Note

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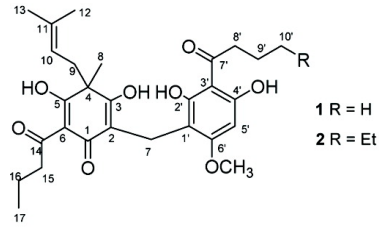

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Molluscicidal Phloroglucinols from the Fern *Elaphoglossum piloselloides*

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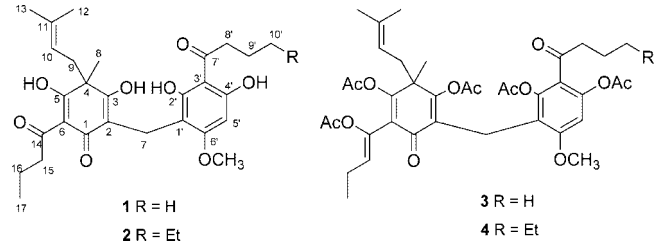
Two new bicyclic phloroglucinols were isolated from the diethyl ether extract of an Argentine collection of the fern *Elaphoglossum piloselloides*. Structures were established by analysis of their spectroscopic data (^{1}H NMR, HRMS, IR) and chemical derivatization. Both compounds show an acute molluscicidal activity against the schistosomiasis vector snail *Biomphalaria peregrina*.

Powdered rhizomes from ferns of the genus *Dryopteris*, especially from the male fern (*D. filix-mas*), and their ether extracts, also known as "crude filicin", have been used as effective anthelmintic agents. However, due to serious side-effects—headache, gastroenteritis, vertigo, amblyopia, convulsions, or even death in severe cases—they are no longer in use.[1,2] The anthelmintic activity has been related to the presence of mixtures of phloroglucinols in these extracts. The occurrence of phloroglucinols is restricted to ferns previously included in the family Aspidiaceae, to which the genera *Elaphoglossum* and *Dryopteris* belonged.[3] In the current system of fern classification, these genera were relocated in the families Lomariopsidaceae and Dryopteridaceae, respectively. Moreover, bioactive prenylated phloroglucinols have been isolated from species of the large genus *Hypericum* (Clusiaceae). They display antibacterial activity against Gram-positive bacteria.[4] Antiplasmodial activity was also reported for prenylated phloroglucinols.[5] Furthermore, synthetic monocyclic phloroglucinols display important antibacterial activity against antibiotic-resistant *Staphylococcus aureus*, with an MIC of around 1 μg/mL.[6]

Previously, we reported the presence of aspidinol and desaspidinol from the Et_2O extract of an Argentine collection of the fern *Elaphoglossum piloselloides* (C. Presl) T. Moore.[7] These compounds could be detected by GC-MS analysis of fractions from the ether extract, but they could not be isolated and purified. Monocyclic phloroglucinols such as aspidinol and desaspidinol are believed to be artifacts formed during the isolation procedure from polycyclic phloroglucinols; therefore we suspected that the plant extract should have at least one compound of this kind. Thus, we reinvestigated the Et_2O extract of *E. piloselloides* and subsequently identified the phloroglucinols **1** and **2**. Phloroglucinol mixtures have been reported as showing strong vermifuge properties, and, since some compounds such as niclosamide displaying anthelmintic activity are also potent molluscicides, we evaluated the molluscicidal effects of **1**, **2**, and those of their acetylated derivatives on the snail *Biomphalaria peregrina* (Orbignyi), a vector of the serious tropical disease schistosomiasis in South America.

The air-dried sterile fronds, rhizomes, and roots were ground and extracted with Et_2O. The resulting extract showed molluscicidal activity against the snail *B. peregrina* with an LD_{50} of 19.6 ppm and $LD_{100} \leq 50$ ppm. In order to isolate the active principles, the extract was dewaxed and processed successively by column chromatography and reversed-phase high-pressure liquid chromatography (RPHPLC), leading to the isolation of phloroglucinols **1** and **2**.

The HREIMS data of compound **1** showed a molecular ion peak $[M]^+$ at *m/z* 500.2415, consistent with the molecular formula



$C_{28}H_{36}O_8$, accounting for 11 degrees of unsaturation. The IR spectrum of this compound showed a broad absorption centered at 3269 cm^{-1} assigned to the O−H stretching, and intense bands between 1637 and 1595 cm^{-1}, which indicated the presence of a 2-hydroxyarylketone or a β-diketone. The ^{1}H NMR spectrum of compound **1** in acetone-d_6 showed a singlet at a very low field (δ 18.6, 3-OH), assigned to an enolizable β-triketonic system, as was previously reported for phloroglucinols isolated from *Hypericum brasiliense* and *H. japonicum*.[4,8,9] In addition, the ^{1}H NMR spectrum showed duplicated peaks, presumably indicating the presence of tautomers in solution.

