Incomptines C and D, two heliangolides from Decachaeta incompta and their antiprotozoal activity

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Materials and Methods

General experimental procedures

Melting points (uncorrected) were determined on a Fisher-Johns apparatus. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. UV spectra were recorded on a Shimadzu UV 160U spectrophotometer. IR spectra were obtained on a Bruker Tensor 27 spectrometer. 1D and 2D NMR experiments were performed on a Varian Unity Plus 500 (\(^1\)H at 500 MHz; \(^{13}\)C at 125 MHz) spectrometer or on a Brucker Avance III (\(^1\)H at 400 MHz; \(^{13}\)C at 100 MHz). Chemical shifts were referred to TMS. EIMS were recorded on a JEOL JMS-AX505HA mass spectrometer. X-ray crystallographic analyses were carried out on a Bruker Smart Apex CCD diffractometer with graphite-monochromated Mo KR radiation (\(\lambda = 0.71073 \text{ Å}\)). The structures were solved by direct methods using the program SHELXS. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were included at calculated positions and were not refined. Column chromatography (CC) was performed on silica gel 60 (Merck G) and assisted with vacuum, while silica gel 230—400 mesh (Macherey–Nagel) was used for flash chromatography. TLC was carried out on precoated Macherey–Nagel Sil G/UV254 plates of 0.25 thickness, and spots were visualized by spraying with 3% CeSO\(_4\) in H\(_2\)SO\(_4\) 2 N followed by heating. HPLC separations were performed on a Waters 2795 Alliance equipped with a Waters 996 detector photodiode array collecting data at 240 nm; a column Waters Spherisorb S5 ODS2 (4.5 x 250 mm, 5 \(\mu\)m) was used with a logarithmic gradient from 96% of
aqueous acetic acid 2% and 4% of CH₃CN to 50% of aqueous acetic acid 2% and 96% of CH₃CN over a period of 50 min at flow rate of 1 mL/min. All the solvents were HPLC grade.

Isolation procedures of 1-4

The extract of CH₂Cl₂ (22.86 g) was subject to vacuum column chromatography (VCC, silica gel 60G, 9.0x11.0 cm, 500 mL) beginning with CHCl₃-EtOAc mixtures: 95:5 (fr. A, 1.53 g), 9:1 (fr. B, 2.57 g) and 8:2 (fr. C, 4.14 g). Fraction A was percolated through a bentonite clay (tionsil) column (1.5x6.0 cm) with CH₂Cl₂ (50 mL) to eliminate colored and polar compounds. After evaporation, the residue crystallized from CH₂Cl₂-hexane yielding incomptine A (1, 370.4 mg). Fraction B was also percolated through a bentonite clay (tionsil) column (2.5x6.0 cm) with CH₂Cl₂ (150 mL), and the residue (2.42 g) was subject to VCC (silica gel 60G, 4.5x7.5 cm, 25 mL) using hexane-EtOAc mixtures: 7:3 (fr. B1), 6:4 (fr. B2), and 4:6 (fr. B3). Fraction B3 (1.37 g) was purified by VCC (silica gel 60G, 3.5x6.5 cm, 25 mL) using as eluents CHCl₃-EtOAc 9:1 to obtain a mixture (0.72 g) of 3 and 4. This mixture was separated by VCC (silica gel 60G, 3.5 x 6.5 cm, 25 mL) with hexane-EtOAc 7:3, giving 3 (62.2 mg) and 4 (93.6 mg). From fraction C, a solid was obtained, which was crystallized from CH₂Cl₂-hexane yielding incomptine B (2, 2.64 g).

The roots of D. incompta, dried and powered (700 g), were extracted by percolation with CH₂Cl₂ (8 L), to obtain 11.3 g of residue.

Crystal data of compounds 3-4

Incomptine C (3): C₁₉H₂₄O₇, Mᵣ 364.38, orthorhombic, space group P 2₁ 2₁ 2₁, a = 6.139(4) Å, α = 90°, b = 13.832(9) Å, β = 90°, c = 22.316(14) Å, γ = 90°, V = 1895(2) Å³, Z = 4, D_c = 1.277 Mg/m³, F(000) = 776, crystal dimensions/shape/color 0.382x0.068x0.062 mm/needle/colorless. Reflections collected
17703, independent reflections 2399. Number of parameters refined 239, final $R$ indices [$I>2\sigma(I)$] $R_1 = 0.0607$, $wR_2 = 0.1095$, $R$ indices (all data) $R_1 = 0.1185$, $wR_2 = 0.1276$.

