Coumarins from *Galipea panamensis* and Their Activity against *Leishmania panamensis*

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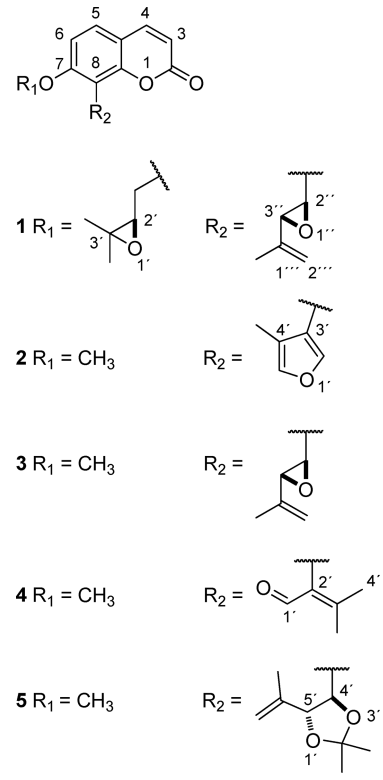
Two new coumarin compounds (**1** and **2**), phebalosin (**3**), its derived artifact murralongin (**4**), and murrangatin acetonide (**5**) were isolated from the leaves of *Galipea panamensis*. The structures of **1** and **2** were assigned as 7-{[(2*R**)-3,3-dimethyloxiran-2-yl]methoxy}-8-[(2*R**,3*R**)-3-isopropenyloxiran-2-yl]-2*H*-chromen-2-one and 7-methoxy-8-(4-methyl-3-furyl)-2*H*-chromen-2-one, respectively, on the basis of their spectroscopic data (primarily NMR and MS). Compounds **1**−**3** were tested against axenic amastigote forms of *Leishmania panamensis* and displayed 50% effective concentrations (EC_{50}) of 9.9, 10.5, and 14.1 μg/mL, respectively. These three compounds also displayed cytotoxicity (IC_{50}) at concentrations of 9.7, 33.0, and 20.7 μg/mL, respectively, on human promonocytic U-937 cells.

Leishmania (Viannia) panamensis is the causal agent of most cutaneous leishmaniasis in Central and South America, accounting for about 50% of all cases in eight endemic countries.[1] Chemotherapy is largely based on antimony compounds such as Pentostam and Glucantime; however, renal and cardiac toxicity[1,2] together with clinical resistance against these commonly used antimonial agents[3] have prompted a search for new chemicals in order to overcome these flaws. Availability of effective pharmaceuticals in remote places is a problem where the use of folk remedies based on medicinal plants for the treatment of leishmaniasis is common practice.[4] Therefore, phytochemical research on these plants is considered essential.

The plant family Rutaceae is comprised of around 160 genera and approximately 1900 species that are primarily distributed in Australia, South Africa, and tropical America.[5] The genus *Galipea*, composed of approximately 20 species, deserves special mention, as some of its members are known to produce highly active metabolites against different *Leishmania* species.[6,7] Phytochemical studies have been reported for only five species in this genus (*G. bracteata*, *G. granulose*, *G. longiflora*, *G. officinalis*, and *G. trifoliate*), affording a diversity of secondary metabolites including chalcones,[8] chromones,[9] coumarins,[10] flavones,[11] and quinoline alkaloids.[6,12−15] *Galipea panamensis*, a small tree found in eastern Panamá and northwest Colombia, which has a distinctive corymbose inflorescence and white fragrant flowers,[16] is a member of the genus for which no phytochemical studies have been published. This paper reports the isolation of two new and three known coumarins from *G. panamensis* and their activity in response to in vitro cultures of *L. panamensis*.

Ethyl acetate extracts of the leaves of *G. panamensis* T. S. Elias (Rutaceae) yielded compounds **1** and **2** and the known coumarins 8-(3-isopropenyloxiran-2-yl)-7-methoxy-2*H*-chromen-2-one (**3**, phebalosin),[17,18] 2-(7-methoxy-2-oxo-2*H*-chromen-8-yl)-3-methylbut-2-enal (**4**, murralongin),[19,20] and 8-(5-isopropenyl-2,2-dimethyl-1,3-dioxolan-4-yl)-7-methoxy-2*H*-chromen-2-one (**5**, *threo*-murrangatin acetonide).[20,21] Compound **4** has been reported as an acidic rearrangement product of phebalosin (**3**).[19,20] We were not able to detect this compound in a freshly prepared extract. Moreover, passing a pure sample of **3** through a silica gel column resulted in complete transformation into murralongin (**4**) after elution with dichloromethane, confirming the artifact nature of **4**. Interestingly, compound **5**, a semisynthetic compound obtained by treatment of *threo*-murrangatin with acetone in acidic media,[20] was recently reported as a possible artifact isolated from *Murraya omphalocarpa*, but with no apparent reason.[21] However, in the present study, compound **5** was isolated without use of acetone during the extraction or purification processes, and it was also detected in freshly prepared ethyl acetate extracts. Therefore, it is unlikely that the acetonide moiety in this compound comes from artificial origin. Compound **3** was obtained as the major compound (38 mg kg^{-1}) followed by compound **1** (35 mg kg^{-1}). The levels of compounds **2** and **5** were 30 and 12 mg kg^{-1}, respectively.



