Terrenolide S, a new antileishmanial butenolide from the endophytic fungus *Aspergillus terreus*

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1. Introduction

Endophytic fungi living asymptptomatically within plant tissues have been found in virtually all plant species (Saikkonen et al. 1998; Bacon & White 2000). Recently, attention has been focused on the biology and chemistry of the endophytic micro-organisms (Geris dos Santos...
& Rodrigues-Fo 2003). They became a hunting ground for novel drug leads (Strobel & Daisy 2003; Larsen et al. 2005). The genus *Aspergillus* represents a diverse group of fungi, which are amongst the most abundant fungi in the world (Krijgsheld et al. 2012). *Aspergillus* is a filamentous, cosmopolitan and ubiquitous fungus commonly found in soil, plant debris and indoor air environment. This genus includes over 185 species and is famous for the production of bioactive secondary metabolites (e.g. antibiotic, mycotoxin, antifungal, β-glucuronidase inhibitor, cytotoxic compounds, etc.) (Balajee 2009; Deng et al. 2013; Haroon et al. 2013; Ibrahim et al. 2015). *Aspergillus terreus* is ubiquitous fungus isolated from both marine and terrestrial environments in tropical or sub-tropical areas (Balajee 2009; Parvatkar et al. 2009). It produces a variety of secondary metabolites that are economically significant, such as the antihypercholesterolemic drug lovastatin and several other metabolites, including sulochrin and terrein which have antibiotic activities (Macedo et al. 2004). In our continued search for biologically active metabolites from natural sources, we have identified a new butenolide derivative: terrenolide S (6), together with six known compounds (1–5 and 7) from the endophytic fungus *A. terreus* isolated from the roots of *Carthamus lanatus* (Figure 1(A)). Their chemical structures were identified by one- and two-dimensional NMR and HRESIMS analyses. In addition, their antimicrobial, antileishmanial, antimalarial and cytotoxic activities have been evaluated.

The fungus was cultured on rice solid medium. The metabolites from the rice culture have been extracted with EtOAc. The latter was concentrated and partitioned between n-hexane and 90% MeOH. The total 90% MeOH extract was subjected to vacuum liquid chromatography (VLC), silica gel, sephadex LH-20 and RP-18 column chromatography to yield a new (6) and six known compounds (1–5 and 7).

### 2. Results and discussion

Compound 6 was obtained as yellow gum. It gave a red spot under UV light (254 nm) and a rose-red spot with *p*-anisaldehyde/H$_2$SO$_4$ spray reagent. Its HRESIMS gave a pseudo-molecular ion peak at *m/z* 385.1285 [M+H]$^+$, consistent with a molecular formula C$_{21}$H$_{20}$O$_7$, requiring 12 degrees of unsaturation. The $^1$H and $^{13}$C NMR spectral data of 6 revealed that 8 of the 12 units of unsaturation were attributed to 2 phenyl moieties. In addition, two carbonyls and two olefinic carbons were accounted for another three degrees of unsaturation. Thus, the remaining unit indicated the presence of an additional aliphatic ring in 6. Its IR spectrum showed absorption bands at 3359 (hydroxyl), 1731 (ester/lactone carbonyl) and 1664 (C-H aromatic) cm$^{-1}$. The $^{13}$C, DEPT and HSQC spectra of 6 showed the presence of 21 carbons: two methyls; two methylenes, one of them for an oxygen-bonded methylene δ$_c$ 60.3 (C-1″′); eight methines; and nine quaternary carbons, including two carbonyls at δ$_c$ 170.7 (C-5) and 168.4 (C-1), two oxygen-bonded aromatic carbons at δ$_c$ 158.2 (C-4′) and 156.6 (C-4″) and an oxygenated aliphatic carbon δ$_c$ 85.5 (C-4) (Supplementary Figure S2). The $^1$H NMR and $^1$H-$^1$H COSY spectra showed eight ortho-coupled aromatic protons characteristic for the presence of two 1,4-di-substituted phenolic moieties at δ$_h$ 7.60 (2H, brd, $J = 8.4$ Hz, H-2′, 6′), 6.93 (2H, brd, $J = 8.4$ Hz, H-3′, 5′), 6.65 (2H, brd, $J = 8.0$ Hz, H-2″, 6″) and 6.57 (2H, brd, $J = 8.0$ Hz, H-3″, 5″) (Figure 1(B)). They correlated with the carbons resonating at δ$_c$ 128.0, 116.1, 131.7 and 115.1, respectively, in the HSQC spectrum. They were established by the observed HMBC correlations of H-2′ and H-6′ to C-3′, C-5′ and C-4″; H-3′ and H-5′ to C-1′, C-4′ and C-6″; H-2″ and H-6″ to C-1″, C-3″ and C-4″; and H-3″ and H-5″ to C-1″ and C-4″ (Figure 1). $^1$H and $^{13}$C
NMR spectra of 6 displayed signals at $\delta_H$ 3.98 (2H, q, $J = 6.5$ Hz, H-1‴)/$\delta_C$ 60.3 (C-1‴) and 1.11 (3H, t, $J = 6.5$ Hz, H-2‴)/$\delta_C$ 14.3 (C-2‴) indicating the presence of an ethoxy group in 6. This was confirmed by the ESIMS fragment ion peaks at $m/z$ 356 [M+H-CH$_2$CH$_3$]$^+$ and 341 [M+H-C$_3$H$_8$]$^+$. Its attachment at C-2 was secured by the 3 $J$ and 4 $J$ HMBC cross peaks of H-1‴ to C-2 and C-1, respectively, in addition to the 4 $J$ HMBC cross peak of H-2‴ to C-2 (Figure 1(B)). Furthermore, a methylene group at $\delta_H$ 3.47 (2H, m, H-6)/$\delta_C$ 38.4 (C-6) was observed. The
HMBC cross peaks of H-6/C-4, C-5 and C-1″ and H-2″ and H-6″/C-6 indicated the connectivity of H-6/C-6 to the quaternary carbons C-4 (δ_C 85.2) and C-1″ (δ_C 123.7). Signals for methoxy group at δ_H 3.71 (3H, s, 5-oCH₃)/δ_C 53.6 (5-oCH₃) were observed in 1H and 13C NMR spectra. It showed HMBC cross peak to carbonyl carbon at δ_C 170.7, establishing its connectivity at C-5. Compound 6 was determined to be 4R-configured based on the biosynthetic ground and similarity of the specific rotation of 6 ([α]D +63.6 (c 0.05, MeOH)) with those of butyrolactones I ([α]D +63.0) and IV ([α]D +55.0), aspernolide B ([α]D +77.0) (Haritakun et al. 2010), aspernolide A ([α]D +88.73) (Parvatkar et al. 2009) and aspernolide D ([α]D + 40.0) (Nuclear et al. 2010) previously isolated from A. terreus and flavipesins A ([α]D +46.0) and B ([α]D +133.0) isolated from A. flavipes (Bai et al. 2014). Thus, the structure of 6 was determined as (R)-methyl 4-ethoxy-2-(4-hydroxybenzyl)-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate and named terrenolide S.

