Supporting Information to:

Antileishmanial Sesquiterpenes from the Brazilian Red Alga

*Laurencia dendroidea*

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General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 341LC polarimeter using a Na lamp at 20 °C. IR spectra were obtained with a Perkin-Elmer spectrometer FT-IR. $^1$H-NMR, $^{13}$C-NMR, DEPT-135, COSY, HMQC, HMBC, and NOESY spectra were measured employing a Bruker Avance instrument operating at 300 MHz for $^1$H-NMR and at 75 MHz for $^{13}$C-NMR in CDCl$_3$. EI-MS spectra were obtained with an Agilent 6890N chromatograph with an Agilent 50973 mass analyzer. HR-EI-MS spectra were recorded on an UlrOTOF (Bruker Daltonics) mass spectrometer. Column chromatography was performed with silica gel 60 (70–230 mesh; Merck), and thin-layer chromatography was carried out with silica gel GF254 plates using a solution of 2% Ce(SO$_4$)$_2$ in H$_2$SO$_4$ followed by heating to visualize spots.

Plant material

The red seaweed Laurencia dendroidea was collected in supralitoral regions at two distinct areas of the southeastern Brazilian coast: Biscaia inlet (23°1'45.36"S, 44°14'9.37"W), Angra dos Reis, Rio de Janeiro State in September 2007 and Manguinhos Beach (23°1'45.36"S, 44°14'9.37"W), Espírito Santo State in March 2008. Botanical identification was made by Mutue T. Fujii and Joel C. de Paula, and voucher specimens (Rio de Janeiro: RFA 12456 and Espirito Santo: RFA 12457) were deposited at the Herbarium of the Rio de Janeiro Federal University, Brazil.

Extraction and isolation

The air-dried specimens of Laurencia dendroidea from Angra dos Reis, Rio de Janeiro (34.3 g) and Manguinhos, Espirito Santo (60.4 g) were extracted with a mixture of dichloromethane–methanol (1:1) at room temperature, yielding the crude extracts A and B, respectively. Extract A was concentrated to give a green residue (1.08 g), which was resuspended in water and partitioned with CH$_2$Cl$_2$–AcOEt and n-BuOH. All the fractions were analyzed by TLC. The CH$_2$Cl$_2$ and AcOEt fractions (448 mg) were assembled and submitted to column chromatography on silica gel (1 × 30 cm), eluting with an increasing gradient of AcOEt (0–100%) in n-hexane (in steps of 10%, 40 mL each), and separated into 15 fractions on the basis of TLC analysis (A–O). Fraction B, eluted with n-hexane–AcOEt (9:1), yielded the sesquiterpene 1 (6 mg). The sesquiterpene 2 (13 mg) was isolated from fraction C (n-hexane–AcOEt 9:1). From fraction E (n-hexane:AcOEt 9:1), after column
chromatography on silica gel (1 × 35 cm) eluting with an increasing gradient of CH2Cl2 (0–100% in steps of 10%, 50 mL each) in n-hexane, the sesquiterpenes 3 (n-hexane–CH2Cl2 6:4, 43 mg) and 4 (n-hexane–CH2Cl2 1:1, 26 mg) were isolated.

Extract B was concentrated to give a green residue (4.54 g), which was partitioned with H2O and CH2Cl2. The resulting CH2Cl2 fraction (1.76 g) was submitted to column chromatography on silica gel 60 (2 × 30 cm; Merck), eluting with an increasing gradient of AcOEt (0–100% in steps of 10%, 120 mL) in n-hexane, and separated into 9 fractions on the basis of TLC analysis (A–I). From fraction D (n-hexane–AcOEt 8:2), after column chromatography on silica gel (1.5 × 30 cm) eluting with an increasing gradient of CH2Cl2 (0–100%, in steps of 10%, 60 mL) in n-hexane, the sesquiterpene 5 (14 mg) was isolated. From fraction E, the sesquiterpene 4 (390 mg) was identified.