In order for NMR signals of compound **1** to be more defined, the potential interconversion among tautomers was blocked by acetylation of the hydroxy groups to afford the pentaacetylated compound **3**. The HRFABMS of compound **3** showed a quasimolecular ion peak $[M + Na]^+$ at *m/z* 733.2853, consistent with the molecular formula $C_{38}H_{46}O_{13}$. The ^{1}H NMR spectrum of **3** (Table 1) showed two signals at δ 3.76 and 3.43 (1H each, d, $J = 15.6$ Hz), indicating the presence of a methylene bridge connecting the two rings of this bicyclic phloroglucinol. In addition, a singlet at δ 6.55 evidenced the presence of a proton on an aromatic ring that was assigned to H-5′, the only proton directly attached to the rings. The presence of a methoxy group linked to the same ring was evident from the singlet at δ 3.78, which showed correlation with H-5′ in the NOESY spectrum. The presence of an acyl group connected to the aromatic ring was inferred by the ^{13}C NMR signal of a ketone carbonyl at δ 200.7 (Table 2). The substitution pattern of ring B was determined by analysis of the HMBC correlations between the ring carbons and protons on C-7 and C-5′. The ^{13}C NMR signal located at δ 183.2 and assigned to C-1, together with the signal at δ 47.6, assigned to C-4, indicated that the second ring was a filicinic acid type ring. Signals assigned to a prenyl moiety were observed at δ 1.48, 1.60, 2.27, 2.43, and 4.73 in the ^{1}H NMR spectrum of **3**. Cross-peaks were detected between the signals assigned to H-9 and H-10, and the C-4 signal in the HMBC spectrum, indicating the location of this prenyl residue at C-4. The ^{13}C NMR spectrum of **3** (δ_C 200.0, 45.6, 17.2, and 13.7) indicated the presence of an oxobutyl moiety that could be located at C-3′ by the NOESY correlations between the signal assigned to the methyl protons of the acetyl residue at C-4′ and the signals assigned to H-5′, H-8′, and H-10′. In addition, the presence of a (1-

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Table 1. ^{1}H NMR Data of Compounds **3** and **4** ($CDCl_3$, 600 MHz)

	δ [ppm], multipicity, J [Hz]	
H	**3**	4
7	3.76, d, $J = 15.6$	3.76, d, $J = 15.4$
	3.43, d, $J = 15.6$	3.43, d, $J = 15.4$
8	1.18, s	1.18, s
9	2.43, dd, $J = 14.3, 8.8$	2.43, dd, $J = 14.3, 8.8$
	2.27, dd, $J = 13.0, 6.3$	2.27, dd, $J = 14.3, 5.8$
10	4.73, br t, $J = 7.4$	4.73, br t, $J = 7.4$
12	1.48, s	1.48, s
13	1.60, s	1.60, s
15	5.40, t, $J = 7.5$	5.40, t, $J = 7.6$
16	2.06, dd, $J = 7.5, 1.0$	2.06, dd, $J = 7.6, 1.1$
	2.05, s	2.05, s
17	1.00, t, $J = 7.5$	1.00, t, $J = 7.4$
5′	6.55, s	6.55, s
8′	2.65, td, $J = 7.3, 1.8$	2.66, td, $J = 7.4, 2.4$
9′	1.64, qd, $J = 7.3, 1.1$	1.64, qd, $J = 7.4, 1.1$
10′	0.92, t, $J = 7.3$	1.34−1.24[a]
11′		1.34−1.24[a]
12′		0.88, t, $J = 7.1$
−O$\underline{C}$H$_3$	3.78, s	3.78, s
$\underline{C}$H$_3$$\underline{C}$=O	2.26, 2.24, 2.19, 2.12, 2.10, *s*	2.26, 2.24, 2.19, 2.12, 2.10, *s*

[a] Overlapping signals.

