SUPPLEMENTARY MATERIAL

Terrenolide S, a new anti-leishmanial butenolide from the endophytic fungus *Aspergillus terreus*

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Terrenolide S, a new butenolide derivative (**6**), together with six known compounds: (22*E*,24*R*)-stigmasta-5,7,22-trien-3- β -ol (**1**), stigmast-4-ene-3-one (**2**), and stigmasta-4,6,8(14),22-tetraen-3-one (**3**), terretonin A (**4**), terretonin (**5**), and butyrolactone VI (**7**) have been isolated from the endophytic fungus *Aspergillus terreus* isolated from the roots of *Carthamus lanatus* (Asteraceae). Their structures were established by extensive spectroscopic analyses (1D, 2D NMR, and HRESIMS), as well as optical rotation measurement and comparison with literature data. Compound **1** displayed a potent activity towards methicillin-resistant *Staphylococcus aureus* (MRSA) and *Cryptococcus neoformans* with IC₅₀ values of 2.29 and 10.68 μ M, respectively. Moreover, **1**, **2**, and **6** exhibited anti-leishmanial activity towards *Leishmania donovani* with IC₅₀ values of 11.24, 15.32, and 27.27 μ M, respectively and IC₉₀ values of 14.68, 40.56, and 167.03 μ M, respectively.

Keywords: *Aspergillus terreus*; terrenolide S; butenolide; stigmasterol derivatives; antimicrobial; anti-leishmanial; anti-malarial; cytotoxic activity

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Experimental

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

Isolation of the fungal material

A. terreus was isolated from the internal tissue of the healthy roots of *Carthamus lanatus* L. (Asteraceae) that collected from the wildy growing plant at Al-Azhar University campus in February 2013. The root was washed under running water and the surface was sterilized with 70% EtOH for 1 min. The root samples were rinsed in sterile water. To distinguish the remaining epiphytic fungi from endophytic fungi, an imprint of the root surface on biomalt agar was performed. The outer layer of the root was removed and small root tissue samples were cut aseptically and pressed onto agar plates containing an antibiotic to suppress bacterial growth. The plates were kept at 27 °C for the isolation of the endophyte. After incubation at room temperature, the fungal strain under investigation was found to grow exclusively out of the root tissues, but not on the agar plates taken from the imprint of the root surface. A fungal strain was maintained on potato-dextrose agar medium (Difco) at 25 °C.

Identification of the fungal material

The strain was identified from the observed morphological features of the fungus on potato-dextrose agar (PDA), sabroud dextrose, and nutrient agar as previously described by Tsuneo Watanabe (Watanabe 2002). The morphology was examined by light microscopy (CX31RBSF, Olympus) and identification was confirmed to be *A. terreus* by Prof. Mohamed Hosam Refaie Kotb, Prof. of Microbiology and Immunology, Department of Microbiology and Immunology, Animal Reproductive Research Institute, Giza, Egypt. From the growing cultures, the pure strain of *A. terreus* was isolated by repeated re-inoculation on PDA agar plates. It was deposited at the Department of Microbiology, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut, Egypt under registration number (AST No. Feb2013).

Cultivation

Mass growth of the fungus for the isolation and identification of its metabolites was carried out in 1 L Erlenmeyer flasks (each flask containing 100 mL of distilled water were added to 100 g

commercially available rice and kept overnight prior to autoclaving). The fungus was grown on rice solid medium (8 flasks) at room temperature under septic conditions for 4 weeks.