The complete 1H- and 13C-NMR data and two-dimension experiments are present for the first time (Figs. 1S–31S).

Compound 1 was isolated as a white solid. The molecular formula C15H23Br2Cl was established by HR-EI-MS. Comparison of the spectroscopic data with the corresponding literature revealed that compound 1 was identical to (+)-obtusane, which was originally described from Laurencia obtusa (Hudson) J.V. Lamouroux, while the (−) enantiomer was isolated from Laurencia nipponica Yamada.

The EI-MS spectra of compound 2 displayed a peak at m/z = 222. Comparison of the spectroscopic data with that in the literature led to the identification of a triquinane sesquiterpene.

Compound 3 was obtained as a colorless oil. The presence of a hydroxy group was evident from an IR absorption at 3458 cm⁻¹. The 1H-NMR spectrum in CDCl3 displayed signals corresponding to hydrogen atoms linked to heterosubstituted carbons at δH = 4.60 (1H, d, J = 3.0 Hz) and 4.14 (1H, ql, J = 4.0 Hz) and two olefinic protons as singlets at δH = 4.79 and 5.12. Signals corresponding to three tertiary methyl groups appeared at δH = 1.70, 1.08, and 1.07. The 13C-NMR spectrum in association with DEPT-135 and HMQC revealed 15 carbons, of which three were due to CH3 groups (δC = 24.2, 20.7, and 19.4); five to CH2 groups (δC =
115.9, 38.6, 38.0, 29.3, and 25.6); two to CH groups ($\delta_C = 72.2, 70.8$); and five to non-hydrogenated carbons ($\delta_C = 140.8, 128.0, 124.1, 49.1$, and 43.1). A $^1$H–$^1$H COSY experiment established two hydrogen sequences: H1–H2–H1–H2–and H8–H9–H10. HMBC data were used to confirm these fragments and to establish the connectivity between them. The correlation of H3-11 and H3-12 to the opposite carbons C-12 and C-13 and the correlation of both to C-6, C-10, and C-11 permitted placement of the gem-dimethyl group at C-11. Correlation of H-14 with C6, C-7, and C-8 established ring A. These data and the HMBC from H-1 to C-7 indicated the presence of a chamigrene structure. HMBCs of H3-15 with C-2, C-3, and C-4 contributed to elucidate ring B. The relative configuration of compound 3 was assigned on the basis of the coupling constants and 2D NOESY experiments. NMR data of 3 show a close resemblance to those of elatol isolated from Chondrophycus cartilagineus (Yamada) Garbary & Harper (as Laurencia cartilaginea), Laurencia elata (C. Agardh) J.D. Hooker & Harvey, Laurencia obtusa (Hudson) J.V. Lamouroux, Laurencia rigida J. Agardh, and Laurencia majuscula (Harvey) A.H.S. Lucas, while the specific rotations are opposite in sign. Elatol was also isolated from a Brazilian algae population of Laurencia microcladia Kützing. These facts suggest that 3 is an enantiomer of elatol, originally found in a Caribbean population of Laurencia obtusa.

Compound 4 was characterized as the known sesquiterpene obtusol, isolated from both sea hare (Aplysia dactylomela Rang) and Laurencia obtusa, while compound 5 was identified as cartilagineol, originally isolated from Chondrophycus cartilagineus (as Laurencia cartilaginea).

$\alpha$-Obtusane (1): White solid; purity 90% (HPLC); Rf = 0.89 (CH$_2$Cl$_2$–Hex 7:3); $[\alpha]_D$: +18.8 (c 0.05, CHCl$_3$); IR (KBr): 2926, 1716, 1456, 911, 870, 804, 738 cm$^{-1}$. NMR data: see Table 1S; EI-MS: m/z (rel. int. %) = 402 (1), 400 (4), 398 (5), 396 (2), 385 (3), 383 (5), 381 (2), 319 (11), 318 (15), 316 (10), 283 (21), 281 (22), 239 (10), 237 (28), 202 (12), 201 (54), 109 (100), 107 (39).