Table 2. ^{13}C NMR Data of Compounds **3** and **4**

	δ [ppm]	
C	**3**	**4**
1	183.2	183.2
2	128.6	128.6
3	160.4	160.4
4	47.6	47.6
5	163.0	163.0
6	125.2	125.2
7	18.3	18.3
8	21.9	21.9
9	35.8	35.8
10	117.5	117.5
11	135.0	135.0
12	17.6	17.6
13	25.8	25.8
14	136.2	136.2
15	127.3	127.3
16	19.7	19.7
17	13.3	13.3
1′	119.8	119.8
2′	147.0	147.0
3′	120.7	120.7
4′	146.7	146.7
5′	103.2	103.2
6′	159.0	159.0
7′	200.7	200.8
8′	45.6	43.8
9′	17.2	23.5
10′	13.7	31.4
11′		22.5
12′		13.9
−O$\underline{C}$H$_3$	56.0	56.0
$\underline{C}$H$_3$$\underline{C}$=O	21.1, 20.8, 20.7, 20.6, 20.5	21.1, 20.8, 20.70, 20.66, 20.5
C=O	168.7, 168.4, 167.4, 166.5, 166.2	168.7, 168.4, 167.4, 166.5, 166.2

acetyloxy)-but-1-enyl group was evident from the ^{1}H NMR signals at δ 1.00, 2.05, 2.06, and 5.40 assigned by key cross-peaks in the ^{1}H−^{1}H COSY and HSQC spectra. The linkage position of this moiety was established through a ^3J correlation observed between H-15 and C-6 in the HMBC spectrum of **3**. Finally, the location of CH_3-8 was clear from the HMBC cross-peak observed between H-8 and C-4. Based on the foregoing spectroscopic data, the structure of **3** could be established, and consequently that of

Table 3. ^{1}H NMR Data of Compounds **1** and **2** (acetone-d_6, 300 MHz)

	δ [ppm], multiplicity, J [Hz]	
H	**1**	**2**
7	3.54, s	3.54, s
8	1.28, s	1.33, s
9	2.66, dd, $J = 12.0, 8.4$	2.66, dd, $J = 12.3, 8.1$
10	4.64, tt, $J = 8.1, 1.5$	4.63, tt, $J = 8.4, 1.5$
12	1.33, s	1.36, s
13	1.39, s	1.39, s
15	3.26−2.90[a]	3.26−2.90[a]
16	1.75−1.60[a]	1.72−1.60[a]
17	0.96, t, $J = 7.5$	0.98, t, $J = 7.2$
5′	6.15, s	6.15, s
8′	3.26−2.90[a]	3.26−2.90[a]
9′	1.75−1.60[a]	1.72−1.60[a]
10′	0.96, t, $J = 7.5$	1.72−1.60[a]
11′		1.40−1.30[a]
12′		0.90, t
-O$\underline{C}$H$_3$	4.06, s	4.06, s
3-O$\underline{H}$	9.06, s	9.05, s
5-OH	18.63, s	18.63, s
2′-OH	11.58, s	11.59, s
4′-OH	13.70, s	13.70, s

[a] Overlapping signals.

Table 4. Molluscicidal Activity of the Phloroglucinols from *E. piloselloides* (**1** and **2**) and of Their Corresponding Acetylated Derivatives (**3** and **4**) on *B. peregrina* Snails after 24 h of Exposure

	% mortality at each concentration [ppm][a]					
compound	40	20	10	5	1	0.5
1				95	10	0
2				100	57	10
3			43	5	0	
4	81	24	0	0	0	
control	0	0	0	0	0	0

[a] For each concentration, $n = 21$.

compound **1** could be deduced as 2-{[2,4-dihydroxy-6-methoxy-3-(1-oxobutyl)phenyl]methyl}-3,5-dihydroxy-4-methyl-4-(3-methylbut-2-enyl)-6-(1-oxobutyl)-2,5-cyclohexadien-1-one. The new compound was named elaphopilosin-A, and its ^{1}H NMR assignments are shown in Table 3. This compound has a stereogenic center at C-4, but we could not define its absolute configuration.

The HREIMS spectrum for compound **2** showed a molecular ion peak $[M]^+$ at m/z 528.2729, consistent with the molecular formula $C_{30}H_{40}O_8$, which indicated a difference of 28 mass units compared to compound **1**. The NMR data for **2** were very similar to those of compound **1**, suggesting that their structures were almost identical. Acetylation of **2** furnished the pentaacetylated derivative **4**. The ^{1}H and ^{13}C NMR patterns of compound **4** were similar to those of compound **3**, differing only in the signals assigned to the acyl group linked to the aromatic ring (Tables 1 and 2). Analysis of the signals permitted determination of its identity as a caproyl residue. Thus, the structure of compound **4** could be determined and consequently that of compound **2** was established as 2-{[2,4-dihydroxy-6-methoxy-3-(1-oxohexyl)phenyl]methyl}-3,5-dihydroxy-4-methyl-4-(3-methylbut-2-enyl)-6-(1-oxobutyl)-2,5-cyclohexadien-1-one. This new compound was named elaphopilosin-B and is the first reported phloroglucinol carrying a caproyl residue as a ring substituent. Elaphopilosin-B has a stereogenic center at C-4, but we could not define its absolute configuration.

