Revista de la Sociedad Química de México, Vol. **47***, Núm. 2 (2003) 143-145*

Investigación

Sesquiterpene Lactone Sequestration by the Tortoise Beetle *Physonota arizonae* (Cassidinae)

Manuel Aregullín and Eloy Rodríguez*

Natural Products Laboratory, Biotechnology 259, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY 14853-4301, USA

In honor of Dr. Alfonso Romo de Vivar for his contributions to the field of phytochemistry the chemistry of sesquiterpene lactones

Recibido el 24 de marzo del 2003; aceptado el 28 de mayo del 2003

Abstract. The phenomenon of sequestration of plant secondary metabolites by herbivorous arthropods and its importance as an arthropod defense strategy is well documented in chemical ecology studies. Damsin (**1**), and the related terpene damsinic acid (**2**), are sesquiterpene lactones sequestered by the tortoise beetle *Physonota arizonae* (Cassidinae) from its host plant *Ambrosia ambrosioides* (Asteraceae) for possible chemical protection.
Keywords: Sequestration, sesquiterpene lactones, Asteraceae, *Ambrosia ambrosioides*, Cassidinae, *Physonota arizonae*, Coleoptera, tortoise beetles.

Resumen. El fenómeno de secuestro de metabolitos secundarios de plantas por artrópodos hervíboros, y su importancia como estrategia de defensa artrópoda, se encuentra bien documentado en estudios de ecología química. Damsina (**1**), y el terpeno ácido damsinico (**2**), son sesquiterpen lactonas secuestradas por el escarabajo tortuga *Physonota arizonae* (Cassidinae) de su planta anfitrión *Ambrosia ambrosioides* (Asteraceae) para su posible protección química.
Palabras clave: Secuestro, sesquiterpen lactonas, Asteraceae, *Ambrosia ambrosioides*, Cassidinae, *Physonota arizonae*, Coleoptera, escarabajos tortuga.

Introducción

The phenomenon of sequestration of natural products is a well documented biological event occurring extensively in the Arthropoda [1, 2]. The uptake of toxic substances of exogenous origin, for purposes of defense, has been dramatically demonstrated in several plant-insect interactions [3, 4]. In the class Insecta, plant-derived chemicals are often stored internally in the hemolymph or in specialized glands, and used in acts of reflex bleeding or active secretion triggered by the encounter with a potential predatorial threat [1].

However, less common are the cases of sequestration in which the toxic chemicals are stored externally (*i.e.*, appendages or whole body coatings). The possible advantage of such a defense posture is its utilization as visual or olfactory warning of chemical protection. It is only recently that the chemistry of these defense strategies or "shields" has been recognized [5-7].

In this study, we investigated the chemistry of an interesting case of external sequestration in the family Cassidinae within the Coleoptera.

One such tortoise beetle species, *Physonota arizonae* [8], is endemic to the southwest United States and northern Mexico and it uses as host plant a commonly occurring shrub from the family Asteraceae, *Ambrosia ambrosioides* (canyon ragweed).

We became very interested in learning the chemical composition of the caudal globe the nymphal stages carry, in an attempt to infer from this composition any potential defensive value.

Because of the external adult morphology (*i.e.*, analogous to the reptilian Chelonia), the beetles in the family Cassidinae are commonly referred to as tortoise beetles (Fig. 1a). The pronotum and elytra in these beetles extend to cover completely the margins of the body, and is an excellent example of mechanical defense for the adult life stage. However, the nymphal stages possess a very different morphology (*i.e.*, platyform with lateral segmental appendages) that cannot be used effectively for protection. The nymphal stages possess a caudal bifurcated process (*i.e.*, urogomphi) where exuvia are accumulated, as the beetle develops, and covered with fecal matter, this assemblage can be best described as a small green caudal globe that can represent from 1 / 5 to 1 / 3 of the overall size of the beetle (Fig. 1b). Because the urogomphi are articulated with muscles, the beetle can raise this caudal globe over the body in an umbrella or shield like fashion and conspicuously display it. Moreover, in the event of a threat, this display is accompanied by a typical defensive behavior in which the caudal globe is actually pointed in the direction of the threat (scorpion syndrome). Thus, we propose that the nymphal stages are protected chemically by these accumulations of fecal matter in combination with a behavioral response, and that this protection represents a case of external sequestration of plant derived chemicals.