Triquinane (2): Colorless oil; purity 96% (HPLC); Rf = 0.62 (CH$_2$Cl$_2$–Hex 7:3); $[\alpha]_D$: −6.75 (c 0.67, CHCl$_3$); IR (mineral oil): 3035, 2932, 2865, 1720, 1457, 1375, 1239, 1165, 1082, 1006, 900 cm$^{-1}$; NMR data: see Table 1S; EI-MS: m/z (rel. int. %) = 222 (3), 207 (1), 189 (1), 135 (37), 86 (100), 85 (14), 81 (32), 79 (10).
(−)-Elatol (3): Colorless oil; purity 99% (HPLC); Rf = 0.52 (CH₂Cl₂–Hex 7:3); [α]D: −66.2 (c 0.13, CHCl₃); IR (mineral oil): 3458, 2970, 2947, 1718, 1676, 898, 817, 736 cm⁻¹; NMR data: see Table 1S; EI-MS: m/z (rel. int. %) = 319 (2), 317 (1), 299 (3), 297 (3), 253 (8), 237 (40), 236 (18), 235 (100), 217 (7), 209 (15), 207 (29), 200 (9), 199 (36).

Obtusol (4): White solid; purity 96% (HPLC); Rf = 0.43 (CH₂Cl₂–Hex 7:3); [α]D: +9.61 (c 0.05, CHCl₃); IR (KBr): 3465, 2969, 1640, 1441, 907, 813, 792 cm⁻¹; NMR data: see Table 1S; EI-MS: m/z (rel. int. %) = 319 (25), 318 (17), 317 (100), 316 (13), 315 (76), 299 (17), 297 (18), 235 (23), 217 (12), 200 (18), 199 (47).

Cartilagineol (5): White solid; purity 98% (HPLC); Rf = 0.45 (CH₂Cl₂–Hex 7:3); [α]D: −83.5 (c 0.14, CHCl₃); IR (KBr): 3410, 2968, 1637, 1448, 969, 902, 736, 712 cm⁻¹; NMR data: see Table 1S; EI-MS: m/z (rel. int. %) = 334 (3), 332 (2), 320 (4), 319 (24), 318 (16), 317 (100), 316 (13), 315 (75), 300 (4), 299 (25), 279 (8), 253 (7), 235 (13), 217 (31), 201 (10), 200 (22), 199 (49), 197 (21).

Antileishmanial assay

Parasites: Leishmania amazonensis (WHOM/BR/75/Josefa) transfected with the gene of green fluorescent protein was used. The parasites were periodically isolated from cutaneous lesions of experimentally infected mice and maintained in culture as the insect-stage promastigote forms at 26 °C in M199 medium (Sigma) supplemented with 10% fetal calf serum (HIFCS; Cultilab) and 40 μg/mL gentamicin (Schering–Plough) for no more than four passages. They were selected periodically.

Anti-promastigote activity: For anti-promastigote activity, fluorescent promastigotes were plated in triplicate at 10⁵ parasites/well with varying concentrations of test compounds (0, 0.1, 1, 10, and 100 μg/mL) in a final volume of 200 μL of medium M199 containing 5% HIFCS and 1% Hybri-Max dimethyl sulfoxide (DMSO; Sigma). After 72 h at 27 °C, the fluorescence intensity of the cultures was measured using a plate-reader fluorometer (Fluoroskan) set at 435 nm excitation/538 nm emission. All cultures were performed in triplicate, and the results were expressed as percentage inhibition in relation to controls cultured in medium alone.
Anti-amastigote activity: For anti-amastigote activity, mouse peritoneal macrophages were harvested from the peritoneal cavities of BALB/c mice in ice-cold DMEM medium (Sigma). The cells were plated at 2 \times 10^6/mL (0.4 mL/well) in Lab-Tek 8-chamber slides (Nunc) and incubated at 37 °C under an atmosphere of 4% CO₂ for 1 h. Non-adherent cells were removed by washing with prewarmed phosphate-buffered saline (PBS). Adherent macrophages were infected with *L. amazonensis* promastigotes (stationary growth phase) at a 5:1 parasite/macrophage ratio and incubated for 1 h at 35 °C, 5% CO₂. The cell monolayers were washed three times with prewarmed PBS to remove free parasites, and 0.4 mL of the test compounds’ complete medium at different concentrations was added in duplicate for a further 72 h. The cultures were then fixed with absolute methanol and stained with Giemsa. The numbers of intracellular amastigotes were determined by counting at least 100 macrophages per sample, and the results were expressed as the 50% effective dose (ED₅₀), which was determined by logarithm regression analysis. The reference drug used was the trivalent antimonial potassium antimony(III) tartrate hydrate (≥99.0% purity; Sigma–Aldrich).

