Supporting Information

Leishmanicidal and multidrug resistance reversal constituents from *Aeonium lindleyi*

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General Experimental Procedure

IR spectra were recorded in CDCl₃ on a Bruker IFS 55 spectrophotometer, and UV spectra were collected in absolute EtOH on a JASCO V-560. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts are given in δ (ppm) with residual CDCl₃ as internal reference, and coupling constants in Hz. Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter, and [α]D values are given in 10⁻¹ deg cm² g⁻¹. EI-MS and HR-EI-MS were recorded on a Micromass Autospec spectrometer. Purification was performed using silica gel 60 for column chromatography (particle size 15-40 and 63-200 µm), POLYGRAM SIL G/UV₂₅₄, used for analytical and preparative TLC, and HPTLC-Platten Nano-Sil 20 UV₂₅₄ were purchased from Macherey-Nagel. Sephadex LH-20 was obtained from Pharmacia Biotech. The spots were visualized by UV light and heating silica gel plates sprayed with H₂O-H₂SO₄-AcH (1:4:20). All solvents used were analytical grade from Panreac. Reagents and ketoconazole were purchased from Sigma Aldrich and used without further purification. Miltefosine was obtained from Zentaris GmbH (Frankfurt am Main, Germany).

Extraction and isolation

Fresh leaves (650 g) of A. lindleyi were extracted three times (3 x 4 L) with 95% EtOH for 12 h each time in a Soxhlet apparatus. Removal of the solvent under reduced pressure provided 56 g of crude extract, which was subjected to a silica gel (500 g) column (10 x 50 cm) eluting with a mixture of hexanes-EtOAc (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8, 0:10; 1000 mL each) and EtOAc-EtOH (10:0, 9:1, 8:2, 5:5, 0:10; 1000 mL each) to afford 48 fractions, which were
reduced to 5 pooled fractions: A (0-20%, hexanes-EtOAc), B (20-40%), C (40-60%), D (60-100%), and E (0-100%, EtOAc-EtOH).

Fr A (4.3 g) was purified by vacuum liquid chromatography on silica gel (4 x 40 cm, 50 g), eluting with a CH$_2$Cl$_2$-Me$_2$CO gradient system (10:0, 9.5:0.5, 9:1, 8.5:1.5 8:2; 200 mL) to give compound 1 ($R_f = 0.45$, CH$_2$Cl$_2$-Me$_2$CO 8.5:1.5, 11.7 mg). Fr B (3.2 g) was subjected to a Sephadex LH-20 (150 g) column (4 x 40 cm), using hexanes-CHCl$_3$-MeOH (2:1:1; 1.5 L) as eluent to yield fractions B1 and B2. Fr B1 (750 mg) was further purified by flash chromatography on a silica gel column (4 x 40 cm, 7g), eluting with a CH$_2$Cl$_2$-Me$_2$CO gradient system (9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3; each 50 mL) to afford 2 ($R_f = 0.41$, CH$_2$Cl$_2$-Me$_2$CO 8.5:1.5, 61.2 mg) and 3 ($R_f = 0.36$, CH$_2$Cl$_2$-Me$_2$CO 8.5:1.5, 12.1 mg), and three additional fractions (B1A, B1B, and B1C) which were further purified by preparative TLC. Similarly, Fr B1A (76.8 mg) was eluted with CH$_2$Cl$_2$ to afford 5 ($R_f = 0.55$, CH$_2$Cl$_2$, 21.3 mg), 9 ($R_f = 0.82$, CH$_2$Cl$_2$-Me$_2$CO 9.8:0.2, 27.8 mg), and 11 ($R_f = 0.70$, CH$_2$Cl$_2$-Me$_2$CO 9.2:0.8, 8.2 mg); Fr B1B (39.2 mg) was eluted with CH$_2$Cl$_2$-Me$_2$CO 9.4:0.6 to yield 4 ($R_f = 0.35$, CH$_2$Cl$_2$-Me$_2$CO 9.5:0.5, 4.3 mg) and 8 ($R_f = 0.55$, CH$_2$Cl$_2$-Me$_2$CO 9.3:0.7, 31.7 mg); and Fr B1C (20.4 mg) was eluted with CH$_2$Cl$_2$-Me$_2$CO 8.2:1.8 to yield 7 ($R_f = 0.52$, CH$_2$Cl$_2$-Me$_2$CO 8:2, 12.6 mg). Fr C (2.6 g) was subjected to wet flash chromatography on a silica gel column (4 x 40 cm, 40 g), using mixtures of CH$_2$Cl$_2$-Me$_2$CO of increasing polarity (9:1, 8.5:2.5, 8:2, 7.5:2.5, 7:3; 100 mL) to afford 37 fractions, which were reduced to 9 fractions (C1-C9) by TLC profiles. Fr C5 (23.6 mg) was further purified by preparative TLC eluting with CH$_2$Cl$_2$-Me$_2$CO (8:2) to afford 6 ($R_f = 0.60$, 6.1 mg) and 10 ($R_f = 0.18$, 4.3 mg). Fr D (2.8 g) was purified by wet flash chromatography on a silica gel column (4 x 40 cm, 40 g), using mixtures of CH$_2$Cl$_2$-MeOH of increasing polarity (10:0, 9.8:0.2, 9.6:0.4, 9.4:0.6, 9.2:0.8, 9:1; 100 mL) as eluent to afford 32 fractions, which were reduced to 8 fractions.
(D1-D8) by TLC profiles. Fr D4 (34.0 mg) was further purified by preparative TLC with CH$_2$Cl$_2$-MeOH (9.5:0.5) to afford 12 ($R_f = 0.15$, 15 mg) and 13 ($R_f = 0.20$, 12 mg).