Compound **1**, $[\alpha]_D$ −40 (23 °C, *c* 0.25, $CHCl_3$), displayed 14 signals in the ^1H NMR spectrum (see Experimental Section and

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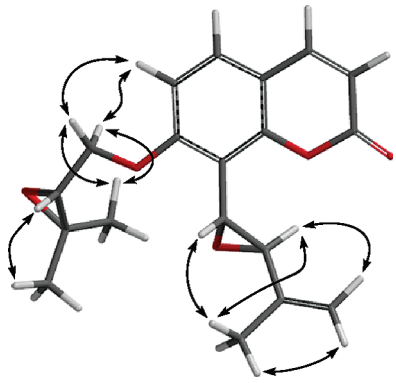

Figure 1. Observed NOESY interactions of compound **1**.

Supporting Information). A pair of doublets [δ 6.27 and 7.61 (each 1H, d, $J = 9.8$ Hz)] confirmed an AB spin system characteristic of H-3 and H-4 of a coumarin nucleus.[10] Two *ortho*-coupled protons [δ 7.40 and 6.93 (each 1H, d, $J = 8.7$ Hz)] were attributed to H-5 and H-6 in the aromatic section. The ^{1}H−^{1}H COSY spectra evidenced correlations between a vinylic methyl group (δ 1.85) and signals at δ 5.08 and 5.29 (each 1H). The latter two signals displayed correlations with a carbon atom at δ 113.6 in the HMQC spectra. This confirmed the presence of an isopropenyl group (−(CH_3)C═CH_2). Further inspection of the ^{1}H NMR spectrum revealed an AB quartet spin system (two oxymethine protons at δ 3.92 and 4.02 with a vicinal coupling constant of 2.1 Hz). This system, found in phebalosin (**3**) and other related coumarins,[10] was consistent with an isopropenyloxyran side chain. This was confirmed by NOESY experiments (Figure 1), in which strong correlations were observed between the oxymethine protons at δ 3.92 and 4.02 with the vinylic methyl group at δ 1.85, the latter also correlating with the vinylic proton at δ 5.08. The five remaining signals in the ^{1}H NMR spectrum were identified as two methyl singlets (δ 1.38 and 1.40), an oxymethine proton centered at δ 3.18 (1H, m), and signals at δ 4.17 and 4.34 (each 1H, m), which displayed cross-peaks in the HMQC spectra with only one carbon atom (δ 68.5), revealing a diastereotopic oxymethylene. The ^{1}H−^{1}H COSY spectrum revealed an ABX spin system relating the protons at δ 4.17 and 4.34 to the oxymethine proton at δ 3.18. Further connection between the ABX system and the methyl groups was observed in the NOESY data (Figure 1), for which strong correlations were evidenced between the oxymethine proton at δ 3.18 and the methyl at δ 1.40 and between the oxymethylene protons and the methyl at δ 1.38, thus establishing the presence of the (3,3-dimethyloxiran-2-yl)methoxy side chain. Assignment of this side chain to C-7 of the coumarin nucleus was confirmed by a strong NOESY correlation between the oxymethylene protons and the aromatic proton (H-6). Thus, **1** was determined to be 7-[(3,3-dimethyloxiran-2-yl)methoxy]-8-(3-isopropenyloxiran-2-yl)-2*H*-chromen-2-one. Further HMBC and HMQC correlations were used to assign the other ^{1}H and ^{13}C signals (see Experimental Section and Supporting Information). The structure was supported by HRTOFESIMS (*m/z* 351.1194, calcd for $C_{19}H_{20}O_5Na$, 351.1208).