Nitric oxide production
After 72 h of treatment, NO production by the infected macrophages was measured by assessing the level of nitrite in the culture supernatants. Briefly, 100 mL of fresh Griess reagent [1% sulfanilamide p-aminobenzene sulfonamide, 5% H₃PO₄, 0.1% n-(1-naphthyl)ethylenediamine dihydrochloride; Sigma] was added to equal volumes of culture supernatants. After 10 min of incubation at room temperature, the optical density at 570 nm was measured. Macrophages stimulated with IFN-γ (10 mg/mL, ≥98.0% purity; Sigma–Aldrich) were used as positive control. The nitrite concentration was determined by using NaNO₂ diluted in DMEM as the standard and DMEM plus Griess reagent alone as the blank.

Cytotoxicity against mammalian cells
For cytotoxicity against mammalian cells, single-cell suspensions of cervical lymph nodes of BALB/c mice were freshly prepared in DMEM supplemented with 40 μg/mL gentamicin sulfate (Schering–Plough), 25 mM HEPES (Sigma), 4.7 μg/mL sodium bicarbonate, and 5 mM/L β-mercaptoethanol and cultured for 72 h at 37 °C with varying concentrations of the test compounds. The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was measured using an assay kit (Doles Reagentes). Maximum and minimum
release values were cells cultured with 2% Triton X-100 or 1% DMSO, respectively. The IC$_{50}$ values were calculated by linear regression analysis. To test for cytotoxicity to macrophages, the supernatants of the infected macrophages treated as above for anti-amastigote activity were assayed as described for lymph node cells {Authors: please verify changes}.

**Statistical analysis**

Data were analyzed by Student’s $t$-test when two groups were compared and by one-way ANOVA for more than two groups, followed by Tukey’s multiple comparisons post-test, using the GraphPad Program. $P$ values of less than 0.05 were considered significant.
### Table 1S