Incomptine D (4): $C_{19}H_{24}O_7$, $M_r$ 364.38, orthorhombic, space group $P 2_1 2_1 2_1$, $a = 11.305(1)$ Å, $\alpha = 90^\circ$, $b = 11.603(1)$ Å, $\beta = 90^\circ$, $c = 14.882(1)$ Å, $\gamma = 90^\circ$, $V = 1957(3)$ Å$^3$, $Z = 4$, $D_c = 1.237$ Mg/m$^3$, $F(000) = 776$, crystal dimensions/shape/color 0.446x0.166x0.148 mm/prism/colorless. Reflections collected 19418, independent reflections 2642. Number of parameters refined 239, final $R$ indices [$I>2\sigma(I)$] $R_1 = 0.0545$, $wR_2 = 0.1216$, $R$ indices (all data) $R_1 = 0.0716$, $wR_2 = 0.1303$.

**Evaluation of antiprotozoal activity**

**Antiprotozoal assays**

*Entamoeba histolytica* strain HM1-IMSS used in all experiments was grown axenically at 37 °C in TYI-S-33 medium supplemented with 10% heat inactivated bovine serum. In the case of *Giardia lamblia*, strain IMSS: 8909:1 was grown in TYI-S-33 modified medium supplemented with 10% calf serum and bovine bile. The trophozoites were axenically maintained and for assays were employed in the log phase of growth. *In vitro* susceptibility tests were performed using a subculture method described previously [21]. Briefly, *E. histolytica* (6 x 10$^3$) or *G. lamblia* (5 x 10$^4$) trophozoites were incubated for 48 h at 37 °C in the presence of different concentrations (2.5–200 μg/mL) of pure compounds 3 and 4 in dimethyl sulfoxide (DMSO). Each test included metronidazole and emetine as standard amoebicidal and giardicidal drugs, a control (culture medium plus trophozoites and DMSO), and a blank (culture medium). After incubation, the trophozoites were detached by chilling, and 50 μL samples of each tube were subcultured in fresh medium for another 48 h, without antiprotozoal samples. The final number of parasites was determined with a haemocytometer, and the percentages of trophozoites growth inhibition were calculated by comparison with the control culture. The results
were confirmed by a colorimetric method: the trophozoites were washed and incubated for 45 min at 37 °C in phosphate buffer saline with MTT (3-[4, 5-dimethylthiazol-2-il]-2, 5-diphenyl tetrazolium bromide) and phenazine methosulfate. The dye produced (formazan) was extracted, and the absorbance was determined at 570 nm. The experiments were performed in duplicate for each protozoan and repeated at least three times.

**Statistical analysis**

Data were analysed using probit analysis. The percentage of trophozoites surviving was calculated by comparison with the growth in the control group. The plot of probit against log concentration was made; the best straight line was determined by regression analysis, and the 50% inhibitory concentration (IC$_{50}$) values were calculated together with the 95% confidence limits.
Chromatographic profile (measured at 240 nm) of pure compounds, CHCl₂ extracts from leaves and roots of *D. incompta*.
$^1$H NMR Spectrum, determined in CDCl$_3$ at 500 MHz.

$^{13}$C NMR Spectrum, determined in CDCl$_3$ at 125 MHz.
DEPT NMR Spectrum, determined in CDCl₃ at 125 MHz.

COSY spectrum, determined in CDCl₃ at 500 MHz.
NOESY spectrum, determined in CDCl₃ at 500 MHz.

HMBC spectrum, determined in CDCl₃ at 500 MHz.
$^1$H NMR Spectrum. determined in CDCl$_3$ at 500 MHz.

$^{13}$C NMR Spectrum. determined in CDCl$_3$ at 125 MHz.
\(^1\)H NMR Spectrum, determined in CDCl\(_3\) at 500 MHz.

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