

New Antifungal Compounds from *Aspergillus terreus* Isolated from Desert Soil

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Two new butyrolactone I derivatives: 3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-5-(–4-hydroxybenzyl)-4-methyl-dihydrofuran-2(3H)-one (1) and (Z)-3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-5-(–4-hydroxybenzylidene)-4-methyl-dihydrofuran-2(3H)-one (2), in addition to the previously described (S)-methyl-4-hydroxy-2-[4-hydroxy-3-(3-methyl-but-2-enyl)-benzyl]-3-(4-hydroxy-phenyl)-5-oxo-2,5-dihydro-furan-2-carboxylic acid methyl ester (3), were identified from a strain of *Aspergillus terreus* Thom (Trichocomaceae) isolated from desert soil. The antifungal activities of both intra- and extracellular metabolites of *A. terreus* grown on yeast extract sucrose and malt extract media were determined. Only the secondary metabolites of *A. terreus* grown on yeast extract sucrose medium were active against *Aspergillus fumigatus* RCMB 002008. The antifungal activity of *A. terreus* was attributed to the presence of the compounds (1) and (2), whose minimum inhibitory concentrations (MIC) against *A. fumigatus* were found to be 32.00 and 16.00 µg/mL respectively. Structure elucidation was carried out using UV spectrometry, electrospray ionization mass spectrometry (ESIMS), high resolution electron impact (HREIMS) spectrometry, ¹H- and ¹³C-NMR, proton–proton correlation spectroscopy (¹H–¹H Cosy), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlations (HMBC) spectroscopy. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: *Aspergillus terreus* Thom; *Aspergillus fumigatus* Fresen; trichocomaceae; antifungal activity; butyrolactone I.

INTRODUCTION

Many fungal metabolites display a broad range of useful antibiotic, antiviral, antitumour, antihypercholesterolaemic and immunosuppressant properties, and the production of fungal secondary metabolites, frequently associated with sporulation processes, has attracted interest for their biotechnological and pharmaceutical applications (Pieckova and Kunova, 2002; Rossano *et al.*, 1999). Hawksworth's studies of fungal biodiversity led to the conclusion that nearly 1.5 million fungal species exist on Earth, with only 5% identified thus far (Fisher *et al.*, 1999). Thus, the potential for fungal secondary metabolite discovery is vast. Furthermore, the discovery of global regulators for fungal secondary metabolite production would allow for manipulation of secondary metabolite production.

Aspergillus terreus Thom (Trichocomaceae), like many filamentous fungi, undergoes morphological and physiological changes when the abundance and complexity of nutrient sources are altered. Under conditions of nutrient limitation, such as deprivation of easily assimilated carbon, nitrogen, or phosphorus sources, the organism enters a period of slower growth, morphological alterations and changes in metabolism known as secondary metabolism (Calvo *et al.*, 2002). Secondary metabolism is widely variable and may or may not have a readily apparent biological function. A variety of clinically beneficial secondary metabolites are produced by fungi, such as the β-lactam antibiotics penicillin and

cephalosporin, the antifungal antibiotic griseofulvin and the ergot alkaloids (Vinokurova *et al.*, 2003).

Aspergillus terreus is an especially prolific producer of secondary metabolites. Compounds produced by *A. terreus* include aspulvinone, asteric acid (Calvo *et al.*, 2002), asterriquinone (Cueto *et al.*, 2002), butyrolactone I (Pieckova and Kunova, 2002), citrinin (Manzoni and Rollini, 2002), emodin (Fisher *et al.*, 1999), geodin (Fisher *et al.*, 1999), itaconate (Rao *et al.*, 2000), lovastatin (Birch, 1997), questrin and terrecyclic acid (Shiao and Don, 1987; Cueto *et al.*, 2002). This study was carried out to investigate the impact of growth media on the production of fungal secondary metabolites both intracellularly (i.e. retained in the fungal mat) and extracellularly (i.e. excreted into the broth) and their biological activity.

