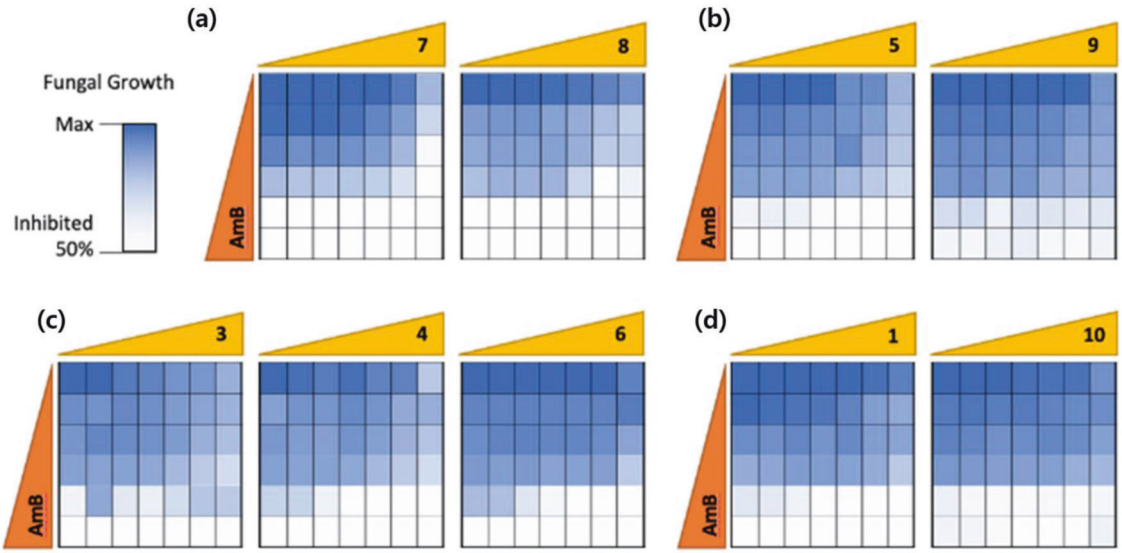

Fig. 5 Checkerboard assay of each compound's synergism with AmB. AmB was diluted 2-fold from 1 µg/mL to 0.03125 µg/mL and each compound was 2-fold diluted from 128 µg/mL to 2 µg/mL. **a**–**d** AmB synergism was confirmed with compounds **7** and **8** (**a** synergistic), compounds **5** and **9** (**b** no interaction), compounds **3, 4**, and **6** (**c** antagonistic), and compounds **1** and **10** (**d** no interaction)

with AmB (Fig. 5d). In summary, the compounds isolated from *P. radicina* FB55 moderately inhibited the growth of human fungal pathogens by single treatment or synergistic effects with AmB and could be optimized to reduce the use of existing antifungal drugs.

Conclusion

Two new compounds (**1** and **2**) and eight known compounds (**3**–**10**) were isolated from *P. radicina* FB55. Nine isolated compounds have a benzopyranone or benzofuranone skeleton, which has often been reported in *Paraphoma* sp. The absolute configuration of compound **1** was established by comparing the optical rotations and ECD with reported values, and the absolute configuration of **2** was determined by Marfey's method. Among these compounds, compounds **3**–**5** and **7**–**9** showed antifungal activity only against *C. neoformans* while compound **7** showed activity against *C. albicans*, *C. glabrata*, *C. auris*, and *C. neoformans*. Although the antifungal activity of the isolated compounds was not potent, co-treatment with compounds **7** and **8** and AmB lowered the IC_{50} values of AmB by synergism against human pathogenic yeast.

Experimental section

General experimental procedures

Optical rotation (OR) values were measured on a JASCO P-2000 polarimeter (JASCO, Easton, PA, USA) at 20 °C with a 10-mm cell. Ultraviolet (UV) absorption spectra were obtained using a UV-VIS spectrometer (Thermo, Orion AquaMate 8100). Infrared spectroscopy (IR) spectra were obtained with a FT-IR/4200 (JASCO Co., Tokyo, Japan). High-resolution electrospray ionization mass spectrometry (HRESIMS) data were recorded using a Q-TOF 6530 mass spectrometer (MS) (Agilent, 1290 Infinity). ^{1}H (400 and 600 MHz) and ^{13}C (100 and 150 MHz) nuclear magnetic resonance (NMR) spectra were obtained using JEOL JNM-ECA-400 and JEOL JNM-ECA-600 spectrometers with deuterated methanol (CD_3OD; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). ECD spectra were recorded on a Chirascan-plus spectrometer in a 2 mm cell (Applied Photophysics Ltd., Leatherhead, England).

