Rapid Communications

Chemical Defense in the Zebra Swallowtail Butterfly, *Eurytides marcellus*, Involving Annonaceous Acetogenins

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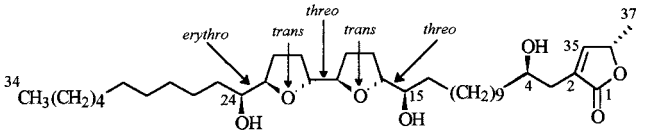
Abstract: Few herbivores feed on the foliage of the North American paw paw tree, *Asimina triloba*; notable exceptions are the larvae of the zebra swallowtail butterfly, *Eurytides marcellus*. Toxic annonaceous acetogenins, produced by *A. triloba*, are responsible for the relative unpalatability of the leaves. Acetogenins found in *A. triloba* extracts are potent pesticidal and antineoplastic agents and have emetic activity in vertebrates. In this study, partitioned aqueous MeOH fractions of the bioactive CH_2Cl_2 extracts, of freeze-dried and pulverized larvae, and of mature butterflies revealed acetogenin content through the use of HPLC coupled to tandem MS (LC-MS/MS). This sensitive technique provides an uncomplicated method for the detection of trace compounds and, in this instance, has confirmed tissue presence of acetogenins that serve a probable role as chemical defense agents against bird predation in zebra swallowtail larvae and adults.

Many organisms are known to produce or sequester toxic compounds as a form of chemical defense. A classic example is illustrated in the relationship between the monarch butterfly, *Danaus plexippus* (L.), and its larval food plant, the milkweed, *Asclepias curassavica* L.; monarch larvae feed on the milkweed and retain cardiac glycosides, found therein, in their body tissues, thereby conferring the adult butterfly unpalatable to potential bird predators.[1] The zebra swallowtail butterfly, *Eurytides marcellus* (Cramer) (Papilionidae), is also known to sequester specific compounds from its larval food source, the North American paw paw tree, *Asimina triloba* (L.) Dunal (Annonaceae); however, the retained compounds, as reported previously, are innocuous flavonoid pigments that have no role in defense.[2]

The only previously known defense mechanism of *E. marcellus* exists in the form of specialized osmaterial glands used to expel butyric acids as a protective aid against ants and other small predators; these glands are present solely in the larval stage and are useless against large predators such as birds.[3] So, how might this species of swallowtail escape bird predation? We report herein that annonaceous acetogenins are retained and sequestered from larval feedings on the leaves of *A. triloba* and thus provide the swallowtails with a form of chemical defense against bird predation.

Chart 1. Structures of Bullatacin (**1**), Asimicin (**2**), Trilobacin (**3**), and Bullatalicin (**4**)[a]



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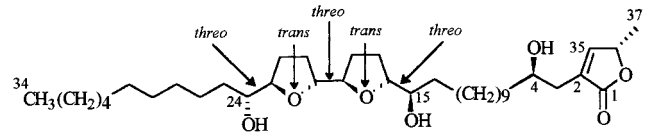

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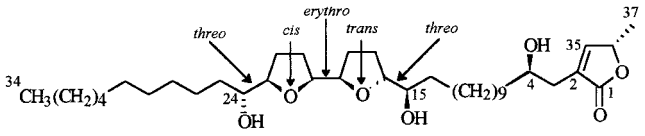

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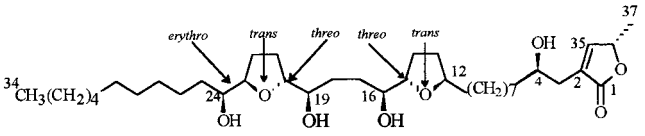

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[a] Compounds **1**−**4** were present in 10% aqueous MeOH fractions of CH_2Cl_2 extracts of *E. marcellus* larvae and wing and body sections.

Annonaceous acetogenins are waxlike fatty acid derivatives of 32 or 34 carbons combined with a propan-2-ol unit at carbon 2 to form a γ-lactone (Chart 1). These compounds are produced exclusively in plants of the family Annonaceae and, in this case, *A. triloba*. Several annonaceous acetogenins have shown in vivo antitumor effects and exhibit extremely potent cytotoxicity, close to one billion times that of the standard reference, adriamycin, against several human tumor cell lines.[4,5] These acetogenins act through the inhibition of the enzyme, NADH-ubiquinone oxidoreductase and, thus, block mitochondrial oxidative phosphorylation at complex I; the ubiquinone-linked NADH oxidase that is elevated in cancerous cells is also suppressed. The result of both actions is a reduction in cellular ATP, which yields potent antineoplastic and pesticidal activity.[5] This unique mechanism of action and the resulting depletion of cellular ATP extend the potential use of these agents to the elimination of neoplastic cells and pests that exhibit ATP-dependent resistance mechanisms.[8b]

Due to the production of acetogenins and the resulting toxic nature of the twigs and foliage, few herbivores or pests

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feed on *A. triloba*. However, zebra swallowtail butterflies make the leaves of this tree their exclusive larval food source. Utilizing paw paw as a food source would seem to be advantageous because the consumption of acetogenins may provide a form of protection from predation by birds; thus, the role of acetogenins in *E. marcellus* seemed worthy of investigation.