We have now shown that *P. arizonae* covers its exuvia with resinous fecal deposits that comprise up to 90 % (w/w) of a mixture of two sesquiterpene lactones, Damsin (1) and Damsinic acid (2), sequestered from *A. ambrosioides*. Flavonoids were also present in the mixture but were not characterized.

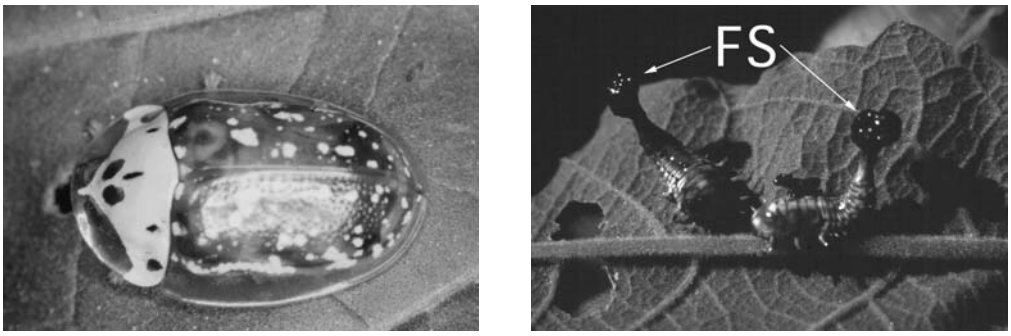


Fig. 1. 1a (*left*) Adult tortoise beetle *Physonota arizonae* with typical Cassidinae morphology.
1b (*right*) Larval stages of *Physonota arizonae* feeding on host plant *Ambrosia ambrosioides* and displaying defensive fecal shields (FS).

Experimental

Collection of Plant Material and Beetles. *Ambrosia ambrosioides* leaf material containing feeding *Physonota arizonae* beetles were collected from wild populations in washes, in the Coyote Mountains off of Hwy 86, approximately 20 mi. west of Tucson, Arizona during the months of July and August. The leaf plant material was brought to the laboratory, and the beetles were removed manually and processed separately. The leaf plant material was air-dried and ground prior to solvent extraction, and from the beetles the exuviae and its resinous coating were collected and extracted fresh with organic solvents.

Ambrosia ambrosioides **Extraction and Chemical Analysis.** Dried and ground leaf plant material (250 g) was extracted with 1500 mL of chloroform and magnetic stirring overnight. The chloroformic extract was filtered and the solvent evaporated to dryness in a rotavapor under vacuum. The residue was retaken in methanol to remove most of the hydrocarbons and filtered. The filtrate was evaporated to dryness to yield 24 g (9.6 %) of methanol soluble crude extract.

The crude extract was dissolved in methanol and applied to a Sephadex LH-20 chromatographic column and eluted with methanol. Fractions from the column were collected according to their fluorescence under longwave light from a portable ultraviolet lamp. Fractions 1 and 2 showed to contain a mixture of hydrocarbons that was not further characterized. Fraction 5 was shown to contain the sesquiterpene lactone Damsin (**1**). Fraction 6 was shown to contain the sesquiterpene lactone Damsinic acid (**2**). The structures of the two sesquiterpene lactones were established by spectroscopic methods (*i.e.*, UV, IR, ^{1}H-NMR and ^{13}H-NMR, and MS), and comparison with authentic samples. Fractions 7-10 were shown to contain a mixture of flavonoids that was not further characterized.