Information about the relative configuration of compound **1** was gathered from careful analysis of NOESY spectra with the aid of quantum chemical calculations. In the case of the isopropenyloxyran side chain, a *trans* configuration for the epoxide was inferred from the vicinal coupling constant of 2.1 Hz for the oxymethine AB spin system. This configuration was also deduced from NOESY spectra due to the previously mentioned correlation between vinylic methyl protons and the oxymethine protons at C-2″ and C-3″, a situation only possible for the *trans* epoxide during rotation of the C(sp^2)−C-3″ bond (Supporting Information). In fact, simulation of NOESY spectra for the four diastereoisomers displayed this double correlation only for 7-(2*R**)-8-(2*R**,3*R**)-**1** and 7-(2*S**)-8-(2*R**,3*R**)-**1** diastereoisomers but not for 7-(2*R**)-8-(2*R**,3*S**)-**1** and 7-(2*S**)-8-(2*R**,3*S**)-**1** (Supporting Information). Differentiation between 7-(2*R**)-8-(2*R**,3*R**)-**1** and 7-(2*S**)-8-(2*R**,3*R**)-**1** isomers is less straightforward, as inspection of molecular models show that all observed NOESY correlations are possible in both structures. However, it is worth mentioning that the 7-(2*S**)-8-(2*R**,3*R**)-**1** isomer allows a closer proximity between the proton at C-2′ and the vinylic methyl protons than the 7-(2*R**)-8-(2*R**,3*R**)-**1** isomer. This proximity is reflected in a NOESY correlation between those protons in the simulated spectra of 7-(2*S**)-8-(2*R**,3*R**)-**1** that is absent in the other isomer. In our case we did not observe any NOESY correlation among these protons, and therefore, the 7-(2*R**)-8-(2*R**,3*R**)-**1** configuration is suggested. However, further experiments are needed to fully differentiate this compound from the 7-(2*S**)-8-(2*R**,3*R**) diastereoisomer.

The ^{1}H NMR spectra of compound **2** displayed 8 signals integrating for 12 protons (see Experimental Section and Supporting Information) attributable to a vinylic methyl group [δ 1.89 (3H, d, $J = 0.5$ Hz)], an OCH_3 group [δ 3.88 (3H, s)], four doublets [δ 6.25, 7.67 (each 1H, d, $J = 10.0$ Hz) and δ 6.94, 7.45 (each 1H, d, $J = 8.5$ Hz)] characteristic of H-3, H-4, H-6, and H-5 in the coumarin nucleus, a multiplet at δ 7.34 (1H, brs), and a doublet at δ 7.48 (1H, d, $J = 1.2$ Hz). ^{13}C NMR and DEPT spectra displayed 15 signals (see Experimental Section and Supporting Information), representing two methyl, six methine, and seven quaternary carbons. Positive ion APCIMS analysis afforded a peak at *m/z* 257 $[M + H]^+$ (100), which together with the ^{1}H and ^{13}C NMR spectra established the molecular formula $C_{15}H_{12}O_4$. The aromatic signals at δ 7.34 and 7.48 in conjunction with the molecular formula revealed the presence of a 3,4-disubstituted furan ring. Mutual HMBC correlations between the OCH_3 protons and C-7 (δ 160.3), and the later with H-5 (δ 7.45), placed the OCH_3 at C-7. Thus, the structure of **2** was 7-methoxy-8-(4-methyl-3-furyl)-2*H*-chromen-2-one. All carbon atoms and protons were assigned with the aid of HMQC, HMBC, and COSY spectra (see Experimental Section and Supporting Information).

Compounds **1**−**4** were tested against axenic amastigote forms of *L. panamensis*. Compound **5** was not assayed due to an insufficient amount available. Compounds **1**−**3** displayed 50% effective concentration (EC_{50}) in the range 10−15 μg/mL, with compounds **1** and **2** being the most active (10 μg/mL each). Compound **4** was inactive in the leishmanicidal assay ($EC_{50} > 100$ μg/mL). Compounds **1**−**3** were cytotoxic to human promonocytic U-937 cells with CC_{50} of 9.7, 33.0, and 20.7 μg/mL, respectively. Murralongin (**4**) showed a CC_{50} of 121.4 μg/mL. This analysis afforded selectivity indexes (defined as CC_{50} U937/EC_{50}) of 1.0, 3.0, and 1.5 for compounds **1**−**3**, respectively. Compound **2**, the most selective, was tested against intracellular amastigotes but displayed an EC_{50} superior to its CC_{50} (>33 μg/mL). Therefore, it is not clear whether the in vitro activity of these metabolites is due to its general cytotoxic activity or if they possess a selective mode of action against *L. panamensis*. However, these results suggest that both cytotoxic and leishmanicidal activities can be differentially modulated by introduction of substituents at positions C-7 and C-8 in chromenone compounds.