Extraction and isolation of metabolites

The metabolites from the rice culture were extracted with EtOAc at room temperature. The obtained crude extract was concentrated under vacuum and partitioned between *n*-hexane and 90% MeOH. The total 90% MeOH extract (11.8 g) was subjected to normal phase vacuum liquid chromatography (VLC) using *n*-hexane, CHCl₃, EtOAc, and MeOH, which were separately concentrated to give AS-1 (1.5 g), AS-2 (2.9 g), AS-3 (2.55 g), and AS-4 (3.72 g), respectively. Fraction AS-2 (2.9 g) was subjected to VLC using *n*-hexane:EtOAc gradient elution to afford seven sub-fractions: AS-2-A (0.32 g), AS-2-B (0.49 g), AS-2-C (0.61 g), AS-2-D (0.29 g), AS-2-E (0.12 g), AS-2-F (0.41 g), and AS-2-G (0.36 g). Sub-fraction AS-2-A (0.32 g) was chromatographed over silica gel column (50 g × 50 × 2 cm) using *n*-hexane:EtOAc (98:2 to 85:15) to obtain **1** (21 mg, white needles). Silica gel column chromatography (80 g × 50 × 2 cm) of sub-fraction AS-2-B (0.49 g) using *n*-hexane:EtOAc (98:2 to 85:15) gave impure **2** (34 mg), which was further purified by RP-18 column (100 g × 50 × 2 cm) using MeOH:H₂O gradient to afford **2** (18 mg, white needles). Subfraction AS-2-C (0.61 g) was subjected to Sephadex LH-20 column chromatography (100 g × 70 × 3 cm) using MeOH:CHCl₃ (90:10) to afford impure **3**, **4**, and **5**. They were further purified by RP-18 column (150 g × 50 × 3 cm) using MeOH:H₂O gradient to obtain **3** (23 mg, white needles), **4** (12 mg, colorless cubic crystals), and **5** (9 mg, colorless cubic crystals). Sub-fraction AS-2-D and E (0.41 g) were collected together based on TLC plates. Similarly, they were chromatographed over Sephadex LH-20 chromatography (100 g × 70 × 3 cm) using MeOH as an eluent to afford impure **6** and **7**. Purification was achieved by repeated silica gel column chromatography using CHCl₃:MeOH gradient to give **6** (6.7 mg, yellow gum) and **7** (11 mg, yellow gum). The other fractions were kept for further investigation.

Spectral data

(22*E*,24*R*)-Stigmasta-5,7,22-trien-3- β -ol (**1**). White needles; mp 151-152 °C; IR (KBr) ν_{max} 3435, 2850, 1665, 1585, 1460, 970 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ_H 2.30 (1H, m, H-1A), 1.36 (1H, m, H-1B), 1.91 (1H, m, H-2A), 1.80 (1H, m, H-2B), 3.62 (1H, m, H-3), 2.48 (1H, dd, *J* = 12.6, 11.4 Hz, H-4A), 2.44 (1H, dd, *J* = 12.6, 4.2 Hz, H-4B), 5.56 (1H, d, *J* = 6.2 Hz, H-

6), 5.37 (1H, d, $J = 6.2$ Hz, H-7), 2.01 (1H, t, $J = 5.8$ Hz, H-9), 1.48 (1H, m, H-11A), 1.22 (1H, m, H-11B), 1.68 (1H, m, H-12A), 1.25 (1H, m, H-12B), 2.27 (1H, t, $J = 7.2$ Hz, H-14), 1.37 (1H, m, H-15A), 1.25 (1H, m, H-15B), 1.82 (1H, m, H-16A), 1.44 (1H, m, H-16B), 1.87 (1H, m, H-17), 0.62 (3H, s, H₃-18), 0.96 (3H, s, H₃-19), 2.07 (1H, m, H-20), 1.03 (3H, d, $J = 6.8$ Hz, H₃-21), 5.14 (1H, dd, $J = 16.8, 6.9$ Hz, H-22), 5.22 (1H, dd, $J = 16.8, 7.2$ Hz, H-23), 1.99 (1H, m, H-24), 1.78 (1H, m, H-25), 0.92 (3H, d, $J = 6.3$ Hz, H₃-26), 0.92 (3H, d, $J = 6.3$ Hz, H₃-27), 1.25 (2H, m, H-28), 0.82 (3H, t, $J = 6.1$ Hz, H₃-29); ¹³C NMR (100 MHz, CDCl₃): δ_C 38.3 (C-1), 31.9 (C-2), 70.4 (C-3), 40.7 (C-4), 139.5 (C-5), 119.8 (C-6), 116.1 (C-7), 141.2 (C-8), 46.2 (C-9), 37.0 (C-10), 21.1 (C-11), 39.1 (C-12), 42.8 (C-13), 54.5 (C-14), 22.9 (C-15), 28.2 (C-16), 55.7 (C-17), 12.1 (C-18), 16.3 (C-19), 40.4 (C-20), 17.6 (C-21), 131.9 (C-22), 135.4 (C-23), 42.8 (C-24), 33.1 (C-25), 19.7 (C-26), 20.0 (C-27), 21.1 (C-28), 12.1 (C-29); ESIMS m/z : 411 [M+H]⁺; HRESIMS m/z : 411.3629 [M+H]⁺ (calcd for C₂₉H₄₇O 411.3627).