| Position | $\delta^1$ | $\delta^2$ (multiplicity)$^b$ | $\delta^3$ (multiplicity)$^b$ | $\delta^4$ | $\delta^5$ (multiplicity)$^b$ | $\delta^6$ | $\delta^7$ (multiplicity)$^b$ | $\delta^8$ | $\delta^9$ (multiplicity)$^b$ | $\delta^{10}$ | $\delta^{11}$ (multiplicity)$^b$ | $\delta^{12}$ | $\delta^{13}$ (multiplicity)$^b$ | $\delta^{14}$ | $\delta^{15}$ (multiplicity)$^b$ | $\delta^{16}$ | $\delta^{17}$ (multiplicity)$^b$ | $\delta^{18}$ | $\delta^{19}$ (multiplicity)$^b$ |
|----------|-----------|-----------------------------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|-----------------------------|-----------|
| 1        | 25.5      | 1.87 (m)                    | 69.9                        | -         | 25.6                        | 1.74 (m)  | 24.3                        | 2.05      | 1.70                        | 2.38      | 3.08                        | 3.38      | 4.45                        | 4.77      | 2.78                        | 4.41      | 1.60                        |
| 2        | 40.4      | 2.26 (m)                    | 85.3                        | -         | 29.3                        | 2.30 (m)  | 32.5                        | 3.28      | 1.82                        | 2.38      | 3.08                        | 3.38      | 4.45                        | 4.77      | 2.78                        | 4.41      | 1.60                        |
| 3        | 68.4      | -                           | 41.0                        | 1.62 (m)  | 128.0                       | -         | 67.6                        | 73.3      | -                           | 73.3      | -                           | 73.3      | -                           | 73.3      | -                           | 73.3      | -                           |
| 4        | 67.9      | 4.71 (dd, 12.4, 4.7)        | 47.3                        | 1.61 (m)  | 124.1                       | -         | 68.1                        | 57.2      | 4.45                        | 57.2      | 4.45                        | 57.2      | 4.45                        | 57.2      | 4.45                        | 57.2      | 4.45                        |
| 5        | 37.2      | 2.27 (m)                    | 49.7                        | 1.96 (dd, 14.4, 12.6)       | 38.6        | 2.59 (dl, 16.1)             | 37.1      | 1.94 (dd, 14.0, 11.7)       | 33.8      | 3.08 (d, 16.0)              | 2.78      | 3.08 (d, 16.0)              | 2.78      | 3.08 (d, 16.0)              | 2.78      | 3.08 (d, 16.0)              |
| 6        | 50.4      | -                           | 49.1                        | 1.46 (m)  | 140.8                       | -         | 141.2                       | 147.2     | -                           | 147.2     | -                           | 147.2     | -                           | 147.2     | -                           | 147.2     | -                           |
| 7        | 145.7     | -                           | 29.0                        | 1.51 (m)  | 124.8                       | -         | 141.2                       | -         | 147.2                       | -         | 147.2                       | -         | 147.2                       | -         | 147.2                       | -         | 147.2                       |
| 8        | 33.5      | 2.32 (m)                    | 57.7                        | 1.50 (m)  | 38.0                        | 2.63 (dd, 14.4, 3.0) | 38.5 | 2.62 (dd, 14.1, 3.1) | 39.4 | 2.70 (t, 11.9) | 2.45 | 2.70 (t, 11.9) | 2.45 | 2.70 (t, 11.9) |
| 9        | 35.8      | 2.27 (m)                    | 42.5                        | 1.38 (m)  | 72.2                        | 4.14 (ql, 3.0) | 71.9 | 4.10 (sl) | 69.6 | 3.68 (dt, 11.9, 4) | 4.64 | 3.68 (dt, 11.9, 4) | 4.64 | 3.68 (dt, 11.9, 4) |
| 10       | 63.5      | 4.44 (dd, 12.7, 4.5)        | 36.3                        | 1.72 (ddd, 11.4, 5.7, 2.3) | 70.8        | 4.60 (d, 3.0)              | 70.1      | 4.47 (d, 3.0)              | 76.4      | 4.41 (q, 3.7, 1.6)         | 76.4      | 4.41 (q, 3.7, 1.6)         | 76.4      | 4.41 (q, 3.7, 1.6)         | 76.4      | 4.41 (q, 3.7, 1.6)         |
| 11       | 43.9      | -                           | 27.9                        | 1.86 (ddd, 13.5, 6.9, 2.3) | 43.1        | -                         | 44.2      | -                           | 43.7      | -                           | 43.7      | -                           | 43.7      | -                           | 43.7      | -                           |
| 12       | 23.5      | 1.14 (s)                    | 22.3                        | 1.10 (s)  | 24.2                        | 1.08 (s)  | 24.2                        | 1.08 (s)  | 24.8                        | 1.31 (s)  | -                           | 1.31 (s)  | -                           | 1.31 (s)  | -                           | 1.31 (s)  | -                           |
| 13       | 17.4      | 0.96 (s)                    | 12.5                        | 0.87 (d, 6.1) | 20.7        | 1.07 (s)  | 20.6                        | 1.08 (s)  | 25.3                        | 1.04 (s)  | -                           | 1.04 (s)  | -                           | 1.04 (s)  | -                           | 1.04 (s)  | -                           |
| 14       | 114.8     | 5.26 (s)                    | 26.7                        | 1.07 (s)  | 115.9                       | 5.12 (sl) | 117.8                       | 5.39 (s)  | 114.7                       | 5.20 (sl) | -                           | 5.20 (sl) | -                           | 5.20 (sl) | -                           | 5.20 (sl) | -                           |
| 15       | 23.8      | 1.83 (s)                    | 19.5                        | 0.97 (d, 6.4) | 19.4        | 1.70 (sl) | 23.9                        | 1.83 (s)  | 33.1                        | 1.75 (sl) | -                           | 1.75 (sl) | -                           | 1.75 (sl) | -                           | 1.75 (sl) | -                           |
| OH       |           |                             |                             |           |                             |           |                             |           |                             |           |                             |           |                             |           |                             |           |                             |           |                             |