Experimental Section

General Experimental Procedures. Optical rotations were obtained using a cell (1.5 mL) with 1 dm path length on a PolAAr 32 polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum RX I FT-IR system in a KBr disk. UV spectra were obtained in MeOH, using a GENESYS 2PC spectrophotometer. ^{1}H NMR (400 and 300 MHz) and ^{13}C NMR (100 and 75 MHz) spectra (all in $CDCl_3$) were recorded on Bruker AMX 400 and/or Bruker AMX 300 NMR spectrometers, using TMS as internal standard. APCIMS and HRTOFESIMS were run on a Waters Micromass LCT mass spectrometer. Silica gel 60 (Merck 0.063−0.200 mesh) was used for column chromatography, and precoated silica gel plates (Merck 60 F_{254} 0.2 mm) were used for TLC. For visualization purposes, TLC plates were sprayed with a mixture of anisaldehyde−sulfuric acid−acetic acid−methanol (0.1:1:2:17) and heated to 100−105 °C.

Plant Material. Healthy plants of *Galipea panamensis* were collected in Apartadó (Antioquia), Colombia, during June 2008. A

voucher specimen is deposited at Jardín Botánico Joaquin Antonio Uribe, Medellin, Colombia (voucher number JAUM-50622).

Extraction and Isolation. Powdered leaves (1.0 kg) of *G. panamensis* were extracted successively with petroleum ether, EtOAc, and MeOH (10 L each) in a percolator at room temperature and concentrated in vacuo to give the corresponding extract (8, 34, and 44 g, respectively). The ethyl acetate extract was subjected to silica gel column chromatography (5 × 80 cm) eluting with a step gradient of *n*-hexane−ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, each 500 mL), to obtain 10 fractions (F1−F10) collected on the basis of their TLC profiles. Fractions F4 and F5 were recognized as the most interesting ones, due to the appearance of blue spots after spraying with anisaldehyde reagent. Compounds **2** (30 mg) and **3** (38 mg) were isolated from F4, and compounds **1** (35 mg) and **5** (12 mg) from F5, by preparative TLC using CH_2Cl_2−EtOAc (4:1), except for compound **2**, for which an *n*-hexane−ethyl acetate (4:1) mixture was employed. Compound **4** appeared during purification of phebalosin (**1**).

7-{[(2*R)-3,3-Dimethyloxiran-2-yl]methoxy}-8-[(2*R**,3*R**)-3-isopropenyloxiran-2-yl]-2*H*-chromen-2-one (1):** white, amorphous powder; $[\alpha]_D$ −40 (23 °C, *c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 214 (2.5), 251 (1.8), 322 (2.3) nm; IR (KBr) ν_{max} 2924, 1735 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.38 (3H, s, 3′-CH_3), 1.40 (3H, s, 3′-CH_3), 1.85 (3H, s, 1‴-CH_3), 3.18 (1H, m, H-2′), 3.92 (1H, m, H-3″), 4.02 (1H, dd, J = 10.9, 2.1 Hz, H-2″), 4.17−4.34 (2H, m, −OCH_2−), 5.08 (1H, s, H-2‴), 5.29 (1H, d, J = 7.8 Hz, H-2‴), 6.27 (1H, d, J = 9.8 Hz, H-3), 6.93 (1H, d, J = 8.7 Hz, H-6), 7.40 (1H, d, J = 8.7 Hz, H-5), 7.61 (1H, d, J = 9.8 Hz, H-4); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.3 (1‴-CH_3), 19.1 (3′-CH_3), 24.5 (3′-CH_3), 51.6 (C2″), 58.3 (C3′), 60.7 (C3″), 61.0 (C2′), 68.5 (−OCH_2−), 108.9 (C6), 113.2 (C4a), 113.3 (C8), 113.6 (C2‴), 113.7 (C3), 128.8 (C5), 141.3 (C1‴), 143.3 (C4), 154.2 (C8a), 160.2 (C2), 160.5 (C7); HRTOFESIMS *m/z* 351.1194 (calcd for $C_{19}H_{20}O_5Na$, 351.1208).