Wild butterflies and larvae of *E. marcellus* were collected, freeze-dried, and separated into three groups: larvae, butterfly wings, and butterfly bodies. Each sample (ca. 1.0−2.0 g) was pulverized separately with mortar and pestle and soaked in ca. 20 mL of CH_2Cl_2 for 24 h. Samples were then filtered with Whatman No. 1 filter paper, and the extraction solvent was evaporated. The resulting crude larval extract was tested for bioactivity via the brine shrimp lethality test (BST) and yielded an LC_{50} value of 943 ppm.[9] Next, the CH_2Cl_2 extract was partitioned between hexane and 10% aqueous MeOH, and the BST was again performed. The bioactivity of the 10% aqueous MeOH fraction increased substantially (LC_{50} = 117 ppm) while that of the hexane fraction decreased (>1000 ppm). This provided evidence that the components of the bioactive extract had been concentrated in the more polar 10% aqueous MeOH fraction, the fraction also known to concentrate acetogenins from extracts of *A. triloba* itself.[10]

In the presence of impurities, acetogenins are difficult to detect through UV spectroscopy and exhibit only weak short-wavelength absorbance (at ca. 220 nm), but they can easily be analyzed by LC-MS/MS in the electrospray positive-ion mode [LC/(+)-ESI-MS]. Thus, the three 10% aqueous MeOH extracts were subjected to HPLC coupled to tandem MS (LC-MS/MS). The power of this procedure for the detection of minute quantities of known compounds in crude extracts was recently demonstrated with the detection of 40 known acetogenins from a MeOH leaf extract of the related annonaceous plant *Rollinia mucosa* (Jacq.) Baill.[11] By coupling reversed-phase HPLC (RP-HPLC) retention times for known acetogenins with MS adduct, fragment ion, and characteristic MS/MS fragment daughter ion chromatograms, the detection of specific trace acetogenins in crude mixtures can easily be achieved.

Methanol combined with a gradient of dilute NH_4OAc buffer (0.01 M, pH 4) was used as the HPLC mobile phase. With this regimen, MS analysis of any acetogenin yields molecular adduct ions and fragment ions resulting from the successive losses of water directly related to the number of hydroxyls in the structure (usually three to five). A collision energy of ca. 20 eV proved best in yielding the characteristic ion pattern, $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - 3H_2O]^+$, etc. Known acetogenin molecular weights and characteristic adduct and fragment ion weights were used to search for specific acetogenins through the use of selected-ion chromatograms (SICs). For example, the acetogenin bullatacin (**1**) (molecular weight 622) yields five characteristic SICs at a known RP-HPLC retention time. Further MS analysis under a collision energy of ca. 45 eV yields characteristic daughter ions; **1** produces such a daughter ion at *m/z* 533, which results from the loss of a 112 amu portion of the $[M + Na]^+$ adduct ion. This combination of known ion patterns resulting from adduct, fragment, and daughter ions at the appropriate RP-HPLC retention time yields conclusive evidence for the presence of specific acetogenins.

The extremely potent acetogenins, bullatacin (**1**), asimicin (**2**), trilobacin (**3**), and bullatalicin (**4**) (Chart 1), were detected in the 10% aqueous methanol extract of *E. marcellus* larvae and in separate extracts of butterfly wings and bodies via these methods. Figure 1 shows an HPLC-MS/MS ion chromatograph obtained from the MeOH fraction of an *E. marcellus* extract and reveals **1**, **4**, and a mixture of **2** and **3**, which are inseparable in this HPLC system. Acetogenins detected in larval extracts could, arguably, be the result of *A. triloba* leaves in the larval gut; however, we suggest these results more likely indicate acetogenin retention in larval tissues. Conversely, the detection of acetogenins in butterfly wing and body extracts can only be reflective of retention and sequestration from larval feeding since the butterflies themselves feed only on nectar. Consequently, the presence of these toxic acetogenins is suspected to provide adequate resistance against bird predation in larvae, and from the results of this study, it is clear that mature butterflies gain resistance through concentration and incorporation of acetogenins into both body and wing tissues.