Compounds 1–5 and 7 were identified as (22E,24R)-stigmasta-5,7,22-trien-3-β-ol (1) (Ha et al. 1982), stigmast-4-ene-3-one (2) (Barla et al. 2006), stigmasta-4,6,8(14),22-tetraen-3-one (3) (Kobayashi et al. 1992), terretonin A (4) (Li et al. 2005), terretonin (5) (Springer et al. 1979, Liu et al. 2013) and butylrolactone VI (7) (San-Martín et al. 2011) by analysis of the spectroscopic data (1D, 2D NMR and MS) and comparison of their data with those in literature.

The isolated compounds 1–7 were evaluated for their antimicrobial, antileishmanial and antimalarial activities. Furthermore, they were tested for cytotoxicity against four human cancer cell lines: malignant melanoma (SK-MEL), epidermoid (KB), ductal (BT-549) and ovarian (SK-OV-3) human carcinomas and two non-cancerous kidney cell lines: pig kidney epithelial (LLC-PK₁) and monkey kidney fibroblast (VERO).

Compound 1 displayed potent activity against MRSA with an IC₅₀ value of 2.29 μM and good antifungal activity against Cryptococcus neoformans with an IC₅₀ value of 10.68 μM compared to ciprofloxacin (IC₅₀ 0.21 μM) and amphotericin B (IC₅₀ 0.37 μM), respectively, as well as it showed weak activity towards Staphylococcus aureus (IC₅₀ 28.54 μM). The resting compounds 2–7 did not show any significant activity.

Compounds 1, 2 and 6 exhibited antileishmanial activity towards L. donovani with IC₅₀ values of 11.24, 15.32 and 27.27 μM, respectively, and IC₉₀ values of 14.68, 40.56 and 167.03 μM, respectively, compared to pentamidine (positive control, IC₅₀ 6.18 and IC₉₀ 28.15 μM) (Table 1). However, none of the tested compounds 1–7 showed antimalarial or cytotoxic activities.

| Compd No. | Leishmania donovani |
|-----------|---------------------|
|           | IC₅₀    | IC₉₀   |
| 1         | 11.24   | 14.68  |
| 2         | 15.32   | 40.56  |
| 3         | 54.26   | 79.41  |
| 4         | >84.75  | >84.75 |
| 5         | >81.97  | >81.97 |
| 6         | 27.27   | 167.03 |
| 7         | >87.34  | >87.34 |
| Pentamidine | 6.18   | 28.15  |

IC₅₀: concentration causing 50% growth inhibition (μM).
IC₉₀: concentration causing 90% growth inhibition (μM).
3. Experimental

