Pseudoguanolide Sesquiterpene Lactones from *Vernoniopsis caudata* and Their in Vitro Antiplasmodial Activities

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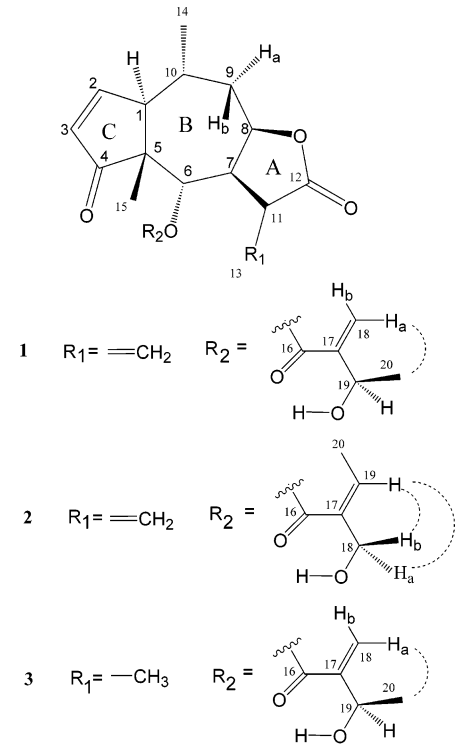
Two new helenanolide sesquiterpene lactones, **1** and **2**, as well as one known related structure, 11α,13-dihydrohelenalin-[2-(1-hydroxyethyl)acrylate] (**3**), together with 4′-*β*-D-*O*-glucopyranosyl-luteolin and ethyl 2,5-dihydroxycinnamate were isolated from an ethyl acetate extract of leaves of *Vernoniopsis caudata* with potent antiplasmodial activity (IC_{50} 1.6 μg/mL) in a preliminary biological screen. The structures of the new compounds were determined by spectroscopic techniques. The three sesquiterpene lactones **1**−**3** displayed strong in vitro antiplasmodial activity, with IC_{50} values of 1, 0.19, and 0.41 μM, respectively. However, these compounds also exhibited considerable cytotoxicity on KB cells (IC_{50} < 1 μM in each case).

The main reasons for the continued and even increasing rates of malaria infections are attributable to the spread of drug-resistant strains of *Plasmodium* malaria and the failure to get existing, effective drugs to be applied in those areas where they can be of most benefit.[1] The need for new and effective antimalarial drugs is therefore an urgent priority to expand existing monotherapy or drug combination regimens. Toward this end, we have screened plant extracts from the biodiversity of Madagascar with the aim of discovering new antimalarial leads.[2] *Vernoniopsis caudata* (Drake) Humbert (Asteraceae) was selected for further investigation because its ethyl acetate extract showed strong in vitro antiplasmodial activity with an IC_{50} value of 1.6 μg/mL. It is used traditionally to treat pulmonary afflictions in Madagascar.[3] Prior to our work, no previous phytochemical or biological studies had been performed on any species in the genus *Vernoniopsis*, which is endemic to Madagascar. Bioassay-guided fractionation of the ethyl acetate extract from the leaves of *V. caudata* led to the isolation of two new pseudoguaianolide sesquiterpene lactones (**1** and **2**) of the helenalin type, together with a known structurally related sesquiterpene lactone, 11α,13-dihydrohelenalin-[2-(1-hydroxyethyl)acrylate] (**3**), and two known phenolic compounds, identified as 4′-*β*-D-*O*-glucopyranosyl-luteolin and ethyl 2,5-dihydroxycinnamate. The present report deals with the structure elucidation of the new sesquiterpene lactones **1** and **2** on the basis of the interpretation of their spectroscopic data, as well as the antiplasmodial and cytotoxic activities of compounds **1**−**3**.