Stigmast-4-ene-3-one (**2**). White needles; mp 96-97 °C; IR (KBr) ν_{max} 2940, 1671, 1453 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ_H 1.70 (1H, m, H-1A), 1.52 (1H, m, H-1B), 2.35 (2H, m, H-2), 5.70 (1H, s, H-4), 2.00 (1H, m, H-6A), 1.80 (1H, m, H-6B), 1.45 (1H, m, H-7A), 1.18 (1H, m, H-7B), 1.41 (1H, m, H-8), 1.15 (1H, m, H-9), 1.57 (1H, m, H-11A), 1.29 (1H, m, H-11B), 1.48 (1H, m, H-12A), 1.24 (1H, m, H-12B), 0.65 (1H, m, H-14), 1.65 (1H, m, H-15A), 1.35 (1H, m, H-15B), 1.60 (1H, m, H-16A), 1.31 (1H, m, H-16B), 0.87 (1H, m, H-17), 0.68 (3H, s, H₃-18), 1.15 (3H, s, H₃-19), 1.65 (1H, m, H-20), 0.89 (3H, d, $J = 6.2$ Hz, H₃-21), 1.48 (2H, m, H-22), 1.35 (2H, m, H-23), 1.22 (1H, m, H-24), 1.25 (1H, m, H-25), 0.83 (3H, d, $J = 6.6$ Hz, H₃-26), 0.83 (3H, d, $J = 6.6$ Hz, H₃-27), 0.76 (3H, t, $J = 6.9$ Hz, H₃-29); ¹³C NMR (100 MHz, CDCl₃): δ_C 35.6 (C-1), 32.9 (C-2), 200.0 (C-3), 123.8 (C-4), 171.8 (C-5), 33.9 (C-6), 33.8 (C-7), 36.1 (C-8), 53.8 (C-9), 38.6 (C-10), 21.0 (C-11), 39.5 (C-12), 42.3 (C-13), 55.8 (C-14), 24.2 (C-15), 28.2 (C-16), 55.9 (C-17), 12.0 (C-18), 17.3 (C-19), 35.6 (C-20), 18.7 (C-21), 32.0 (C-22), 27.0 (C-23), 45.7 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.0 (C-28), 11.9 (C-29); ESIMS m/z : 413 [M+H]⁺; HRESIMS m/z : 413.3780 [M+H]⁺ (calcd for C₂₉H₄₉O 413.3783).

Stigmasta-4,6,8(14),22-tetraen-3-one (**3**). White needles; mp 118-119 °C; ¹H NMR (400 MHz, CDCl₃): δ_H 2.02 (1H, m, H-1A), 1.82 (1H, m, H-1B), 2.54 (1H, m, H-2A), 2.48 (1H, m, H-2B), 5.72 (1H, brs, H-4), 6.58 (1H, d, $J = 9.5$ Hz, H-6), 6.00 (1H, d, $J = 9.5$ Hz, H-7), 2.13 (1H,

m, H-9), 1.71 (1H, m, H-11A), 1.63 (1H, m, H-11B), 1.68 (1H, m, H-12A), 1.25 (1H, m, H-12B), 2.48 (1H, m, H-15A), 2.37 (1H, m, H-15B), 1.76 (1H, m, H-16A), 1.53 (1H, m, H-16B), 1.26 (1H, m, H-17), 0.94 (3H, s, H₃-18), 0.97 (3H, s, H₃-19), 2.11 (1H, m, H-20), 1.03 (3H, d, $J = 6.0$ Hz, H₃-21), 5.18 (1H, dd, $J = 16.5, 6.3$ Hz, H-22), 5.25 (1H, dd, $J = 16.5, 6.7$ Hz, H-23), 1.76 (1H, m, H-24), 1.48 (1H, m, H-25), 0.91 (3H, d, $J = 7.0$ Hz, H₃-26), 0.91 (3H, d, $J = 7.0$ Hz, H₃-27), 1.21 (2H, m, H-28), 0.82 (3H, t, $J = 6.2$ Hz, H₃-29); ¹³C NMR (100 MHz, CDCl₃): δ_C 34.1 (C-1), 18.9 (C-2), 199.6 (C-3), 122.8 (C-4), 164.5 (C-5), 124.4 (C-6), 134.1 (C-7), 124.8 (C-8), 44.2 (C-9), 36.8 (C-10), 25.3 (C-11), 34.1 (C-12), 44.0 (C-13), 156.3 (C-14), 35.5 (C-15), 27.7 (C-16), 55.6 (C-17), 16.6 (C-18), 19.0 (C-19), 39.3 (C-20), 21.2 (C-21), 132.5 (C-22), 134.9 (C-23), 42.8 (C-24), 33.1 (C-25), 19.6 (C-26), 20.0 (C-27), 29.7 (C-28), 17.6 (C-29); ESIMS m/z : 407 [M+H]⁺; HRESIMS m/z : 407.3311 [M+H]⁺ (calcd for C₂₉H₄₃O 407.3314).