Fungal materials

Fungal strain FB55 was isolated from marine sediment collected from the Beaufort Sea in North Alaska. Identification of the fungal strain was achieved by analyzing the rRNA internal transcribed spacer sequences using Macrogen (Seoul, Korea). Sequencing data were identical to those of *Paraphoma radicina* (accession no. LT796835.1). Human pathogenic yeast strains were cultured and maintained in a yeast extract peptone dextrose medium. *Cryptococcus neoformans* H99 (ATCC), *Candida albicans* SC5314 (ATCC), *Candida auris* B8441, and *Candida glabrata* BG2 were used for the fungal competition, minimal inhibitory concentration (MIC), and checkerboard assays [20, 21].

Fungal competition assay

For the fungal competition assay, *P. radicina* FB55 was inoculated on potato dextrose agar (PDA) plates. After

12 days of incubation at room temperature, yeast cells were identified near the *P. radicina* colonies and further incubated.

Cultivation and extraction of the fungal strain

P. radicina was cultured on potato dextrose agar (PDA) at room temperature for 14 days. After 14 days, agar plugs were cut into small pieces and inoculated onto the PDA medium. PDA medium was prepared with 12 g potato dextrose broth, 11.5 g agar, and 500 mL distilled water per 2800 ml PYREX flask. Ten flasks were prepared and incubated at 28 °C for 21 d. The cultured PDA medium was immersed in 1000 ml of ethyl acetate for 1 d, and the extracts were collected three times. The extracts were evaporated under reduced pressure at 30 °C to obtain 1.2 g of the total extract.

Isolation of compounds

The crude extract of the PDA medium (1.2 g) was fractionated by medium-pressure liquid chromatography (MPLC) with silica gel using a gradient solvent system of *n*-Hexane/$CHCl_3$/CH_3OH (*n*-Hexane: $CHCl_3$ from 10:0 to 0:10; $CHCl_3$: CH_3OH from 10:0 to 0:10) at a flow rate of 50 ml min^{-1} to obtain fractions A-K. Fraction B (110 mg) was separated by reverse-phase high-performance liquid chromatography (HPLC, Phenomenex Luna Phenyl-Hexyl column, 5 µm, 250 × 10.0 mm; 20 °C; Flow rate, 2.0 ml min^{-1}) by elution with a gradient solvent system of 42:58 to 85:15 of CH_3CN-H_2O (acidified with 0.1% TFA) over 30 min to gain compound **3** [3.5 mg, Retention time (R_t) = 18 min], compound **4** (1.0 mg, R_t = 27 min), and compound **5** (1.2 mg, R_t = 30 min). Fraction C (270 mg) was subjected to reverse-phase HPLC (J'sphere ODS-H80 C18, 4 µm, 250 × 10.0 mm; 20 °C; flow rate, 2.0 ml min^{-1}) using a gradient of aqueous CH_3CN from 40% to 70% over 40 min to yield compounds **6** (4.5 mg, R_t = 9 min) and **7** (5 mg, R_t = 30 min). Fraction D (81 mg) was separated by reverse-phase HPLC by elution of an isocratic system of 35% aqueous CH_3CN to yield subfractions D1 and D2. D1 and D2 were purified by reverse-phase high-performance liquid chromatography (HPLC) using an isocratic solvent system of 50% aqueous MeOH. Subfractions D1 and D2 produced compounds **1** (3.1 mg, R_t = 15 min) and **8** (1.9 mg, R_t = 18 min), respectively by semi-preparative HPLC (J'sphere ODS-H80 C18, 4 µm, 250 × 10.0 mm; 20 °C; flow rate, 2.1 ml min^{-1}; 35% aqueous acetonitrile for D1 and 50% aqueous methanol for D2]. Fraction F (170 mg) was subjected to reverse-phase HPLC (J'sphere ODS-H80 C18, 4 µm, 250 × 10.0 mm; 20 °C; flow rate, 2.1 ml min^{-1}, 35% aqueous methanol) to yield compounds **9** (10.1 mg, R_t = 22 min) and **10** (1.7 mg, R_t = 31 min). Fraction I (45 mg) was separated by reverse-phase HPLC (Luna Phenyl Hexyl, 5 µm, 250 × 10.0 mm; 20 °C; flow rate, 2.0 ml min^{-1}) using a gradient solvent system of 42 to 70% CH_3CN in water over 45 min to obtain compound **2** (1.0 mg, R_t = 30 min).

3-Acetyl-5,7-dihydroxy-3,4-dimethylisobenzofuran-1(3*H*)-one (**1**): white solid; $[\alpha]_D^{20} = +235.5$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) = 216 (4.26), 262 (3.90), 302 (3.64) nm; IR ν_{max} (ATR) 3224, 2929, 1722, 1614, 1252 cm^{-1}; ECD (5.30×10^{-4} M, MeOH) λ_{max} (Δε) 291 (+6.41), 241 (+6.32), 220 (−12.77) nm; for ^1H and ^{13}C NMR data (400 and 100 MHz in CD_3OD), see Table 1; HMBCs (CD_3OD, H-# → C-#) H-6 → C-1, C-4, C-5, C-7, and C-7a; H-9 → C-3 and C-8; H-10 → C-3, C-4a, and C-8; H-11 → C-4, C-4a, and C-5; (+)HRESIMS *m/z* 237.0752 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$, 237.0757).