A combination of silica gel column chromatography and preparative TLC of the ethyl acetate extract of *V. caudata* leaves afforded three helenanolide-type sesquiterpenes, **1**−**3**. Compound **1** was obtained as a colorless oil. Its



TOFMS showed a $[M + H]^+$ ion peak at *m/z* 361, and the molecular formula was determined as $C_{20}H_{24}O_6$ by HRDCIMS (obsd *m/z* 361.1655, calcd *m/z* 361.1651) as well as by ^{13}C NMR. As evident from the ^1H NMR spectrum (Table 1), three methyl group signals were observed: one as singlet at δ 1.02 and two as doublets at δ 1.21 (J = 6.4 Hz) and 1.31 (J = 6.7 Hz), respectively. In the olefinic region, the combined interpretation of the ^1H NMR spectrum and the long-range $^1H-^1H$ COSY spectra enabled signals to be assigned to two methylene groups [δ 6.13, 6.38 (1H each, d, J = 2.9 Hz); δ 5.87 (broad triplet, 1H, J = 1.2 Hz),

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Table 1. NMR Spectral Data (δ, ppm) of Compounds **1** and **2** in CD_3OD[a]

	compound **1**			compound **2**		
position	δ_H (mult.)[b]	J (Hz)	δ_C	δ_H (mult.)[b]	J (Hz)	δ_C
1	3.11 (ddd)	1.8, 3.0, 11.4	55.5	3.11 (br d)	11.5	55.5
2	7.90 (dd)	3.0, 6.2	165.6	7.87 (d)	5.4	165.8
3	6.06 (dd)	1.8, 6.2	130.8	6.06 (dd)	3.0, 5.4	130.5
4			212.4			211.9
5			57.3			57.3
6	5.45 (s)		79.5	5.49 (s)		79.1
7	3.63 (d)	7.6	49.7	3.68 (br d)	7.5	49.3
8	5.02 (dt)	2.4, 7.6, 7.6	80.5	5.02 (dt)	7.5, 7.5, 2.3	80.4
9a	1.80 (ddd)	2.4, 8.1, 15.4	41.5	1.82 (ddd)	15.3, 8.1, 2.3	41.4
9b	2.37 (ddd)	3.8, 7.6, 15.4	41.5	2.36 (ddd)	15.3, 7.5, 4.0	41.4
10	2.15 (m)		28.0	2.14 (m)		28.0
11			140.3			140.2
12			171.6			171.9
13a	6.13 (d)	2.9	125.0	6.14 (d)	2.8	124.7
13b	6.38 (d)	2.9	125.0	6.38 (d)	2.8	124.7
14	1.31 (d)	6.7	20.7	1.30 (d)	6.6	20.3
15	1.02 (s)		19.5	1.02 (s)		18.9
16			166.7			167.2
17			147.0			133.7
18a	5.87 (br t)		124.9	4.06 (d)	13.1	64.3
18b	6.04 (s)		124.9	4.11 (d)	13.1	64.3
19	4.53 (br q)	6.4	67.2	6.34 (q)	7.2	140.9
20	1.21 (d)	6.4	23.8	1.94 (d)	7.2	16.0

[a] Assignments based on ^1H, ^{13}C, HMQC, and HMBC. [b] Multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet.