Moreover, phloroglucinols **1** and **2** share structural features with phloroglucinols isolated from *H. brasiliense* and *Dryopteris* ferns.[4,8,10−13] Our results indicated that the acetylation of phloroglucinols showing keto−enol tautomerism is a convenient strategy for their structural identification.

Table 5. Lethal Concentrations of Isolated Phloroglucinols and Acetylated Derivatives Evaluated against *B. peregrina* Adults

	LD_{50} (CI_{95})[a]		LD_{90} (CI_{95})[a]	
compound	[ppm]	[μM]	[ppm]	[μM]
1	2.90 (2.22; 3.74)	5.8 (4.4; 7.5)	4.48 (3.65; 5.88)	9.0 (7.3; 11.8)
2	0.940 (0.792; 1.22)	1.8 (1.5; 2.3)	1.37 (1.13; 2.27)	2.6 (2.1; 4.3)
3	10.6 (9.04; 14.7)	14.9 (12.7; 20.7)	14.7 (12.1; 25.6)	20.7 (17.0; 36.0)
4	30.0 (25.8; 35.1)	40.6 (35.0; 47.6)	43.1 (37.5; 53.4)	58.4 (50.8; 72.4)

[a] CI_{95}: 95% confidence interval.

Niclosamide, a commonly used synthetic molluscicide, shows an $LD_{50} \leq 0.5$ ppm for schistosomiasis vector snails *B. pfeifferi*, *B. alexandrina*, and *Oncomelania hupensis*.[14−16] Despite its high activity, some undesirable effects such as toxicity to nontarget organisms and long-term persistence in the environment encourage the search for newer options to control harmful aquatic snails.

In our search for bioactive natural products from ferns, we evaluated the toxicity of the Et_2O extract of *E. piloselloides* on *B. peregrina*, the potential vector of schistosomiasis in Argentina. Guidelines on the screening of plant extracts for molluscicidal activity prescribe that extracts that kill 90% of the aquatic snail population exposed to a solution containing 100 ppm of crude extract for 24 h are of potential use as molluscicides. The ether extract of *E. piloselloides* showed a promising $LD_{100} \leq 50$ ppm. We performed a bioassay-guided fractionation of this extract to isolate phloroglucinols **1** and **2**. Molluscicidal activities of the ether extract of *E. piloselloides* and of pure compounds against *B. peregrina* snails were determined according to the standard World Health Organization protocol,[17,18] with 24 h exposure and recovery periods, respectively, with mortality rates of *B. peregrina* adult snails shown in Table 4. Secretion of mucus, complete retraction into their shell, and loss of hemolymph were observed in snails exposed to lethal and sublethal concentrations of phloroglucinols, compound **2** being the most active with an $LD_{100} \leq 5$ ppm. No mortalities were recorded for any of the negative control groups.

The calculated LD_{50} and LD_{90} values for evaluated compounds are given in Table 5. The table shows that the snails were more susceptible to the free phloroglucinols, **1** and **2**, than to their corresponding acetylated derivatives, **3** and **4**. Compounds **1** and **2** showed an acute molluscicidal effect against the schistosomiasis vector snail *B. peregrina* (Tables 4 and 5) with LD_{50} values of 2.90 and 0.940 ppm (5.8 and 1.8 μM), respectively. The activity of the acetylated derivatives **3** and **4** was nearly 3 and 30 times lower than that of the corresponding free phloroglucinols, indicating that the blockage of the keto−enol tautomerism greatly reduces the molluscicidal activity of phloroglucinols.

Phloroglucinol **2**, with an LD_{90} of 1.37 ppm, is the most active substance, and although not as potent as niclosamide, it is one of the most potent natural molluscicides.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1030 digital polarimeter. Infrared spectra were recorded on a Shimadzu FT/IR-8400S spectrophotometer by the diffuse reflectance method. MS analyses were conducted on a JEOL JMS AX-500 spectrometer. NMR spectra were measured at 600 or 300 MHz for ^1H and 150 MHz for ^{13}C on a Varian Unity 600 and a Varian Unity 300, using $CDCl_3$ as solvent and TMS as internal standard. Column chromatography was carried out over silica gel 60 (70−230 mesh, Merck), using an *n*-hexane−EtOAc gradient as mobile phase. The evolution of the column was monitored by TLC on aluminum precoated plates F_{254}, and Godin reagent was used to visualize the spots.[19] Preparative HPLC was carried out on Gilson equipment, using a C_{18} column (Luna; 5 μm, 250 × 10 mm i.d.), and ultraviolet and refractive index detectors in parallel.