MATERIALS AND METHODS

Fungal isolation and growth conditions

The fungal strain was isolated from desert soil samples collected in clean plastic bags, at a depth of 5–20 cm from different sites, including wadi-beds, terraces and upstream, of various habitats, including rocky, clay and sandy soils. Malt extract agar (MEA; malt extract, 20 g; peptone, 1 g; dextrose, 20 g; agar, 20 g; distilled water, 1 L) and yeast extract sucrose agar (YES; yeast extract, 20 g; sucrose, 150 g; agar, 20 g; distilled water, 1 L) media were used for isolation, cultivation and identification of the fungal isolate. The same media (without agar) were used for production and determination of secondary metabolites. Each medium was prepared by dissolving

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the solid ingredients in 1 L of cold distilled water, and then heating to 60–70 °C with stirring. Media were sterilized by autoclaving at 121 °C for 15 min and, when cool, poured into sterilized 9 mm diameter Petri dishes. For maintenance of stock cultures, 10 molten agar were poured into slants, autoclaved, and then tilted to provide slopes for stock culture. *Aspergillus terreus* was cultured on yeast extract sucrose and malt extract media for 10 days at 28 ± 2 °C. The fungal isolate was identified by Professor Mohamed E. Zain, Professor of Fungal taxonomy, College of Science, King Saud University, KSA as *Aspergillus terreus* according to Travis *et al.* (2002) and confirmed at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

Antifungal screening

In vitro antifungal screening was carried out by the disc diffusion method (Dash *et al.*, 2005). Here, 20 mL of MEA were plated in a petri dish with 0.2 mL of a 10^{-2} dilution of each fungal culture. Filter paper discs (13 mm in diameter), soaked in ether, chloroform, ethyl acetate and butanol extracts of *A. terreus* grown on MEA and YES media and left to dryness from solvent, were placed on test organism-seeded plates. The test organisms *Candida albicans* RCMB 005002, *Saccharomyces cerevisiae* RCMB 006001, *Aspergillus flavus* RCMB 002002, *Aspergillus fumigatus* RCMB 002008, *Aspergillus niger* RCMB 002017 and *Penicillium italicum* RCMB 001018 were obtained from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The activity was determined after 72 h of incubation at 30 °C. The diameter of the zone of inhibition produced by the extract was then compared with the standard antibiotic amphotericin B, 30 µg/disc. Each sample was used in triplicate for the determination of antifungal activity.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MICs) of all extracts and isolated compounds has been determined by microdilution techniques as described by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2002). All extracts (ether, chloroform, ethyl acetate, butanol) and isolated compounds were prepared as 1.024 mg/mL stock solutions, in sterile distilled water. The prepared stock solution was sequentially diluted with growth medium (4 mL added to 16 mL medium) to give a concentration of 204.8 µg/mL, (2 mL added to 18 mL medium) 102.4 µg/mL, (1 mL added to 19 mL medium) 51.2 µg/mL, (1 mL added to 29 mL medium) 34.13 µg/mL, (1 mL added to 39 mL medium) 25.6 µg/mL, and so on to concentration of 20.48 µg/mL, 17.06 µg/mL and 14.62 µg/mL.

Stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar (Difco) following NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2008). Stock suspensions were adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (80–82% transmittance)

and contained conidia or sporangiospores and hyphal fragments. The diluted (two times) inoculum sizes ranged from 0.9×10^4 to 4.7×10^4 cfu/mL, as demonstrated by a quantitative colony count on Sabouraud dextrose agar. A total of 0.5 mL of the suspension was added to each extract dilution and incubated at 35 °C for 3 days. The MIC was defined as the lowest drug concentration that resulted in complete inhibition.