6.04 (1H, broad singlet)], along with two *cis*-coupled unsaturated protons (δ 6.06, 1H, dd, J = 6.2/1.8 Hz; δ 7.90, 1H, dd, J = 6.2/3.0 Hz). Full characterization of the structure of compound **1** was accomplished by the concerted examination of the COSY, HMQC, HMBC, and NOESY NMR spectral data, in addition to the conventional 1D-NMR technique (Table 1). In effect, proton H-7 (δ 3.63) showed COSY correlations with H-8 (CH, δ_H 5.02, δ_C 80.5) and H-6 (CH, δ_H 5.45, δ_C 79.5) as well as with the two methylene protons H-13$_a$ (δ_H 6.13, δ_C 125.0) and H-13$_b$ (δ_H 6.38, δ_C 125.0). In the HMBC spectrum, the same proton displayed three-bond connectivities with C-12 (δ 171.6), C-13 (δ 125.0), C-5 (δ 57.3), and C-9 (δ 41.5) and two-bond connectivities with C-11 (δ 140.3) and C-6 (δ 79.5). These data indicated unambiguously the presence of the α-methylene-γ-lactone unit (A ring) and allowed this to be linked to the B ring. In the next step, the olefinic proton H-2 (δ_H 7.90, δ_C 165.6) exhibited HMBC correlations with C-3 (δ 130.8), C-4 (δ 212.4), C-1 (δ 55.5), and C-5 (δ 57.3). These results allowed the α,β-unsaturated cyclopentenone C ring to be defined. Correlations of H-6 (δ_H 5.45, δ_C 79.5) with C-8 (δ 80.5) and C-11 (140.3) on one hand, and with C-1 (55.5), C-5 (57.3), and C-4 (212.4), on the other hand, permitted the A and C rings to be assembled with the B ring. The side chain was identified as 2-(1-hydroxyethyl)-acrylate by HMBC correlations between H-19 and C-16 (δ 166.7), C-17 (δ 147.0), C-18 (δ 124.9), and C-20 (δ 23.8), and the observation of a three-bond connectivity between H-6 and C-16 provided evidence for its linkage to C-6. In the mass spectrum, an intense fragment peak appearing at *m/z* 263 indicated the loss of the side chain unit from the intact helenalin-type sesquiterpene lactone,[3] further confirming the proposed structure. The relative stereochemistry of compound **1** was established by the interpretation of the NOESY spectrum. Thus, the observed cross-peaks between H-1/H-8 and H-7/H-8 indicated a relative α-configuration for these three protons. This was further supported by the observation of a coupling constant of 7.6 Hz, which was characteristic of the *cis* relationship of H-7 and H-8. Furthermore, the observation of a NOESY correlation between CH_3-15, H-6, and H-10 was indicative of their relative β-configuration. No correlation was observed between H-1 and CH_3-15, and this further supported the *trans* junction of the B and C rings. The stereochemistry of the side chain was determined by NOESY experiments. The observation of correlation between H-18$_a$ and H-20, and the absence of correlation between H-18$_a$, H-18$_b$, and H-19, permitted to assign its stereochemistry, as indicated in the structure, and suggested a hydrogen bonding between the carbonyl and the hydroxyl groups in this preferred conformation. Compound **1** was therefore identified as helenalin-[2-(1-hydroxyethyl)acrylate].

The second new sesquiterpene lactone (**2**) isolated in this study had a $[M + H]^+$ ion peak at *m/z* 361 in the positive TOFMS, and this was in agreement with the molecular formula $C_{20}H_{24}O_6$ determined by HRCIMS (obsd *m/z* 378.1913 $[M + NH_4]^+$, calcd *m/z* 378.1917). It was found to be structurally related to compound **1**. On analysis of its ^1H NMR spectrum (Table 1), in comparison with that of compound **1**, the signals assigned to the side chain of the latter compound were modified, and there was the appearance of an AB system at δ 4.06 (1H, d, J = 13.1 Hz) and 4.11 (1H, d, J = 13.1 Hz), along with a set of signals attributable to a vinylidene group (δ 6.34, 1H, q, J = 7.2 Hz; δ 1.94, 3H, d, J = 7.2 Hz). These preliminary observations suggested a 2-hydroxyethyl-3-methylacrylate unit for the side chain of compound **2**. In the HMBC spectrum, the observation of two- and three-bond connectivities between H-19 and C-17, C-20, C-16, and C-18 supported the proposed side chain structure, and the observation of a cross-peak between H-6 and C-16 provided evidence for its linkage to C-6. The relative configuration of compound **2** was established as in compound **1** by means of NOESY data. In particular, the cross-peak between H-18 and H-19 clearly indicated a *Z* configuration for the double bond of the 2-hydroxyethyl-3-methylacrylate unit. The molecular modeling studies of this side chain with ChemBats3D Ultra indicated the existence of a hydrogen bonding between the carbonyl and the hydroxyl groups, which resulted in the equal distance orientation of the two H-18 protons with respect to H-19. This was in agreement with the observation of a NOESY correlation between H-18$_a$, H-18$_b$, and H-19, as displayed in the structure. Compound **2** was therefore identified as helenalin-[(2-hydroxyethyl-3-