Plant Material. *E. piloselloides* was collected near La Banderita, Tucumán, Argentina, in December 2005. A voucher specimen (LIL 607856) was deposited at the Herbarium of the Fundación Miguel Lillo, Tucumán, Argentina.

Extraction and Isolation. The air-dried plant material (660 g), consisting of rhizomes, roots, and sterile fronds, was ground and extracted with Et_2O. The ether extract (20.4 g) was dewaxed by overnight maceration with MeOH (400 mL) at 4 °C. The wax-free extract (6.2 g) obtained by this procedure was submitted to column chromatography over silica gel using an *n*-hexane−EtOAc gradient as mobile phase, and fractions were submitted to the molluscicidal bioassay to give one active fraction (987 mg), a portion of which (397 mg) was further processed by RPHPLC (98:1:1 MeOH−H_2O−HOAc, 2.0 mL/min) to afford compounds **1** (136 mg) and **2** (50 mg).

Elaphopilosin-A (1): orange oil; $[\alpha]_D^{21.4}$ −5.8 (*c* 10.0, $CHCl_3$); IR ν_{max}^{neat} cm^{-1} 3269, 2731, 2664, 1637, 1622, 1595; ^1H NMR data (300 MHz, acetone-d_6) in Table 3; HREIMS, 75 eV, *m/z* 500.2415 (calcd for $C_{28}H_{36}O_8$ 500.2411).

Elaphopilosin-B (2): orange oil; $[\alpha]_D^{22.2}$ −6.3 (*c* 10.0, $CHCl_3$); IR ν_{max}^{neat} cm^{-1} 3261, 2729, 2661, 1630, 1599; ^1H NMR data (300 MHz, acetone-d_6) in Table 3; HREIMS, 75 eV, *m/z* 528.2729 (calcd for $C_{30}H_{40}O_8$ 528.2724).

Acetylation of Elaphopilosin-A. A 38 mg amount of **1** was dissolved in 2 mL of pyridine, 2 mL of Ac_2O was added, and the mixture was stirred overnight at room temperature. The reaction mixture was dried and the product was purified by normal-phase HPLC (1:1 *n*-hexane−EtOAc, 2.5 mL/min), leading to the isolation of 10.8 mg of **3**.

Compound 3: colorless oil; $[\alpha]_D^{20.6}$ +21.0 (*c* 10.0, $CHCl_3$); IR ν_{max}^{neat} cm^{-1} 1774, 1694, 1649, 1611, 1366, 1180; ^1H NMR data (600 MHz, $CDCl_3$) in Table 1; ^{13}C NMR data (150 MHz, $CDCl_3$) in Table 2; HRFABMS *m/z* 733.2853 (calcd for $C_{38}H_{46}O_{13}Na$ 733.2830).

Acetylation of Elaphopilosin-B. A 20 mg amount of **2** was acetylated using the procedure employed for **1**. The residue was purified by normal-phase HPLC (3:2 *n*-hexane−EtOAc, 2.5 mL/min), furnishing 16.4 mg of **4**.

Compound 4: colorless oil; $[\alpha]_D^{22.7}$ +15.6 (*c* 10.0, $CHCl_3$); IR ν_{max}^{neat} cm^{-1} 1774, 1691, 1649, 1610, 1366, 1190; ^1H NMR data (600 MHz, $CDCl_3$) in Table 1; ^{13}C NMR data (150 MHz, $CDCl_3$) in Table 2; FABMS *m/z*: 761 $[M + Na]^+$.

Molluscicidal Activity. A methanol solution of the sample to be tested was added to distilled H_2O in order to obtain the desired concentrations and a final volume of 20 mL. The proportion of MeOH should not exceed 5% v/v. Control experiments were carried out using the same volume of solvent added to H_2O. Seven individuals of *B. peregrina* were placed inside the solution. Each test concentration was set in triplicate. After 24 h exposure, snails were removed from both test and control systems and washed thoroughly in clean, dechlorinated tap water. They were then transferred to beakers containing dechlorinated tap water and lettuce (*Lactuca sativa*) leaves (natural diet) for a 24 h recovery period. Thereafter, mortality was assessed as described by Adenusi et al.[14] Finally, LD_{50} and LD_{90} values were calculated by analysis of the mortality data and logarithm concentration, using a probit analysis computer software program.[20] A 10 ppm solution of $CuSO_4$ was employed as positive control and produced 100% mortality of the snail population after 6 h of exposure. The snails used in this assay were field-collected and laboratory acclimatized.

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