Molluscicidal Sesquiterpene Lactones from Species of the Tribe *Vernonieae* (Compositae)

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Schistosomiasis is caused by parasitic flatworms of the genus *Schistosoma*, and some snails, particularly of the genus *Biomphalaria* (Planorbidae), are directly implicated in the transmission of the disease. Continuing with our investigations of bioactive plant constituents, we evaluated and report in the present article, the molluscicidal effects of 16 sesquiterpene lactones, as well as the commercial reagents tetrahydrofuran, furfural, and furfuryl alcohol, on an adult population of *B. peregrina*. The natural sesquiterpene lactones tested are characteristic constituents of species of the tribe *Vernonieae*, family Asteraceae. Compounds **1**–**3** and **7** came from a Bolivian collection of *Vernonanthura pinguis*, compounds **4** and **5** from an Argentine collection of *Cyrtocymura cincta* var. *cincta*, **6** was obtained from a Bolivian collection of *Eirmocephala megaphylla*, **8**–**14** from an Argentine collection of *Centratherum punctatum* ssp. *punctatum*, and compounds **15** and **16** were obtained by chemical derivatization from **5** and **14**, respectively. Ten of the sesquiterpene lactones displayed moderate molluscicidal activity (LD_{50} < 100 μg/ml). Commercial reagents were inactive.

Introduction. – Schistosomiasis, commonly known as bilharzia, is endemic to *ca.* 75 countries throughout South America, Africa, and the Far East. About 250 million people are annually infected. It is caused by parasitic flatworms of the genus *Schistosoma* [1], and some snails, particularly of the genus *Biomphalaria*, are directly implicated in the transmission of the disease. Incidence of schistosomiasis is increasing as a result of the construction of dams and the introduction of irrigating schemes, which inadvertently provide ideal breeding sites for the snail vectors. Chemotherapy is one way of controlling this disease, though a disadvantage of the method is the high cost of the drugs and the possibility of re-infection. The intermediate host (the mollusc) constitutes the weakest link in the cycle of transmission and thus is the logical point of attack to control the disease with molluscicidal agents interrupting the parasite's life cycle and preventing infection of people in contact with water in high-risk areas. There is an urgent need for more selective and efficient molluscicides for the control of the snail vector, especially because the presently available compounds or formulations tend to be general biocides, affecting many of the plants or animals (or both) in the snail environment [2]. The *World Health Organization* (*WHO*) has given plant molluscicides a high priority as tools for the integrated control of schistosomiasis due to their low cost and rapid biodegradability [3].

During the course of our search for biologically active compounds from plants, we noticed that many species belonging to the tribe *Vernonieae* (Asteraceae) have been used in traditional medicine [4], and have been investigated in relation to their biological activity. They have shown to possess insecticidal [5], analgesic, anti-ulcerogenic [6], antiparasitic [7][8], antibacterial [9], antifungal [10], cytotoxic [11], and molluscicidal actions [12][13]. Continuing with our investigations of bioactive plant constituents, we evaluated and report, in the present article, the toxicity of the sesquiterpene lactones **1**–**14** isolated previously in our laboratory from species of the tribe *Vernonieae* [14–16] on an adult population of the mentioned snail. To find the structural requirements for activity, the synthetic derivatives **15** and **16** as well as the reagents **17**–**19** were tested for their molluscicidal effects under the same experimental conditions.

Results and Discussion. – *Compounds.* The sesquiterpene lactones evaluated for their molluscicidal action are 14 highly oxygenated germacranolides characteristic of the tribe *Vernonieae*. In addition, two synthetic derivatives and three commercial reagents were also evaluated. Compounds **1**–**16** belong to four structural types: hirsutinolides, **1**–**4** and **15**, glaucolides, **5**–**7**, goyazensolides, **8**–**10** and **16**, and isogoyazensolides, **11**–**14**. Hirsutinolides and glaucolides (*Figs. 1* and *2*) are widespread in the subtribe *Vernoniinae* of the tribe *Vernonieae*, and their molecules carry a C(7)=C(11) bond (endocyclic), a C(13)–OAc group, and an ester chain at C(8). Goyazensolides and isogoyazensolides (*Figs. 3* and *4*), common metabolites in species of the subtribes *Centratherinae* and *Lychnophorinae* of the tribe *Vernonieae*, possess furanone and exomethylidene-γ-lactone rings in their structures [17]. Compound **15** (*Fig. 5*), obtained by chemical derivatization of **5** in our laboratory, was previously isolated as a natural product [15]. The new compound **16** (*Fig. 5*) was obtained by reduction of the isogoyazensolide **14** with $NaBH_4$, and its structure was determined by spectroscopic techniques. The molecular weight of compound **16** was established by HR-CI-MS, which showed a quasimolecular ion peak at *m/z* 379.1759 ($[M+H]^+$) consistent with the molecular formula $C_{20}H_{26}O_7$. The presence of a OH group, an α,β-unsaturated ketone, and two esters was deduced from the FT-IR bands at 3400, 1690, 1735, and 1760 cm^{-1}, respectively. The molecular formula accounted for eight degrees of unsaturation, two of them were HC=C moieties that were inferred from the typical ^{1}H-NMR signals at δ(H) 5.72 and 6.18, assigned to H–C(2) and H–C(3′), the vinyl H-atom of the angeloyloxy (=2-methylbut-2-enoyloxy) residue at C(8), respectively.