Beetle Resinous Coating Extraction and Chemical Analysis. Approximately 50 to 100 exuvia covered with feces were removed from the beetles and extracted fresh with chloroform. The chloroformic extract was filtered, and the solvent evaporated to dryness in a rotavapor under vacuum. The residue was redissolved in methanol, the insoluble fraction was discarded, and the filtrate evaporated to dryness to yield a gummy residue.

The residue was dissolved in methanol and applied to a Sephadex LH-20 chromatographic column and eluted with methanol. Several fractions from the column were collected according to their different fluorescence under longwave ultraviolet light. Two fractions showed to contain each one a discrete sesquiterpene lactone that upon spectral analysis were shown to be Damsin (**1**) and Damsinic acid (**2**). The structures of (**1**) and (**2**) were established by spectroscopic analysis and comparison with authentic samples.

Results and discussion

Field observations have revealed the occurrence of the tortoise beetle *Physonota arizonae* on wild populations of canyon ragweed (*Ambrosia ambrosioides*), suggesting high host-plant specificity for this Asteraceae. All four life stages of the beetle (*i.e.*, eggs, larvae, pupae and adults) were present on the host plant. Field observations also suggested that the appearance of the beetle on the plant is closely associated with the initiation of the regional monsoon season that usually starts in July-August [9]. We suspected that the highly conspicuous accumulation of fecal matter (primarily derived from plant material), in combination with a discrete defensive behavior by the nymphal stages, could be implicated in the chemical protection of the beetle from potential predators (*i.e.*, birds, lizards, etc.).

P. arizonae larvae, feeding on the plants, were collected from wild populations of *A. ambrosioides* to obtain enough material for chemical analysis. It was found that the fecal excretions covering the urogomphi were essentially lipophilic and that dissolved in chloroform rather easily. Thus, the chloroformic solution of the excretions was analyzed preliminary by tlc using a standard solvent system for terpenic chemicals (*i.e.*, chloroform-acetone, 9:1) [10], the tlc plates were sprayed with a vanillin spray reagent highly specific for terpenoids [11]. This preliminary analysis revealed the presence of two major components in the excretion.

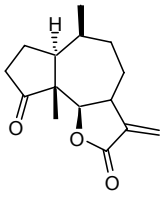
In further analysis, the chloroform washings were combined and the solvent evaporated to yield a green resinous material. The green resinous material was dissolved in methanol to remove the lipid fraction, and filtered. The filtrate was concentrated and applied to a Sephadex LH-20 chromatographic column and eluted with methanol. Several fractions were collected and tlc analysis revealed that fractions 5 and 6 contained the two major constituents of the fecal excretions identical to the ones originally detected by tlc. Final purification of the constituents of fractions 5 and 6 was achieved by preparative tlc. The ^{1}H-NMR spectra of fractions 5 and 6 had a very important diagnostic value in determining that fraction 5 contained a sesquiterpene lactone (*i.e.*, doublets at 6.27 and 5.57 ppm with a coupling constant of approximately 3 Hz corresponding to the two protons in the exocyclic double bond of the γ-butyrolactone), and that fraction 6 contained a structurally related compound. This initial finding suggested that the origin of these sesquiterpene lactones was actually the host-plant. The sesquiterpene lactone chemistry of *A. ambrosioides* has been previously reported [12-14] and it is known that there are geographical variations in the chemistry of *A. ambrosioides* [15]. Following the same isolation procedure as the one used in the case of the beetle excretions our study revealed that the population sampled, contained the sesquiterpene lactones Damsin (**1**) and Damsinic acid (**2**). Moreover, we determined by ^{1}H- and ^{13}C-NMR that these sesquiterpene lactones are identical to the compounds in fractions 5 and 6 from the beetle secretions.