Extraction

The mat of *A. terreus*, grown on YES (700 g), was extracted by percolation in methanol (4 L) at room temperature for 2 days and filtered. The residue was re-percolated and the process repeated four times. The combined methanol extracts were concentrated under reduced pressure at a temperature not exceeding 35 °C to yield a dry extract (120 g). The broth was extracted by partitioning with *n*-butanol saturated with water and the butanol extract concentrated in the same way to yield 150 g. The intracellular (i.e. from the mat) and extracellular (i.e. from the broth) extracts were dissolved in water (400 mL) and successively extracted with diethyl ether, chloroform, ethyl acetate and *n*-butanol respectively. All extracts were dried over anhydrous sodium sulphate and concentrated under reduced pressure at a temperature not exceeding 35 °C to yield dry extracts (5.5, 6, 18.5 and 40 g for intracellular) and (6.5, 4.5, 11.5 and 50 g for extracellular). All extracts were tested for their antifungal activity.

Isolation of active compounds

The ether and chloroform extracts for intra- and extracellular growth of *A. terreus* on both media (MEA and YES) were found to be identical (in number and colours of spots) when chromatographically investigated (chloroform:methanol 95:5) and visualized under UV light. Both were active against *A. fumigatus*, so were combined (20 g from both intra- and extracellular) and further fractionated using a silica gel (450 g) column and eluted gradually with ether–chloroform–methanol. One hundred fractions were collected (60 mL each) and those that were chromatographically similar were combined to produce four main fractions. These were concentrated under reduced pressure to yield 2.13, 3.41, 2.6 and 3.21 g, respectively. The ethyl acetate and butanol fractions were also found to be identical chromatographically, but were inactive and therefore discarded.

Instruments

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected; the mass spectrometry (electrospray negative ion) sample was dissolved in acetonitrile for use in a Micromass Quattro spectrometer. ^1H - and ^{13}C -NMR spectra, using external electronic referencing through the deuterium resonance frequency of the solvent, were determined at 600.17 or 150.91 MHz respectively with a JEOL ECA 600 spectrometer fitted with an automatic 5 mm X/H probe. Carbon atom types were established in the ^{13}C -NMR spectrum by using a

combination of broad- and proton-decoupled and distortionless enhancement by polarization transfer (DEPT) experiments with 64000 data points over a spectrum width of 17605.6 Hz. [$^1\text{J}_{\text{C-H}}$, $^2\text{J}_{\text{C-H}}$ and $^3\text{J}_{\text{C-H}}$]. ^1H - ^{13}C correlations were established by using HMQC and HMBC pulse sequences respectively. ^1H - ^1H correlations were by double quantum filtered COSY.

RESULTS AND DISCUSSION

A vast number of fungi have been utilized for the biotransformation process and many more remain to be exploited for isolation of potential compounds. According to Demain (1999) fungi are well known to show antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free-radical scavenging activities. Findings of the present investigation revealed the inhibitory action of fungal secondary metabolites in both crude and purified forms tested against the fungus *A. fumigatus*. Of the three isolated compounds, only compounds (**1**) and (**2**) were active against *A. fumigatus*, with MICs of 34.13 and 17.06 $\mu\text{g/mL}$, respectively (see Table 2). The antifungal activity of intra- and extracellular extracts of *A. terreus* cultured on the two types of media against some unicellular and filamentous fungi are shown in Table 1. Extracts from *A. terreus* cultures grown on yeast extract sucrose medium showed remarkable inhibition (Table 1) of the growth of *A. fumigatus* (38 and 36 mm inhibition zone for intra- and extracellular extracts respectively). For isolation of the active compound(s), 2 L of yeast extract sucrose medium (in 10 flasks; 500 mL) were inoculated with *A. terreus* and incubated at 28 °C for 10 days. Both the fungal mat and the growth medium were collected and subjected to successive extraction using ether, chloroform, ethyl acetate and *n*-butanol solvents. Because the antifungal activity of *A. terreus* extract was the highest on *A. fumigatus*, all extracts were tested on this microorganisms. The ether and chloroform extracts showed the most activity against *A. fumigatus* (MICs 102.40 and 204.80 $\mu\text{g/mL}$, respectively; Table 2). The chloroform extract was more active than ether, and had a higher content of compounds **1–3** as shown chromatographically by the intensity of spots under UV light. The ether and chloroform extracts were rechromatographed on a silica gel column and eluted with ether:chloroform (9:1), from which compounds