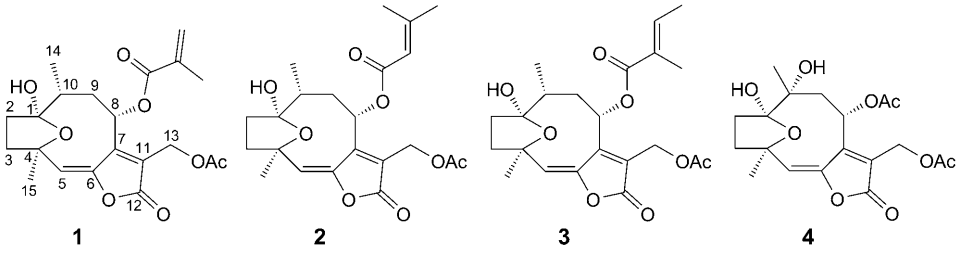


Fig. 1. *Chemical structures of natural hirsutinolides* **1**–**4**

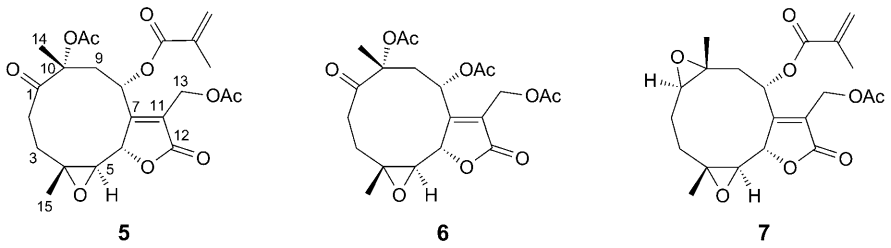


Fig. 2. *Chemical structures of natural glaucolides* **5**–**7**

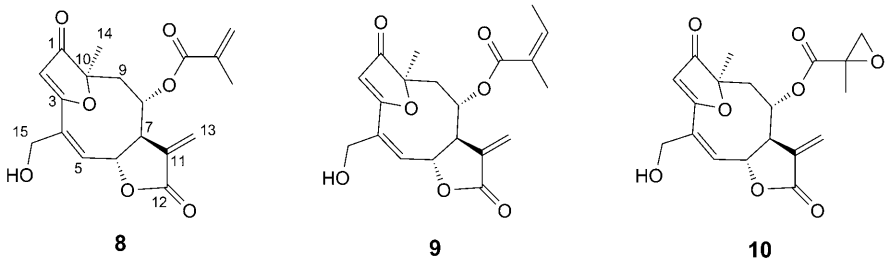


Fig. 3. *Chemical structures of natural goyazensolides* **8**–**10**

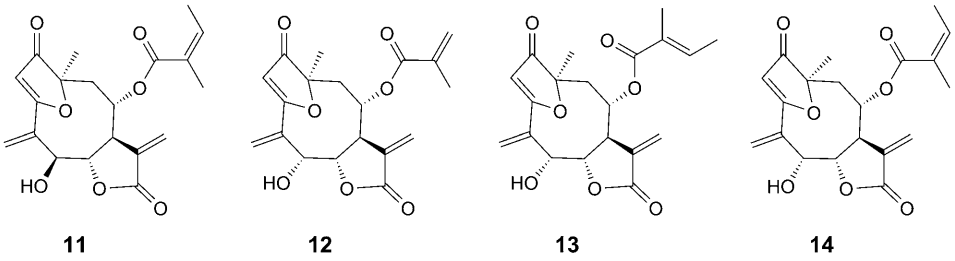


Fig. 4. *Chemical structures of natural isogoyazensolides* **11**–**14**

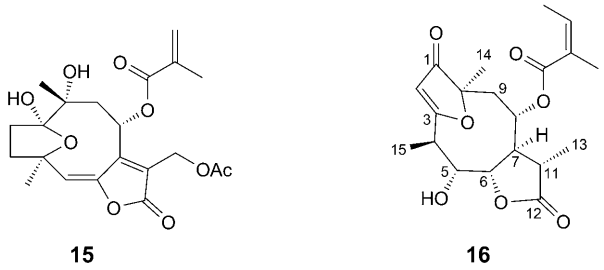


Fig. 5. *Synthetic derivatives* **15** *and* **16**

Because three additional degrees of unsaturation are related to the presence of three C=O groups (^{13}C-NMR signals at 204.4, 166.7, and 177.8), the compound must be tricyclic. The ^{13}C-NMR and HSQC (^{1}H,^{13}C correlation) spectra showed the presence of five Me groups (two more than compound **14**, the synthetic precursor), one CH_2 group, eight CH groups, and six quaternary C-atoms. Additional evidence for the presence of an angeloyloxy chain was provided by the CI-MS spectrum in which the base peak at *m/z* 279 was assigned to the loss of angelic acid (=2-methylbut-2-enoic acid) from the ($[M+H]^+$) ion (for full spectral data, see *Exper. Part*). The NOESY spectrum provided information on the relative configuration, as depicted in *Fig. 5*. The β-orientation of Me(15), H–C(5), and H–C(6) was evident from the correlations observed in the NOESY spectrum (*Fig. 6*) among the signals of the mentioned H-atoms. NOESY Correlations also showed that H–C(8) and H–C(11) are located on the same side of the molecule (β), therefore, Me(13) should be α-oriented as shown in *Fig. 6*.

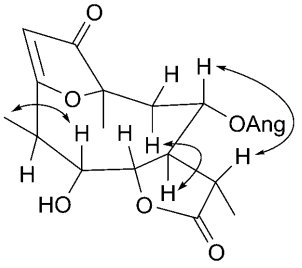
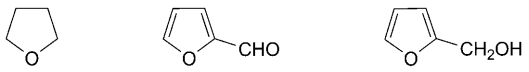


Fig. 6. *Partial NOEs observed for compound* **16**

Molluscicidal Effects. Sesquiterpene lactones from *Vernonieae* were previously reported as natural molluscicides against collections of the Brazilian snail *B. glabrata*, whereas we selected, for the evaluation of the molluscicidal activity of our sesquiterpene lactones, the snail *B. peregrina*, widespread in the north of Argentina. The bioactivity