Terretonin A (**4**). Colorless cubic crystals, mp 233-234 °C; IR (KBr) ν_{max} 3369, 1736, 1712, 1683, 1105 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ_H 2.14 (1H, dd, $J = 14.0, 9.0$ Hz, H-1A), 1.72 (1H, dd, $J = 14.0, 10.4$ Hz, H-1B), 2.65 (1H, dd, $J = 13.6, 9.0$ Hz, H-2A), 2.50 (1H, m, H-2B), 1.54 (1H, dd, $J = 12.2, 3.0$ Hz, H-9), 2.42 (1H, dd, $J = 13.8, 12.2$ Hz, H-11A), 2.28 (1H, dd, $J = 13.8, 3.0$ Hz, H-11B), 2.75 (1H, brs, H-14), 1.38 (3H, s, H₃-18), 1.40 (3H, s, H₃-19), 1.76 (3H, s, H₃-20), 1.05 (3H, s, H₃-21), 5.05 (1H, brs, H-22A), 4.92 (1H, brs, H-22B), 1.36 (3H, s, H₃-23), 1.65 (3H, s, H₃-24), 3.76 (3H, s, H₃-1'), 6.18 (1H, s, 6-OH); ¹³C NMR (100 MHz, CDCl₃): δ_C 34.2 (C-1), 32.6 (C-2), 214.1 (C-3), 47.9 (C-4), 137.1 (C-5), 138.9 (C-6), 197.7 (C-7), 45.7 (C-8), 52.8 (C-9), 36.8 (C-10), 29.0 (C-11), 142.7 (C-12), 50.6 (C-13), 49.1 (C-14), 166.8 (C-15), 85.6 (C-16), 201.1 (C-17), 20.9 (C-18), 23.6 (C-19), 16.5 (C-20), 16.8 (C-21), 112.5 (C-22), 23.5 (C-23), 22.0 (C-24), 168.2 (C-25), 53.8 (C-1'); ESIMS m/z : 473 [M+H]⁺; HRESIMS m/z : 473.2176 [M+H]⁺ (calcd for C₂₆H₃₃O₈, 473.2175).

Terretonin (**5**). Colorless cubic crystals; mp 259-260 °C; IR (KBr) ν_{max} 3410, 2935, 1729, 1711, 1685, 1252, 1150 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ_H 2.41 (1H, m, H-1A), 1.76 (1H, m, H-1B), 2.65 (1H, dd, $J = 14.0, 8.0$ Hz, H-2A), 2.44 (1H, m, H-2B), 2.91 (1H, brd, $J = 15.0$ Hz, H-11A), 2.27 (1H, brd, $J = 15.0$ Hz, H-11B), 3.53 (1H, s, H-14), 1.40 (3H, s, H₃-18), 1.37 (3H, s, H₃-19), 1.86 (3H, s, H₃-20), 1.15 (3H, s, H₃-21), 5.35 (1H, s, H-22A), 5.02 (1H, s, H-22B), 1.40 (3H, s, H₃-23), 1.64 (3H, s, H₃-24), 3.76 (3H, s, H₃-1'), 6.19 (1H, s, 6-OH); ¹³C NMR (100 MHz,

CDCl₃): δ_C 28.0 (C-1), 32.6 (C-2), 214.5 (C-3), 47.8 (C-4), 131.4 (C-5), 138.6 (C-6), 197.1 (C-7), 51.1 (C-8), 77.6 (C-9), 43.1 (C-10), 34.9 (C-11), 139.5 (C-12), 49.3 (C-13), 44.4 (C-14), 168.0 (C-15), 85.3 (C-16), 201.4 (C-17), 21.2 (C-18), 23.5 (C-19), 19.6 (C-20), 18.5 (C-21), 116.5 (C-22), 23.5 (C-23), 21.1 (C-24), 168.3 (C-25), 53.7 (C-1'); ESIMS m/z : 489 [M+H]⁺; HRESIMS m/z : 489.2127 [M+H]⁺ (calcd for C₂₆H₃₃O₉ 489.2125).