1–3 (Fig. 1) were isolated. The first new compound was identified as 3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-5-(–4-hydroxybenzyl)-4-methyl-dihydrofuran-2(3H)-one (**1**). It was obtained as white crystals from methanol, m/z 366.12, mp. 110–111 °C, UV (MeOH) λ_{max} (log ϵ) 245 (5.13), 285 (4.22), 310 (0.46) nm; IR (KBr) ν_{max} 3400, 2850, 1750, 1701, 1640, 1560. 1100 cm^{-1} . ^{13}C -NMR (CD_3OD); 14.80 (C-12), 17.81 (C-24), 25.64 (C-23), 27.24 (C-20), 37.38 (C-13), 44.87 (C-4), 57.74 (C-3), 86.49 (C-5), 113.13 (C-7), 116.13 (C-16 and C-18), 119.11 (C-21), 123.21 (C-11), 126.87 (C-9), 128.44 (C-15 and C-19), 129.63 (C-10), 132.12 (C-14), 136.44 (C-6), 132.46 (C-22), 155.10 (C-8), 155.96 (C-17), 174.38 (C-2). ^1H -NMR (CD_3OD); 1.55 (3H, s, 24-H), 1.64 (3 H, d, J = 6.6 Hz, 12-H), 1.88.74 (3H, s, 23-H), 2.12, 2.97 (2H, ABX syst. dd, J_{AB} = 14.9, J_{BX} = 8.1, and J_{AX} = 3.7 Hz, 13-H), 3.15 (2H, d, J = 8.8 Hz, 20-H), 3.31 (1H, d, J = 2.2 Hz, 3-H), 3.76 (1H, m, 4-H), 4.69 (1H, q, 5-H), 5.12 (1H, d, 8.8 Hz, 21-H), 6.41 (1 H, d, J = 2.2 Hz, 7-H), 6.49 (1H, d, J = 8.1 Hz, 10-H), 6.57 (1H, dd, J = 8.1, 2.2 Hz, 11-H), 6.96 (2H, dd, J = 8.8, 2.1 Hz, 16-H and 18-H) and 7.67 (2H, dd, J = 8.9, 1.9 Hz, 15-H and 19-H). Elemental analysis C (75.38%), H (7.15%) and O (17.46%). HREIMS m/z (M^+) 366.1231- (calculated for $\text{C}_{27}\text{H}_{28}\text{O}_4$, 366.1236).

The second new compound was identified as: (Z)-3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-5-(–4-hydroxybenzylidene)-4-methyldihydrofuran-2(3H)-one (**2**). It was obtained as white crystals from methanol (Table 3), mp. 120–121 °C, UV (MeOH) λ_{max} (log ϵ) 265 (4.13), 275 (4.22), 315 (0.46) nm; IR (KBr) ν_{max} 3400, 2850, 1750, 1701, 1640, 1560. 1100 cm^{-1} , m/z 364.434.

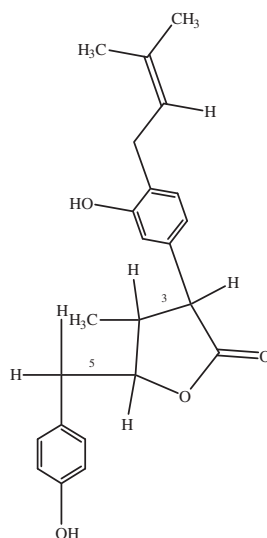
Table 2. Minimum inhibitory concentration (MIC) for the different extracts and isolated compounds of *Aspergillus terreus* against *Aspergillus fumigatus*