of these compounds have many times been related to the presence of an exomethylidene-γ-lactone moiety which may attack SH groups of proteins in *Michael* addition reactions [18]. Among the compounds reported herein, the natural goyazensolides **8**–**10** and the isogoyazensolides **11**–**14** possess an exomethylidene-γ-lactone group, while the remaining lactones **1**–**7**, **15**, and **16** lack this structural feature. A quick glance at the *Table* that contains the results of molluscicidal activity indicates that not all the exomethylidene-γ-lactones are active; therefore, this structural feature may be necessary but not enough for the molluscicidal activity. Our results (*Table*) show that all the isogoyazensolides **11**–**14** tested displayed strong effects with LD_{50} values between 27.99 and 50.13 μg/ml, while, in hirsutinolides, glaucolides, and goyazensolides, the activity varies depending on the functional groups attached to the backbone of each compound. In isogoyazensolides, the exomethylidene C(4)=C(15) bond together with the exomethylidene-γ-lactone moiety that carries a C(7)=C(11) bond, play an important role for the activity, as can be inferred from the loss of the molluscicidal effect that occurred in the synthetic derivative **16** ($LD_{50} \geq$ 100 μg/ml), in which both C=C bonds were chemically reduced. In goyazensolides, the

effects depended on the ester chain at C(8), the methacrylate **8** being the most active. Glaucolides **5** and **6** with a C(1)=O group are more active than the diepoxide **7** (*Table*) that lacks that C=O group. Interestingly, the active hirsutinolides **4** and **15** are those which possess two vicinal OH groups on C(1) and C(10). Finally, to check the influence of the furan moiety on the activity, we investigated the molluscicidal effects of the furan-containing reagents **17**, **18**, and **19** (*Fig. 7*) and found that all three were inactive. Therefore, we conclude that the activity depends on a combination of structural features including the type of the germacranolide skeleton and the functional groups present in the molecule.