Values are ppm downfield from TMS, and assignments were made by HMQC experiments.

\[ J \] values (in Hz) in parentheses.
Fig. 1S $^1$H-NMR (300 MHz, CDCl$_3$) spectrum for compound 1.
Fig. 2S $^{13}$C-NMR (50 MHz, CDCl$_3$) spectrum for compound 1.
Fig. 3S DEPT-135 (50 MHz, CDCl₃) spectrum for compound 1.
Fig. 4S HMOC (300 MHz, CDCl₃) spectrum for compound 1.
Fig. 5S COSY (300 MHz, CDCl₃) spectrum for compound 1.
Fig. 6S HMBC (300 MHz, CDCl₃) spectrum for compound 1.
Fig. 7S $^1$H-NMR (300 MHz, CDCl$_3$) spectrum for compound 2.
Fig. 8S $^{13}$C-NMR (50 MHz, CDCl$_3$) spectrum for compound 2.
Fig. 9S DEPT-135 (50 MHz, CDCl₃) spectrum for compound 2.
Fig. 10S HMQC (300 MHz, CDCl₃) spectrum for compound 2.
Fig. 11S COSY (300 MHz, CDCl₃) spectrum for compound 2.
Fig. 12S HMBC (300 MHz, CDCl₃) spectrum for compound 2.
Fig. 13S ¹H-NMR (300 MHz, CDCl₃) spectrum for compound 3.
Fig. 14S $^{13}$C-NMR (50 MHz, CDCl$_3$) spectrum for compound 3.
Fig. 15S DEPT-135 (50 MHz, CDCl₃) spectrum for compound 3.
Fig. 16S HMQC (300 MHz, CDCl₃) spectrum for compound 3.
Fig. 17S COSY (300 MHz, CDCl3) spectrum for compound 3.
Fig. 18S HMBC (300 MHz, CDCl₃) spectrum for compound 3.
Fig. 19S NOESY (300 MHz, CDCl₃) spectrum for compound 3.
Fig. 20S $^1$H-NMR (300 MHz, CDCl$_3$) spectrum for compound 4.
Fig. 21S $^{13}$C-NMR (50 MHz, CDCl$_3$) spectrum for compound 4.
Fig. 22S DEPT-135 (50 MHz, CDCl₃) spectrum for compound 4.
Fig. 23S HMOC (300 MHz, CDCl₃) spectrum for compound 4.
Fig. 24S COSY (300 MHz, CDCl3) spectrum for compound 4.
Fig. 25S HMBC (300 MHz, CDCl₃) spectrum for compound 4.
Fig. 26S $^1$H-NMR (300 MHz, CDCl$_3$) spectrum for compound 5.
Fig. 27S $^{13}$C-NMR (50 MHz, CDCl$_3$) spectrum for compound 5.
Fig. 28S DEPT-135 (50 MHz, CDCl₃) spectrum for compound 5.
Fig. 29S HMQC (300 MHz, CDCl₃) spectrum for compound 5.
Fig. 30S COSY (300 MHz, CDCl₃) spectrum for compound 5.
Fig. 31S HMBC (300 MHz, CDCl₃) spectrum for compound 5.