7-Methoxy-8-(4-methyl-3-furyl)-2*H*-chromen-2-one (2): white, amorphous powder; UV (MeOH) λ_{max} (log ε) 208 (2.2), 260 (1.4), 325 (1.9) nm; IR (KBr) ν_{max} 2900, 1728 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.89 (3H, d, J = 0.5 Hz, 4′-CH_3), 3.88 (3H, s, −OCH_3), 6.25 (1H, d, J = 10.0 Hz, H-3), 6.94 (1H, d, J = 8.5 Hz, H-6), 7.34 (1H, brs, H-5′), 7.45 (1H, d, J = 8.5 Hz, H-5), 7.48 (1H, d, J = 1.2 Hz, H-2′), 7.67 (1H, d, J = 10.0 Hz, H-4); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 8.8 (4′-CH_3), 56.2 (−OCH_3), 107.5 (C6), 109.9 (C8), 113.1 (C4a), 113.3 (C3), 115.7 (C3′), 121.0 (C4′), 128.0 (C5), 139.7 (C5′), 142.2 (C2′), 143.6 (C4), 152.9 (C8a), 160.3 (C7), 161.0 (C2); APCIMS *m/z* 257 $[M + H]^+$ (100).

2-(7-Methoxy-2-oxo-2*H*-chromen-8-yl)-3-methylbut-2-enal, Murralongin (4). Phebalosin (**3**, 5.0 mg), dissolved in CH_2Cl_2 (0.5 mL), was absorbed in silica gel (50 mg) and submitted to column chromatography (Pasteur pipet, silica gel) using CH_2Cl_2 as eluent to give 4.7 mg (94%) of murralongin (**4**) as a white powder: UV (MeOH) λ_{max} (log ε) 205 (1.8), 241 (1.4), 325 (1.5) nm; IR (KBr) ν_{max} 1733, 1656, 1606 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.78 (3H, s, −CH_3), 2.42 (3H, s, −CH_3), 3.86 (3H, s, −OCH_3), 6.22 (1H, d, J = 9.6 Hz, H-3), 6.90 (1H, d, J = 8.7 Hz, H-6), 7.44 (1H, d, J = 8.7 Hz, H-5), 7.68 (1H, d, J = 9.6 Hz, H-4), 10.20 (1H, s, −CHO); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 19.8 (−CH_3), 24.9 (C4′), 56.2 (−OCH_3), 107.6 (C6), 113.0 (C3), 113.1 (C4a), 113.2 (C8), 128.6 (C5), 129.2 (C2′), 143.6 (C4), 152.5 (C8a), 159.7 (C3′), 160.1 (C7), 161.8 (C2), 188.9 (C1′); APCIMS *m/z* 259 $[M + H]^+$ (100).

Computational Methods. Initial equilibrium geometries for all diastereoisomers of compound **1** were calculated using an AM1 semiempirical method starting from a MMFF minimal energy conformer. These geometries were used as a starting point for refinement using a RB3LYP/6-311G(d) calculation. Single-point energy and NMR (NOESY) spectra were obtained using the same RB3LYP/6-311G(d) method. All calculations were performed within Spartan'08 (Spartan'08 Wavefunction, Inc., Irvine, CA) using default settings. See details of results in the Supporting Information).

Bioassays. To estimate the 50% effective concentrations (EC_{50}) of compounds **1**−**4** in axenic amastigote forms of *L. panamensis* (M/HOM//87/UA140 *epir*GFP) strain, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) enzymatic micromethod was used.[22] Briefly, axenic amastigotes (1 × 10^6 parasites/mL) were cultured at 32 °C with test compounds in Schneider's Drosophila medium (pH 5.2, Sigma) containing 20% heat-inactivated fetal calf serum. Six concentrations in the range 3.1−100 μg/mL were evaluated for each compound dissolved in DMSO. After 72 h of incubation, 20 μL of MTT (5 mg/mL) was added to each well. Plates were further incubated for 4 h. The enzymatic reaction was stopped by addition of 100 μL/well of 50% 2-propanol−10% sodium dodecyl sulfate solution. Optical density at 570 nm was measured using an ELISA plate reader (Bio Rad). Parasites treated with DMSO or in the absence of treatment but maintained under the same conditions were used as controls. Amphotericin B was used as a reference drug. Two independent experiments were conducted in triplicate, and results were expressed as EC_{50} and calculated by Probit analysis.

Cytotoxicity of compounds **1**−**4** against human U937 (CRL-1593.2) cells and evaluation of leishmanicidal activity of compound **2** on intracellular amastigotes were performed following previously reported methods.[22,23]

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Supporting Information Available: NMR spectra of compounds **1** and **2**, calculated geometries, and predicted NOESY correlations of all diastereoisomers of compound **1**, and table of antileishmanial activity against axenic amastigote forms of *L. panamensis* for all compounds and extracts tested. This material is available free of charge via the Internet at http://pubs.acs.org.

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