3.1. General experimental procedures

Melting points were carried out using an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering Ltd., Essex, England). Optical rotation was measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan) at 25 °C at the sodium D-line (589 nm). UV spectrum was recorded on a Hitachi 300 spectrometer (Hitachi High-Technologies Corporation, Kyoto, Japan). The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer (Shimadzu, Kyoto, Japan). ESIMS spectra were obtained with a LCQ DECA mass spectrometer (Thermo Finnigan, Bremen, Germany) coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. HRESIMS spectra measurements were performed on a Micromass QTof 2 mass spectrometer (Bruker, Rheinstetten, Germany). 1D and 2D NMR spectra were determined on BRUKER Unity INOVA 400 instruments (400 MHz for 1H and 100 MHz for 13C NMR) (Bruker BioSpin, Billerica, MA, USA) using CDCl3 and DMSO-d6 as solvents. Column chromatographic separations were performed on silica gel 60 (0.04–0.063 mm, Merck, Darmstadt, Germany), sephadex LH-20 (0.25–0.1 mm, Merck, Darmstadt, Germany) and RP-18 (0.04–0.063 mm, Merck, Darmstadt, Germany). TLC analysis was performed on precoated TLC plates with silica gel 60 F254 (0.2 mm, Merck, Darmstadt, Germany). Spots were visualised by UV absorption at λmax 255 and 366 nm, followed by spraying with p-anisaldehyde/H2SO4. The solvent systems used for TLC analyses were n-hexane:EtOAc (85:15, S1) and n-hexane:EtOAc (80:20, S2).

3.2. Isolation of the fungal material, identification, cultivation and isolation of metabolites

A. terreus was isolated from the internal tissue of the healthy roots of Carthamus lanatus L. (Asteraceae) that was collected from the wildly growing plant at Al-Azhar University campus in February 2013. The strain was identified from the observed morphological features of the fungus by light microscopy (CX31RBSF, Olympus) (Watanabe 2002). Moreover, the identification was confirmed by Prof. Mohamed Hosam Refaie Kotb, Prof. of Microbiology and Immunology, Department of Microbiology and Immunology, Animal Reproductive Research Institute, Giza, Egypt. It was deposited at the Department of Microbiology, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut, Egypt under registration number (AST No. Feb 2013). The fungus was grown on rice solid medium at room temperature. The metabolites from the rice culture were extracted with EtOAc at room temperature and concentrated to afford a residue. The latter was partitioned between n-hexane and 90% MeOH. The total 90% MeOH extract (11.8 g) was subjected to VLC using n-hexane, CHCl3, EtOAc and MeOH to yield four fractions. Fraction AS-2 (2.9 g) was subjected to VLC, silica gel and RP-18 column to obtain compounds 1–7. The details of the cultivation and isolation of metabolites have been mentioned in Supplementary materials.

3.3. Spectral data

Terrenolide S (6). Yellow gum; [α]D + 63.6 (c 0.05, MeOH); UV (MeOH) λmax (log ε): 213 (3.98), 249 (3.62), 296 (0.55) nm; IR (KBr) νmax 3359, 2989, 1731, 1664, 1255, 1166 cm−1; 1H NMR (400 MHz, DMSO-d6): δH 3.47 (2H, m, H-6), 7.60 (2H, brd, J = 8.4 Hz, H-2′, 6′), 6.93 (2H, brd, J = 8.4 Hz, H-3′,
5′), 6.65 (2H, brd, J = 8.0 Hz, H-2″, 6″), 6.57 (2H, brd, J = 8.4 Hz, H-3″, 5″), 3.71 (3H, s, 5-oCH3), 3.98 (3H, q, J = 6.5 Hz, H-1‴), 1.11 (2H, t, J = 6.5 Hz, H-2‴), 10.01 (1H, s, 4’-OH), 9.31 (1H, s, 4″-OH); 13C NMR (100 MHz, DMSO-d6): δC 168.4 (C-1), 138.5 (C-2), 128.0 (C-3), 85.2 (C-4), 170.7 (C-5), 38.4 (C-6), 121.5 (C-1′), 128.0 (C-2′, 6′), 158.2 (C-4′), 116.1 (C-3′, 5′), 123.7 (C-1″), 131.7 (C-2″, 6″), 156.6 (C-4″), 115.1 (C-3″, 5″), 53.6 (5-OCH3), 60.3 (C-1‴), 14.3 (C-2‴); ESIMS m/z: 384 [M+H]+, 356, 341; HRESIMS m/z: 385.1285 [M+H]+ (calcd for C21H21O7, 385.1287).

3.4. Antimicrobial, antileishmanial, antimalarial and cytotoxicity assays

The antimicrobial activity of the isolated compounds 1–7 was assessed as described previously (Ibrahim et al. 2012; Al-Musayeib et al. 2014). Also, they were evaluated for their antileishmanial and antimalarial activities, as well as their cytotoxicity towards SK-MEL, KB, BT-549, SK-OV-3, LLC-PK1, and VERO cell lines (Borenfreund et al. 1990; El-Shanawany et al. 2011; Al-Musayeib et al. 2014). The details of the assays are mentioned in the Supplementary material.

4. Conclusion

A new butenolide derivative (6) along with six known compounds (1–5 and 7) have been isolated from the endophytic fungus A. terreus. Their structures were determined on the basis of extensive spectroscopic analyses. Compound 1 displayed potent activity against MRSA and C. neoformans. Moreover, 1, 2 and 6 exhibited antileishmanial activity towards L. donovani.

Supplementary material

A detailed experimental section, NMR spectral data of compounds 1–5 and 7, and NMR spectra of 6 are available as Supplementary material.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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