Extracts	MIC ($\mu\text{g/mL}$)	
	Intra-	Extra-
Ether	102.40	102.40
Chloroform	204.80	204.80
Ethyl acetate	–	–
Butanol	–	–
Compound 1	34.13	
Compound 2	17.06	
Compound 3	–	

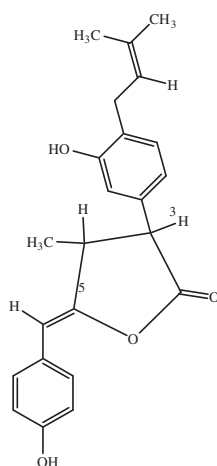
Table 1. Antifungal activity of *Aspergillus terreus* extracts

Fungi:	Test organism	Zone of inhibition (mm) 50 $\mu\text{g/disc}$					
		YES		MEA		Amphotericin B (30 $\mu\text{g/disc}$)	
		Intra-	Extra-	Intra-	Extra-		
Unicellular	<i>Candida albicans</i>	16	17	15	16	19	
	<i>Saccharomyces cirivisae</i>	18	20	17	17	21	
Filamentous	<i>Aspergillus flavus</i>	14	16	7	15	19	
	<i>Aspergillus fumigatus</i>	38	36	0	0	23	
	<i>Aspergillus niger</i>	15	17	16	16	20	
	<i>Penicillium chrysogenum</i>	10	12	9	11	17	

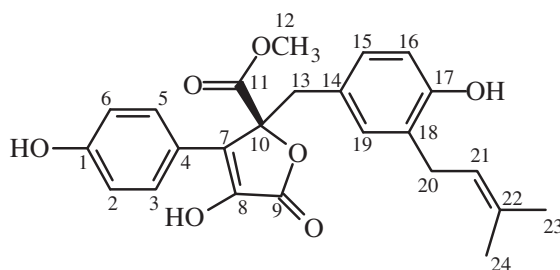
MEA, malt extract agar; YES, yeast extract sucrose agar; Intra-, intracellular; Extra-, extracellular.

**2**

5-(4-Hydroxy-benzyl)-3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-4-methyl-dihydro-furan-2-one

**3**

5-(4-Hydroxy-benzylidene)-3-[3-hydroxy-4-(3-methyl-but-2-enyl)-phenyl]-4-methyl-dihydro-furan-2-one

**Figure 1.** The compounds isolated from *A. terreus*.

^{13}C -NMR (CD_3OD); 17.81 (C-24), 16.94 (C-12), 25.66 (C-23), 27.24 (C-20), 57.82 (C-3), 77.51 (C-4), 104.45 (C-13), 113.97 (C-7), 115.97 (C-16 and C-18), 119.78 (C-21), 124.65 (C-11), 126.86 (C-9), 130.24 (C-10), 130.87 (C-14), 131.76 (C-15 and C-19), 132.01 (C-22), 156.15 (C-8), 156.67 (C-5), 158.27 (C-17), 169.54 (C-2). ^1H -NMR (CD_3OD); 1.76 (3H, s, 24-H), 1.16 (3H, d, $J=6.8$ Hz, 12-H), 1.64 (3H, s, 23-H), 3.02 (2H, d, $J=9.1$ Hz, 20-H), 3.98 (1H, d, $J=4.2$ Hz, 3-H), 3.36 (1H, m, 4-H), 5.34 (1H, t, $J=9.1$, 3.5 Hz, 21-H), 6.30 (1H, s, 13-H), 6.39 (1H, d, $J=1.2$ Hz, 7-H), 6.38 (1H, d, $J=8.3$ Hz, 10-H), 6.51 (1H, dd, $J=8.3$, 1.2 Hz, 11-H), 6.96 (2H, dd, $J=8.7$, 2.0 Hz, 16-H and 18-H) and

7.67 (2H, dd, $J=8.7$, 1.9 Hz, 15-H and 19-H). Elemental analysis C (75.80%), H (6.64%) and O (17.56%). HREIMS m/z 364.4341 (calculated for $\text{C}_{23}\text{H}_{24}\text{O}_4$, 364.4314).