Butyrolactone VI (7). Yellow gum; $[\alpha]_D + 73$ (c 0.5, MeOH); IR (KBr) ν_{max} 3440, 1735, 1660, 1055 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 3.42 (1H, d, J = 16.0 Hz, H-6A), 3.32 (1H, d, J = 16.0 Hz, H-6B), 7.50 (2H, brd, J = 8.0 Hz, H-2', 6'), 6.83 (2H, brd, J = 8.0 Hz, H-3', 5'), 6.59 (1H, d, J = 2.0 Hz, H-2''), 6.41 (1H, d, J = 8.0 Hz, H-5''), 6.49 (1H, dd, J = 8.0, 2.0 Hz, H-6''), 2.63 (1H, dd, J = 16.0, 3.6 Hz, H-7''A), 2.28 (1H, dd, J = 16.0, 8.0 Hz, H-7''B), 3.25 (1H, dd, J = 8.0, 3.6 Hz, H-8''), 1.04 (3H, s, H₃-10''), 1.05 (3H, s, H₃-11''), 3.76 (3H, s, 5-OCH₃), 10.52 (1H, s, 4'-OH), 9.98 (1H, s, 4''-OH), 9.22 (1H, d, J = 5.6 Hz, 8''-OH); ¹³C NMR (100 MHz, DMSO-*d*₆): δ_C 168.5 (C-1), 138.6 (C-2), 128.1 (C-3), 85.2 (C-4), 170.2 (C-5), 38.4 (C-6), 121.5 (C-1'), 129.2 (C-2', 6'), 158.2 (C-4'), 116.2 (C-3', 5'), 123.6 (C-1''), 133.4 (C-2''), 126.6 (C-3''), 154.8 (C-4''), 115.0 (C-5''), 129.0 (C-6''), 32.6 (C-7''), 68.4 (C-8''), 77.1 (C-9''), 26.2 (C-10''), 25.1 (C-11''), 53.8 (5-OCH₃); ESIMS m/z : 459 [M+H]⁺; HRESIMS m/z : 459.1658 [M+H]⁺ (calcd for C₂₄H₂₇O₉ 459.1655).

Antimicrobial, anti-malarial, anti-leishmanial, and cytotoxicity assays

Anti-microbial assay

All the isolated compounds were tested for antimicrobial activity against *Candida albicans* ATCC 90028, *Candida glabrata* ATCC90030, *Candida krusei* ATCC 6258, *Asperigillus fumigates* ATCC 90906, methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Cryptococcus neoformans* ATCC 90113, *Staphylococcus aureus* ATCC 2921, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068 as described previously (Al-Musayeib et al. 2014, Ibrahim et al. 2012). Ciprofloxacin and amphotericin B were used as positive standards.

Anti-leishmanial assay

The anti-leishmanial activity of the isolated metabolites was tested *in vitro* against a culture of *Leishmania donovani* promastigotes as previously outlined (Al-Musayeib et al., 2014; El-Shanawany et al., 2011). Pentamidine was used as positive standard.

Anti-malarial assay

The isolated compounds were tested on chloroquine sensitive (D6, Sierraleon) and resistant (W2, Indo-china) strains of *Plasmodium falciparum* using previously reported method (Al-Musayeib et al. 2014; El-Shanawany et al. 2011). Artemisinin and chloroquine were included in each assay as anti-malarial drug controls.

Cytotoxicity assay

The *in vitro* cytotoxic activity was determined against a panel of four human cancer cell lines: malignant melanoma (SK-MEL), epidermoid (KB), ductal (BT-549), and ovarian (SK-OV-3) carcinomas and two noncancerous kidney cell lines: pig kidney epithelial (LLC-PK₁₁) and monkey kidney fibroblast (VERO). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were seeded at a density of 25,000 cells/well and incubated for 24 h. Test samples were added at different concentrations and cells were again incubated for 48h. At the end of incubation, the cell viability was determined using Neutral Red dye according to a modification of the procedure of Borenfreund *et al* (Borenfreund et al., 1990). Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

Figure S1. ^1H NMR spectrum of compound **6** (400 MHz, $\text{DMSO-}d_6$).

Figure S2. ^{13}C NMR spectrum of compound **6** (100 MHz, $\text{DMSO-}d_6$).

Figure S3. DEPT ^{13}C NMR spectrum of compound **6**.

Figure S4. ^1H - ^1H COSY spectrum of compound **6** ($\text{DMSO}-d_6$).

Figure S5. HSQC spectrum of compound **6** (DMSO- d_6).

Figure S6. HMBC spectrum of compound **6** (DMSO- d_6).

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