Table 2. In Vitro Antiplasmodial and Cytotoxic Activities of Compounds **1**−**3**

test compound	antiplasmodial test[a] IC_{50}[c] (μM)	cytotoxicity test[b] IC_{50}[c] (μM)
1	1.00 ± 0.28	0.33 ± 0.08
2	0.19 ± 0.04	0.93 ± 0.20
3	0.41 ± 0.07	0.05 ± 0.01
chloroquine[d]	0.11 ± 0.05	
adriamycin[d]		0.27 ± 0.04

[a] The chloroquine-resistant strain FcB1 was used for the antiplasmodial test. [b] The cytotoxicity test was evaluated against KB cells. [c] Tests were done in triplicate in three independent experiments. Values are means ± SD. [d] Chloroquine and adriamycin were used as positive controls.

methyl)acrylate]. The low quantity available of compound **2** did not allow us to run its ^{13}C NMR spectrum, and the chemical shifts were instead deduced from the HMQC and HMBC spectra.

One known related structure identified as 11α,13-dihydrohelenalin-[2-(1-hydroxyethyl)acrylate] (**3**),[4] together with two known phenolic compounds, 4′-β-D-*O*-glucopyranosyl-luteolin[5] and ethyl 2,5-dihydroxycinnamate,[6] were also isolated and identified by spectral data in comparison with those reported from the literature.

The five compounds obtained from *V. caudata* were tested for their ability to inhibit *Plasmodium falciparum* growth using the chloroquine-resistant strain FcB1, and the three sesquiterpene lactones were evaluated for their toxicity against KB cells. The inhibitory concentration fifty percentage (IC_{50}) values are reported in Table 2. Compounds **1**−**3** exhibited strong antiplasmodial activity with IC_{50}'s of 1, 0.19, and 0.41 μM, respectively, but were associated with potent cytotoxicity on KB cells. The known compounds 4′-β-D-*O*-glucopyranosyl-luteolin and ethyl 2,5-dihydroxycinnamate exhibited IC_{50} values of >10 μM in the in vitro antiplasmodial assay.

The sesquiterpene lactone activities are mediated chemically by the occurrence of α,β-unsaturated carbonyl structures, such as an α-methylene-γ-lactone, an α,β-unsaturated cyclopentenone, or a conjugated ester, which react with nucleophiles by a Michael-type addition.[7] Investigation of structure−cytotoxicity relationships has revealed that an α,β-unsaturated ketonic moiety was more important than an α-methylene-γ-lactone system for the maintenance of a high level of cytotoxicity,[8] and this is in line with the observed low IC_{50} value for the cytotoxicity of compound **3**. The presence of the bi- or trifunctional α-β-unsaturated carbonyl units conferred strong antiplasmodial activity to compounds **1**−**3**, in accordance with previous similar results.[9] The simple modification of the side chain of compound **1** led to a significant increase in antiplasmodial activity and decrease in cytotoxicity. Related compounds with better cytotoxicity/antiplasmodial ratios may occur in plants in the unique Madagascan biodiversity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 22 °C. IR spectra were obtained on a Perkin-Elmer RXI FT-IR spectrophotometer. NMR spectra were recorded at 400.13 MHz on a Bruker AMX and at 600.13 on a Bruker DRX spectrometer with CD_3OD as solvent. The ^1H (δ_H) and ^{13}C (δ_C) chemical shifts are expressed in ppm relative to TMS with coupling constants (*J*) given in Hz. Mass spectral data were collected with an electrospray time-of-flight mass spectrometer (TOFMS) operating in the positive mode (QSTAR Pulsar I, Applied Biosystems). HRMS were recorded on a JEOL MS700 apparatus. Merck silica gel 60 (70−230 mesh) and Kieselgel 60 F_{254} were used for analytical and preparative TLC.