**Acetylation of 2.** A mixture of acetic anhydride (4 drops) and compound 2 (10 mg) in pyridine (2 drops) was stirred at room temperature for 16 h. The mixture was evaporated to dryness, and the residue was purified by preparative TLC (hexanes-AcOEt, 7:3) to give compound 2a ($R_f = 0.61$, 9.5 mg).

**Bioassays**

*Leishmanicidal activity*

The *in vitro* leishmanicidal activity was evaluated against promastigote forms of *Leishmania braziliensis* (MHOM/PE/95/LQ2) and *Leishmania tropica* (MON 58/LEM 2578) strains. For the *in vitro* studies, samples were dissolved in dimethyl sulphoxide (DMSO), and further dilutions were made with RPMI 1640 medium. Promastigote forms of *L. braziliensis* and *L. tropica* were adapted for growth at 22 ºC in RPMI 1640 modified medium (Gibco) and supplemented with 20% heat-inactivated foetal bovine serum (fFBS). Logarithmic phase cultures were used for experimental purposes, and the *in vitro* susceptibility assay was performed in 24-well microtiter plates (Corning™). To these wells were added $2.5 \times 10^4$well (500 µL/well) parasites and the drug concentration to be tested. Growth of promastigotes was monitored after 48 hours by density determination on a Coulter Counter model Z1. The 50% inhibitory concentration (IC$_{50}$) was determined by linear regression analysis with 95% confidence limits. Tests were performed at least in triplicate. Ketoconazole for both strains and miltefosine for *L. braziliensis* were used as positive controls.
Axenic amastigote cultures, obtained by *in vitro* transformation of promastigotes (Ueda-Nakamura et al., 2001) were incubated at 32°C with 5% CO₂ in Schneider’s insect medium (Sigma Chemical Co., St. Louis, Missouri, USA), pH 5.5, with 20% fetal bovine serum. Assays were performed in 96-well microtiter plates, with each well containing 10 µL of the compound dilution and 200 µL of parasite inoculum (1 x 10⁴ amastigotes/well). Parasite multiplication was compared to that for untreated controls (100% growth). After 24 h of incubation, Alamar Blue® Assay (Biosource) was added and cell viability was measured fluorometrically using a test wavelength of 570 nm and a reference wavelength of 630 nm. The results were expressed as the percentage of reduction in the parasite amount compared to that in untreated control wells, and the 50% inhibitory concentration (IC₅₀) was calculated by Probit analysis. At least three independent replicates were performed for each observation. Miltefosine was used as positive control.

*In vitro reversion of multidrug resistance assays in Leishmania*

Flavonoids have been tested for efficacy as potential modulators of Pgp in a *L. tropica* line according to an established protocol. Briefly, we used a wild-type of *L. tropica* (LCR strain) and a MDR *L. tropica* line highly resistant to daunomycin (DNM), that was maintained in presence of 150 µM DNM. This resistant line possesses a MDR phenotype similar to that of tumor cells, with a cross resistance to different unrelated drugs. Promastigote forms were grown at 28 °C in RPMI 1640-modified medium and supplemented with 20% iFBS. The reversal activity of flavonoids was determined by the calculation of the parasite growth inhibition (GI) using the MTT colorimetric assay after 72 h incubation at 28 °C in presence of increasing concentrations of compounds.