17 **18** **19**

Fig. 7. *Chemical structures of commercial heterocyclic compounds* **17**–**19**

Table. *Molluscicidal Activity of Compounds* **1**–**19** *against* Biomphalaria peregrina

Compound	LD_{50} [μg/ml] (95% CL)[a])	Compound	LD_{50} [μg/ml] (95% CL)[a])
1	>100	**11**	27.99 (13.67, 37.72)
2	>100	**12**	35.35 (27.93, 41.57)
3	>100	**13**	50.13 (37.45, 65.80)
4	56.48 (42.21, 72.05)	**14**	28.53 (10.68, 46.70)
5	38.18 (15.81, 54.75)	**15**	68.48 (45.83, 99.78)
6	78.70 (44.52, 119.68)	**16**	>100
7	>100	**17**	>100
8	48.68 (35.22, 63.43)	**18**	>100
9	88.25 (63.85, 132.84)	**19**	>100
10	>100		

[a]) Mortality was recorded after 24 h of exposure to the chemical. Values [μg/ml] are given as the lethal dose 50 with its 95% confidence limit (CL).

Even when our sesquiterpene lactones are not as potent molluscicides as saponins [2], plants of the tribe *Vernonieae* are widespread in the north of Argentina and are easily available for the native population in large amounts for the preparation of extracts containing active compounds. The present investigation represents a new step towards the use of plant-derived molluscicides, in the hope that locally available plant material might be applied to the control of schistosomiasis in endemic areas.

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Experimental Part

Plants and Compounds. Plant font, isolation, and identification of the sesquiterpene lactones employed in the bioassays were previously reported in [14–16]. Purity of tested compounds was assessed by UV, ^{1}H-NMR, and MS analysis of samples purified by HPLC. Hirsutinolides are often mixed with

glaucolides in species of the subtribe *Vernoniinae*. It is important to point out that only hirsutinolides can be easily detected by UV spectroscopy due to their absorption, in MeOH/H_2O solns., at λ_{max} of 285 nm (ε 10000); therefore, UV spectroscopy is an excellent technique to differentiate hirsutinolides from glaucolides. On the other hand, it is also possible to differentiate goyazensolides from isogoyazensolides. In the UV spectrum, the former absorb at λ_{max} 268–269 nm and the latter at λ_{max} 279–282 nm.

Compounds **1**–**3** came from a Bolivian collection of *V. pinguis* [14]; compound **4** from an Argentine collection of *C. cincta* var. *cincta* [15]. Compounds **5** and **6** were present in a Bolivian collection of *E. megaphylla* [15]; **7** came from a Bolivian collection of *V. pinguis*; **8**–**14** from an Argentine collection of *C. punctatum* ssp *punctatum* [16]. The hirsutinolide **15** was obtained by chemical derivatization from the glaucolide **5**, while **16** was obtained by chemical reduction of **14** with $NaBH_4$.

Reagents. All of them *p.a.* grade. *Tetrahydrofuran* (**17**) was purchased from *Sintorgan*, and *furan-2-carbaldehyde* (**18**) and *furan-2-methanol* (**19**) were from *Sigma-Aldrich*.

Synthesis of **15** *from* **5**. A soln. of **5** (160 mg) in $CHCl_3$ (40 ml) was shaken in the presence of silica gel (SiO_2; 4.8 g) during 48 h at r.t. [19]. When no *glaucolide A* (**5**) was detected by TLC in the reaction mixture, the soln. was filtered to eliminate SiO_2, and the solvent was then evaporated under vacuum. The residue was processed by HPLC on a *Beckman Ultrasphere RP-8* column (5 µm, 10 × 150 mm) using MeOH/H_2O 4 :3, with a flow rate of 2 ml/min to yield 34.8 mg of **15** (t_R 32 min). Identification of **15** was accomplished by comparison of its spectral data with those of an authentic sample from natural origin [15].

Synthesis of **16** *from* **14**. To a soln. of **14** (34.2 mg, 0.1 mmol) and $CoCl_2 \cdot 6\,H_2O$ (37 mg, 0.15 mmol) in CH_2Cl_2/MeOH 1 :1 (2 ml) at −20°, $NaBH_4$ (4.2 mg, 0.1 mmol) was added, and the mixture was stirred for 6 h [20]. The mixture was then allowed to warm to r.t., the reaction was quenched by addition of brine (2.5 ml) and extracted twice with CH_2Cl_2 (2 ml each time). The org. layers were combined and dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue was purified by HPLC using a *Beckman Ultrasphere RP-18* column (5 µm, 10 × 150 mm) and MeOH/H_2O 4 :3 with a flow rate of 2 ml/min to yield 11.4 mg of **16**, identified through its spectral features.