(2*E*,4*E*)-6-((1-Hydroxy-3-methylpentan-2-yl)amino)-5-methyl-6-oxohexa-2,4-dienoic acid (**2**): colorless solid; $[\alpha]_D^{25} = +11.45$ (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) = 202 (3.62), 270 (3.97) nm; IR ν_{max} (ATR) 3351, 2923, 1688, 1618, 1399, 1208, 1139 cm^{-1}; ECD (4.90×10^{-4}M, MeOH) λ_{max} (Δε) 204 (−0.70) nm; for ^1H and ^{13}C NMR data (600 and 150 MHz in CD_3OD), see Table 2; HMBCs (CD_3OD, H-# → C-#) H-2 → C-1 and C-4; H-3 → C-1, C-4, and C-5; H-4 → C-2, C-3, C-6, and C-7; H-7 → C-4, C-5, and C-6; H-1′ → C-2′ and C-3′; H-2′ → C-6, C-1′, C-3′, C-4′, and C-6′; H-3′ → C-1′, C-2′, C-4′, and C-6′; H-4′ → C-1′, C-2′, C-3′, and C-5′; H-5′ → C-3′ and C-4′; H-6′ → C-2′, C-3′, and C-4′; (+)HR-ESI-MS *m/z* 256.1537 $[M + H]^+$ (calcd for $C_{13}H_{22}NO_4$, 256.1543).

Marfey's method

(Hydrolysis) Compound 2 (0.4 mg) was hydrolyzed with 6 N HCl (0.8 ml) at 120 °C for 3 h under stirring. The solution was then dried by evaporation under reduced pressure. The residue was dissolved in 0.2 mL of water with 80 µl of 1 M $NaHCO_3$ and 1% 1-fluoro-2,4-dinitrophenyl-5-D, L-leucine-amide (400 µl) in acetone. After the solution was incubated at 37 °C for 1 h, the sample was added to 80 µl of 1 N HCl. The sample was diluted with 810 µl of methanol and filtered before HPLC analysis. The 1-fluoro-2,4-dinitrophenyl-5-leucine amide (FDLA) derivatives were analyzed by LC-MS using a gradient of CH_3CN in H_2O from 20 to 70% over 30 min (Column; Phenomenox Luna C18 100 A, 3.0 × 100 mm, 3.0 µm, room temperature).

(Preparation of L-FDLA derivatives of isoleucinol standard) L-Isoleucinol (50 mM) was dissolved in 50 µl water, 80 µl of 1 M $NaHCO_3$ and 400 µl of 1% L-FDLA in acetone. After treatment at 37 °C for 1 h, the sample was neutralized with 80 µl of 1 N HCl. The sample was diluted with 810 µl of methanol and filtered before HPLC analysis.

(Racemization) L-Isoleucinol (6 mg) was dissolved in glacial acetic acid (180 µl) containing 5 µl of salicylaldehyde and heated at 100 °C for 1 h under stirring. The solution was then evaporated under reduced pressure.

Minimum inhibitory concentration (MIC) assay

The MIC assay was performed according to the EUCAST guidelines. The optical density of the PBS buffered cell suspension was synchronized to 1.0 at 600 nm. The cells were inoculated into fresh RPMI medium at 1/100 volume of the final required volume. Samples were distributed in flat-bottomed 96-well plates. The drugs were dissolved in DMSO, and the stock concentration was 5.12 mg ml^{-1}. The final DMSO concentration for inoculation was 2.5% and the highest drug concentration was 128 μg ml^{-1} in 100 μl of the cell suspension. The plates were cultured for 48 h in a 35 °C incubator and the absorbance was read with a microplate reader (Epoch2, BioTek) at 600 nm.