Plant Material. *Vernoniopsis caudata* was collected in August 2000, in the eastern forest of Madagascar (Andasibe region). The plant material was identified by Armand Rakotozafy by comparison with an authentic specimen deposited at the Department of Botany of the Parc Botanique et Zoologique de Tsimbazaza, Antananarivo. A voucher specimen has been kept at the Institut Malgache de Recherches Appliquées under the collection ID AR/MORA-051.

Extraction and Isolation. The dried leaves of *V. caudata* (210 g) were powdered and extracted with EtOH (1 L, 3 × 24 h). The combined ethanol solutions were evaporated to dryness under reduced pressure and partitioned between ethyl acetate and water. Each fraction was tested against *P. falciparum* using the chloroquine-resistant strain FcB1. The ethyl acetate extract (5 g) showed significant in vitro antiplasmodial activity (IC_{50} 1.6 μg/mL) and was chromatographed on silica gel eluted with gradients of cyclohexane−AcOEt−MeOH. A total of 20 fractions were collected and respectively tested for in vitro antiplasmodial activity. Three fractions displayed significant activity, namely, F_8 (IC_{50} 2.4−2.5 μg/mL), F_{14} (IC_{50} 2.9−3.2 μg/mL), and F_{15} (IC_{50} 1.4−1.9 μg/mL). F_{18} had a moderate activity (IC_{50} 9.2−9.9 μg/mL), while the remaining fractions had IC_{50} values > 10 μg/mL. Fractions 15 (484.3 mg, 9.7%) and 14 (739.3 mg, 9.8%) were submitted successively to column chromatography and preparative TLC using appropriate combinations of cyclohexane−AcOEt−CH_2Cl_2−MeOH as developing system. From fraction 15, compounds **1** (5.5 mg, 0.11%), **2** (0.5 mg, 0.01%), and **3** (1.1 mg, 0.02%) were obtained after column chromatography over silica gel (CH_2Cl_2−MeOH, 99:1) followed by preparative TLC (cyclohexane−AcOEt, 70:30). From fraction 14, ethyl 2,5-dihydroxycinnamate (24.3 mg, 0.48%) was isolated as active constituent by the same technique using cyclohexane−AcOEt (50:50) as eluent. The low quantity of F_8 (51 mg, 1%) did not allow carrying out any further fractionation.

From fraction 18 (1439 mg, 28.8%), the major constituent, 4′-β-D-*O*-glucopyranosyl-luteolin (7 mg, 0.13%) was isolated by silica gel column chromatography using AcOEt-MeOH, 80:20 as eluent.

Helenalin-[2-(1-hydroxyethyl)acrylate] (1): colorless oil; $[\alpha]^{22}_D$ −165° (*c* 0.1, MeOH); IR ($CHCl_3$), ν_{max} 3535 (OH), 1781 (γ-lactone), 1735 (C=C, C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; TOFMS *m/z* 361 $[M + H]^+$; HRCIMS (obsd *m/z* 361.1655, calcd *m/z* 361.1651).

Helenalin-[(2-hydroxyethyl-3-methyl)acrylate] (2): colorless oil, $[\alpha]^{22}_D$ −243° (*c* 0.1, MeOH); IR ($CHCl_3$), ν_{max} 3530 (OH), 1780 (γ-lactone), 1740 (C=C, C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; TOFMS *m/z* 361 $[M + H]^+$, 263 $[(M - COR) + H]^+$, 245 $[(M - RCOOH) + H]^+$; HRCIMS (obsd *m/z* 378.1913 $[M + NH_4]^+$, calcd *m/z* 378.1917).