The third isolated compound was identified as: butyrolactone I [(S)-methyl-4-Hydroxy-2-[4-hydroxy-3-(3-methyl-but-2-enyl)-benzyl]-3-(4-hydroxy-phenyl)-5-oxo-2,5-dihydro-furan-2-carboxylic acid methyl ester] (**3**) by comparing its data with those previously published (Cueto *et al.*, 2002). In accordance with our findings, Samuel *et al.* (2011) found that the bioactive metabolites from solvents and crude extract of fungal species such as *Aspergillus flavus*, *Geotrichum candidum*, *Penicillium*

Table 3. NMR Data (600 MHz, Methanol-*d*₄) of compounds **2** and **3**

Compound 2				Compound 3			
No.	δ (J in Hz)	δC mult.	HMBC (H to C)	No.	δ (J in Hz.)	δC mult.	HMBC (H to C)
2	—	174.38		2	—	169.54	
3	3.31 d (2.2)	57.74	2, 4, 5, 6, 7, 11	3	3.98 d (4.2)	57.82	2, 4, 5, 6, 7, 11
4	3.76 m	44.87	2, 3, 5, 6, 12, 13	4	3.36 m	77.51	2, 3, 5, 6, 12, 13
5	4.69 q	86.49		5	—	156.67	13, 14.
6	—	136.44	3, 7, 11	6	—	137.20	3, 7, 11.
7	6.41 d (2.2)	113.13	3, 6, 8, 11.	7	6.39 d (1.2)	113.97	3, 6, 8, 11.
8	—	155.10		8	—	156.15	
9	—	126.87		9	—	126.86	20
10	6.49 d (8.1)	129.63		10	6.38 d (8.3)	130.24	20
11	6.57 dd (8.1, 2.2)	123.21	3, 6, 7.	11	6.51 dd (1.2, 8.3)	124.65	3, 6, 7.
12 CH ₃	1.64 d (6.6)	14.80	4	12 CH ₃	1.16 d (6.8)	16.94	4
13a	2.79,	37.38	5, 14.	13	6.30 s	104.45	5, 14.
13b	2.12 dd (14.9, 8.1, 3.7)						
14	—	132.12	13	14	—	130.87	13
15	7.67 dd (8.8, 1.9)	128.44		15	7.67 dd (8.7, 2.0)	131.76	
16	6.96 dd (8.8, 2.1)	116.13		16	6.96 dd (8.7, 1.6)	115.17	
17	—	155.96		17	—	158.27	
18	6.96 dd (8.9, 2.1)	116.13		18	6.96 dd (8.3, 1.6)	115.17	
19	7.67 dd (8.9, 1.9)	128.44		19	7.67 dd (8.3, 2.0)	131.76	
20	3.15 d (8.8)	27.24	21, 9, 10	20	3.02 d (9.1)	27.24	21, 9, 10
21	5.12 d (8.8)	119.11	20	21	5.34 t (9.1, 3.5)	119.78	20
22	—	132.46		22	—	132.01	
23	1.88 s	25.64		23	1.64 s	25.66	
24	1.55 s	17.81		24	1.76 s	17.81	

leuteum, *Penicillium granulatum* and *Acremonium* species were screened for its antibacterial activity.

In conclusion, the newly isolated compounds were produced by filamentous fungi, namely *A. terreus*, and had antifungal activity against another fungus, *Aspergillus fumigatus*. Secondary metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities and according to Siddhardha *et al.* (2009) many of the products currently used for human/animal therapy, in animal husbandry and in agriculture are produced by microbial fermentation or derived from chemical modifications of a microbial product. Hence the present study suggests that these metabolites and

fungal strains can be further utilized for biotechnological applications in medicine and agriculture.

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Declaration of Interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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