Checkerboard assay

Two drugs were sequentially diluted in RPMI medium containing *C. neoformans* cells and measured in accordance with the MIC method as previously described [22]. For the *X*-axis of the 96-well plate, AmB was diluted 2-fold from 1 μg ml^{-1} to 0.03125 μg ml^{-1}. For the *Y*-axis of the 96-well plate, each compound was diluted 2-fold from 128 μ to 2 μg ml^{-1}. The plates were then incubated for 48 h at 35 °C.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

References

1. Bebber DP, Gurr SJ. Crop-destroying fungal and oomycete pathogens challenge food security. Fungal Genet Biol. 2015;74:62–4.
2. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. Sci Transl Med. 2012;4:165rv13.
3. Perfect JR. The antifungal pipeline: a reality check. Nat Rev Drug Discov. 2017;16:603–16.
4. Mesa-Arango AC, Scorzoni L, Zaragoza O. It only takes one to do many jobs: amphotericin B as antifungal and immunomodulatory drug. Front. Microbiol. 2012;3:286.
5. Barrett D. From natural products to clinically useful antifungals. Biochim Biophys Acta Mol Basis Dis. 2002;1587:224–33.
6. Liu J, Liu G. Analysis of secondary metabolites from plant endophytic fungi. Methods Mol Biol. 2018;1848:25–38.
7. Coleine C, Stajich JE, Selbmann L. Fungi are key players in extreme ecosystems. Trends Ecol Evol. 2022;37:517–28.
8. Aveskamp MM, de Gruyter J, Woudenberg JHC, Verkley GJM, Crous PW. Highlights of the didymellaceae: a polyphasic approach to characterise *Phoma* and related pleosporalean genera. Stud Mycol. 2010;65:1–60.
9. El-Elimat T, Raja HA, Figueroa M, Falkinham JO, Oberlies NH. Isochromenones, isobenzofuranone, and tetrahydronaphthalenes produced by *Paraphoma radicina*, a fungus isolated from a freshwater habitat. Phytochemistry. 2014;104:114–20.
10. Chinworrungsee M, Kittakoo P, Isaka M, Chanphen R, Tanticharoen M, Thebtaranonth Y. Halorosellins A and B, unique isocoumarin glucosides from the marine fungus *Halorosellinia oceanica*. J Chem Soc Perkin Trans 1. 2002:2473–6.
11. Furuta T, Fukuyama Y, Asakawa Y. Polygonolide, an isocoumarin from *Polygonum hydropiper* possessing anti-inflammatory activity. Phytochemistry. 1986;25:517–20.
12. Tayone WC, Kanamaru S, Honma M, Tanaka K, Nehira T, Hashimoto M. Absolute stereochemistry of novel isochromanone derivatives from *Leptosphaeria* sp. KTC 727. Biosci Biotechnol Biochem. 2011;75:2390–3.
13. Tayone WC, Honma M, Kanamaru S, Noguchi S, Tanaka K, Nehira T, Hashimoto M. Stereochemical investigations of isochromenones and isobenzofuranones isolated from *Leptosphaeria* sp. KTC 727. J Nat Prod. 2011;74:425–9.
14. Li LY, Sun BD, Zhang GS, Deng H, Wang MH, Tan XM, Zhang XY, Jia HM, Zhang KW, Zhang T, Zou ZM, Ding G. Polyketides with different post-modifications from desert endophytic fungus *Paraphoma* sp. Nat Prod Res. 2018;32:939–43.
15. Gerea AL, Branscum KM, King JB, You J, Powell DR, Miller AN, Spear JR, Cichewicz RH. Secondary metabolites produced by fungi derived from a microbial mat encountered in an iron-rich natural spring. Tetrahedron Lett. 2012;53:4202–5.
16. Luo X, Lin X, Salendra L, Pang X, Dai Y, Yang B, Liu J, Wang J, Zhou X, Liu Y. Isobenzofuranones and isochromenones from the deep-sea derived fungus *Leptosphaeria* sp. SCSIO 41005. Mar Drugs. 2017;15:204.
17. Fujii K, Ikai Y, Oka H, Suzuki M, Harada K. Nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: combination of Marfey's method with mass spectrometry and its practical application. Anal Chem. 1997;69:5146–51.
18. Vijayasarathy S, Prasad P, Fremlin LJ, Ratnayake R, Salim AA, Capon RJ. C_3 and 2D C_3 Marfey's methods for amino acid analysis in natural products. J Nat Prod. 2016;79:421–7.
19. Yamada S, Hongo C, Yoshioka R, Chibata I. Method for the racemization of optically active amino acids. J Org Chem. 1983;48:843–6.
20. Kim JS, Lee KT, Lee MH, Cheong E, Bahn YS. Adenylyl cyclase and protein kinase A play redundant and distinct roles in growth, differentiation, antifungal drug resistance, and pathogenicity of *Candida auris*. mBio. 2021;12:e0272921.
21. Cormack BP, Falkow S. Preservation of duplicate genes by complementary, degenerative mutations. Genetics. 1999;151:979–87.
22. Lee Y, Lee KT, Lee SJ, Beom JY, Hwangbo A, Jung JA, Song MC, Yoo YJ, Kang SH, Averette AF, Heitman J, Yoon YJ, Cheong E, Bahn YS. In vitro and in vivo assessment of FK506 analogs as novel antifungal drug candidates. Antimicrob Agents Chemother. 2018;62:e01627–18.