It is well known that sesquiterpene lactones are plant secondary metabolites with a wide array of very important biological activities (*i.e.*, antifungal, insecticidal, allergenic, antitumoral, etc.) on different biological systems [16-18]. It is reasonable to suggest that for purposes of insect chemical protection, the sesquiterpene lactones occurring naturally in plants should be effective deterrents of predators.

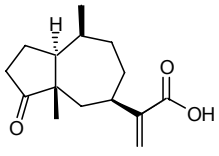
We are currently conducting experiments to demonstrate that arthropods that sequester and use sequiterpene lactones as chemical defenses are capable of deterring more effectively potential predators (*i.e.*, birds, lizards, and other arthropods), and parasites (*i.e*., wasps).

In summary, this is the first instance in which sesquiterpene lactones are shown to be in the repertoire of plant chemicals that are of defensive value to arthropods. Moreover, the study of sequestration as presented by the tortoise beetles should provide us with better insights into the evolution of sequestration. Further study of tortoise beetles species within the genus *Physonota*, its related genera, and their associations with other plant species will shed light on the plant-insect coevolutionary and adaptive aspects and the insect-predator and insect-parasite relationships.

It is now necessary to evaluate the defensive value to the insect of this new type of sequestered substance in order to understand better the efficacy of its deterring activity.



(1) Damsin



(2) Damsinic acid

Acknowledgements

The authors wish to thank Dr. William S. Bowers and Dr. Floyd G. Werner for their support and technical assistance. The authors also wish to thank NIH grant TW000076-0751, and Cornell Hatch funds for financial support.

References

1. Blum, M.S. Chemical Defenses of Arthropods. Academic Press, London, **1981**.
2. Duffey, S.S. Sequestration of Plant Natural Products by Insects. *Annual Review of Entomology* **1980**, *25*, 447.
3. Rothschild, M. Secondary plant substances and warning colourations in insects, In H.F. Van Emden (ed.). Insect / Plant Relationships, *Symp. R. Entomol. Soc. London 6*, Blackwell Sci., Oxford, **1972**. Pg. 59-83.
4. Schilknecht, H. *Endeavour* **1970**, *30*, 136.
5. Olmstead, K.L.; Denno, R.F. *Ecology* **1993**, *74*, 1394.
6. Gomez-Nelida, E.; Witte, L.; Hartmann, T. *J. Chem. Ecol.* **1999**, *25*, 1007.
7. Vencl, F.V.; Morton, T.C.; Mumma, R.O.; Schultz, J.C. *J. Chem. Ecol.* **1999**, *25*, 549.
8. Sanderson, M.W. *Ann. Entomol. Soc. Am.* **1948**, *XLI*, 468.
9. Werner, F. Personal communication.
10. Yoshioka, H.; Mabry, T.J.; Timmermann, B.N. Sesquiterpene Lactones. Chemistry, NMR and Plant Distribution. University of Tokyo Press, Japan, **1973**.
11. Picman, A.K.; Ranieri, R.L.; Towers, G.H.N.; Lam, J. *J. Chrom.* **1980**, *189*, 187.
12. Doskotch, R.W. and Hufford, C.D. *J. Org. Chem.* **1970**, *35*, 486.
13. Higo, A.; Hammam, Z.; Timmermann, B.N.; Yoshioka, H.; Lee, J.; Mabry, T.J. *Phytochemistry* **1971**, *10*, 2241.
14. Romo, J.; Romo de Vivar, A.; Velez, A.; Urbina, E. *Can. J. Chem.* **1968**, *46*, 1535.
15. Seaman, F.C. *Bot. Rev.* **1982**, *48*, 121.
16. Rodríguez, E.; Towers, G.H.N.; Mitchell, J.C. *Phytochemistry* **1976**, *15*, 1573.
17. Picman, A.K. *Biochem. Syst. Ecol.* **1986**, *14*, 255.
18. Robles, M., Aregullin, M., West, J., and Rodriguez, E. *Planta Medica* **1995**, *61*, 199.