3,10-Epoxy-5α-hydroxy-8α-[(2-methylbut-2-enoyl)oxy]-1-oxo-germacra-2-ene-11,6-lactone (**16**). Light yellow oil. $[\alpha]_D^{27} = +101.08$ ($c = 0.0037$, $CHCl_3$). IR (film): 3400 (OH), 1760 (C=O), 1735 (ester C=O), 1690 (C=C). ^{1}H-NMR (500 MHz, $CDCl_3$; δ in ppm, J in Hz): 6.18 (*qq*, $J = 7.5, 1.5$, H−C(3′)); 5.72 (*d*, $J = 1.5$, H−C(2)); 4.76 (*ddd*, $J = 10.5, 3, 1.5$, H−C(8)); 4.54 (*d*, $J = 7$, H−C(6)); 4.19 (*dd*, $J = 7, 1.5$, H−C(5)); 3.14 (*qt*, $J = 7.5, 1.5$, H−C(4)); 3.00 (*ddd*, $J = 10.5, 7.5, 3$, H−C(7)); 2.84 (*d*, $J = 7$, OH); 2.41 (*dq*, $J = 14, 10.5$, H−C(11)); 2.34 (*dd*, $J = 13.5, 10.5$, H_α−C(9)); 2.17 (*dd*, $J = 13.5, 1.5$, H_β−C(9)); 1.98 (*dq*, $J = 7, 1.5$, Me−C(2′)); 1.85 (*quint*, $J = 1.5, 3$ H−C(4′)); 1.51 (*s*, Me(14)); 1.44 (*d*, $J = 7.5$, Me(15)); 1.35 (*d*, $J = 7$, Me(13)). ^{13}C-NMR (125 MHz, $CDCl_3$; δ in ppm): 204.4 (C(1)); 192.6 (C(3)); 177.8 (C(1′)); 166.7 (C(12)); 141.6 (C(3′)); 126.2 (C(2′)); 104.9 (C(2)); 89.6 (C(10)); 82.7 (C(6)); 77.0 (C(5)); 68.7 (C(8)); 48.3 (C(9)); 46.0 (C(7)); 39.7 (C(4)); 37.9 (C(11)); 20.7 (Me(14)); 20.4 (Me(4′)); 16.2 (Me(13)); 15.9 (*Me*−C(2′)); 14.9 (Me(15)). HR-CI-MS: 379.1759 ($[M+H]^+$, $C_{20}H_{27}O_7^+$; calc. 379.1757).

Molluscs. The fresh water snail *B. peregrina* employed for the molluscicidal assay was taken from our laboratory stock culture maintained for 4 years. The snails were reared in aquaria with dist. H_2O at 26 ± 2°, pH 7.2, under laboratory lighting conditions with normal diurnal light changes and fed on fresh leaves of *Lactuca sativa* L. For water mineralization, 0.005 g/l of $Ca_3(PO_4)_2$ were monthly added to the aquaria.

Molluscicidal Activity. Bioassay was assessed against *B. peregrina* adults according to the method specified by *WHO* [21] and modified by the authors. The snails, uniform in age and size (average diameter of the shell, 7 mm), were maintained for 24 h without feeding before the experiment. Compounds were dissolved in MeOH and diluted with dist. H_2O to reach conc. of 100, 50, and 25 µg/ml (H_2O/MeOH 98 :2). Solns. of each compound (20 ml) were then poured into 100-ml flasks, and five snails were then placed in each flask. Control experiments were performed placing five snails in a mixture of dist. H_2O/MeOH 98 :2. After 24 h, snails were removed from the flasks, and the heart beat was observed in a stereoscopic microscope. The mortality was then recorded. To confirm mortality of the snails, they are placed in a beaker containing dist. H_2O alone. After 24 h, their condition is re-checked. A 10 µg/ml H_2O soln. of $CuSO_4$ was used as positive control, since it produced a 100% mortality of the snail population. Experiments were conducted in duplicate. Data were analyzed with the *Finney* computer program to determine the LD_{50} value at a 95% confidence interval [22].

Supplementary Information Available. CI-MS, and ^{1}H- and ^{13}C-, ^{1}H,^{1}H-COSY, HSQC, and NOESY NMR spectra of compound **16** are available from the corresponding author.

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