In Vitro Antiplasmodial Tests. The in vitro antiplasmodial test was based on the inhibition of [G-^{3}H]-hypoxanthine uptake by *P. falciparum* cultured in human blood. Briefly, the FcB1 strain of *P. falciparum* parasites was maintained in culture in a complete medium composed of RPMI-1640, 25 mM HEPES, 25 mM $NaHCO_3$, and 10% pooled human serum, with uninfected human red blood cells at 2.5% haematocrit. The cell suspension (1% parasitaemia) was distributed at 0.2 mL per well into flat-bottomed 96-well plates containing serial concentrations of test compounds in triplicate alongside untreated controls. The culture was then incubated at 37 °C for 18 h under microaerophilic conditions obtained with the candle jar method. Tritiated hypoxanthine was then added to each well (0.5 μCi per well) and incubation continued at 37 °C in the required atmosphere for a further 24 h. The contents of the well were then frozen at −23 °C and unfrozen at 50 °C to lyse the cells, harvested by filtration on glass filter papers using a Skatron apparatus, and finally washed several times with water. Thereafter, the disks were dried and added to toluene scintillator in vials, and the radioactivity incorporated into parasites was estimated in an LKB Wallac 1214 Rackbeta liquid scintillation counter. The IC_{50} values were determined

by the linear regression method. Three separate experiments were carried out for each test.

In Vitro Cytotoxicity Assay. The cytotoxicity assays were carried out in 96-well microtiter plates in triplicate against human nasopharynx carcinoma KB cell lines (3×10^3 cells/mL) using a modification of the published method.[10] KB cells were maintained in Dulbecco's D-MEM medium, supplemented with 10% fetal calf serum, $NaHCO_3$ (3.7 g/L), L-glutamine (2 mM), and penicillin G (100 units/mL). After 72 h incubation at 37 °C in air/CO_2 (95/5) with or without test compounds, cell growth was estimated by colorimetric measurement of stained living cells by neutral red. Optical density was determined at 540 nm on a Titertek Multiscan photometer. The IC_{50} values were defined as the concentration of sample necessary to inhibit the cell growth to 50% of the control. Three separate experiments were carried out for each test.

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Supporting Information Available: Table of HMBC and NOESY correlations of compounds **1** and **2**; molecular modeling of the side chains of compounds **1** and **2**. These data are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Carter, R.; Mendis K. N. *Clin. Microbiol. Rev.* **2002**, *15*, 564−594.
(2) Rasoanaivo, P.; Ramanitrahasimbola, D.; Rafatro, H.; Rakotondramanana, D.; Robijaona, B.; Rakotozafy, A.; Ratsimamanga-Urverg, S.; Labaïed, M.; Grellier, P.; Allorge, L.; Mambu, L.; Frappier, F. *Phytother. Res.* **2004**, *18*, 742−747.
(3) Rasoanaivo, P.; Ramanitrahasimbola, D.; Rafatro, H.; Rakotondramanana, D.; Robijaona, B.; Rakotozafy, A.; Ratsimamanga-Urverg, S.; Labaïed, M.; Grellier, P.; Allorge, L.; Mambu, L.; Frappier, F. *Phytother. Res.* **2004**, *18*, 742−747.
(4) Boiteau, P. *Dictionnaire des Noms Malgaches des Végétaux*; Alzieu Edts: Grenoble, 1999; Tome 3, p 304.
(5) Bohlmann, F.; Zdero, C.; King, R. M.; Robinson, H. *Liebigs Ann. Chem.* **1984**, 503−511.
(6) Yoshizaki, M.; Fujino, H.; Masuyama, M.; Arisawa, M.; Morita, N. *Phytochemistry* **1987**, *26*, 2557−2558.
(7) Harayama, T.; Ohtani, M.; Oki, M.; Inubushi, Y. *Chem. Pharm. Bull.* **1973**, *21*, 25−33.
(8) Schmidt, T. J. *Bioorg. Med. Chem.* **1997**, *5*, 645−653.
(9) (a) Beekman, A. C.; Woedernbag, H. J.; Uden, W. V.; Pras, N.; Konings, A. W. T.; Wikstrom, H. V.; Schmidt, T. J. *J. Nat. Prod.* **1997**, *60*, 252−257. (b) Lee, K.-H.; Furukawa, H.; Huang, E.-S. *J. Med. Chem.* **1972**, *15*, 609−611.
(10) François, G.; Passreiter, C. M. *Phytother. Res.* **2004**, *18*, 184−186.
(11) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.

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