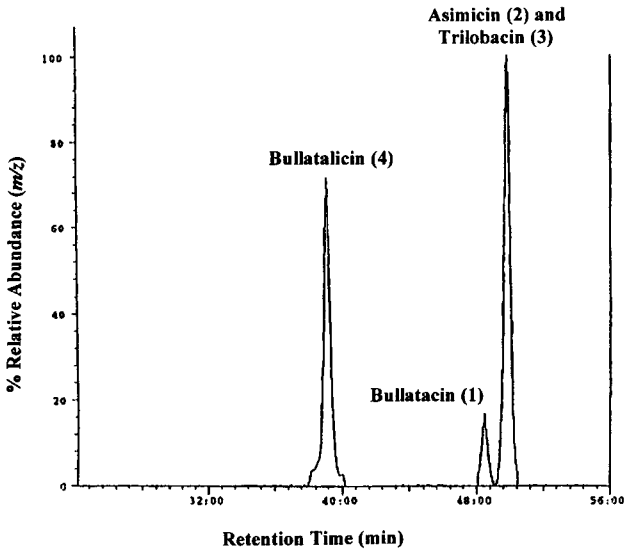

Figure 1. Ion chromatogram showing the presence of compounds **1**−**4** in larval extracts of *E. marcellus*.

Fluid extracts of *A. triloba* seeds were marketed as a fast-acting emetic in the late 1800s by the Eli Lilly Co.[12] The acetogenins possess inherent emetic activity similar to that of the cardiac glycosides found in monarch butterflies. Emesis ensues, in blue jays that consume the glycoside-rich monarchs, with approximately one half the doses causing death; this dose−response relationship proves useful in those birds that survive and, thus, become conditioned against feeding on monarchs after one incident.[1] In our experiment, captive starlings with diets restricted to mature swallowtails eventually resorted to feeding on the butterflies, which sometimes resulted in death. It is now clear that annonaceous acetogenins are present in both zebra swallowtail larvae and the wing and body tissues of mature butterflies and that these compounds may act to provide a form of chemical defense similar to that conferred upon monarchs by the cardiac glycosides of *Asclepias*. There is no previous record of either the retention of annonaceous acetogenins, from insect larval feedings, or the use of these compounds as agents of chemical defense.

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References and Notes

(1) Brower, L. P. *Sci. Am.* **1969**, *220*, 22−29.
(2) Wilson, A. *Phytochemistry* **1986**, *25*, 1309−1313.
(3) Damman, H. *Ecol. Entomol.* **1986**, *11*, 261−265. In his discussion of *E. marcellus*, the author states, "No instances of bird predation were seen over four years of field work, even though several bird species search the leaves of understory plants for insects."
(4) (a) Zhao, G.-X.; Chao, J.-F.; Zeng, L.; Rieser, M. J.; McLaughlin, J. L. *Bioorg. Med. Chem.* **1996**, *4*, 25−32. (b) Zhao, G.-X.; Miesbauer, L. R.; Smith, D. L.; McLaughlin, J. L. *J. Med. Chem.* **1994**, *37*, 1971−1976.
(5) (a) Lewis, M. A.; Arnason, J. T.; Philogene, B. J. R.; Rupprecht, J. K.; McLaughlin, J. L. *Pestic. Biochem. Physiol.* **1993**, *45*, 15−23. (b) Morre, J. D.; de Cabo, R.; Farley, C.; Oberlies, N. H.; McLaughlin, J. L. *Life Sci.* **1995**, *56*, 343−348. (c) Ahammadsahib, K. I.; Hollingworth, R. M.; McGovren, J. P.; Hui, Y.-H.; McLaughlin, J. L. *Life Sci.* **1993**, *53*, 1113−1120.
(6) (a) Goldstein, L. J. *Curr. Prob. Cancer* **1995**, *19*, 65−123. (b) van der Heyden, S.; Gheuens, E.; DeBruijn, E.; Van Oosterom, A.; Maes, R. *Crit. Rev. Clin. Lab. Sci.* **1995**, *32*, 221−264.
(7) (a) Simon, S. M.; Schindler, M. *Proc. Natl. Acad. Sci.* **1994**, *91*, 3497−3504. (b) Reutz, S.; Gros, P. *Trends. Pharm. Sci.* **1994**, *15*, 260−263. (c) Gottesman, M. M. *Annu. Rev. Biochem.* **1993**, *62*, 385−427. (d) Ling, V. *Cancer Res.* **1992**, *69*, 2603−2609.
(8) (a) Oberlies, N. H.; Chang, C.-j.; McLaughlin, J. L. *J. Med. Chem.* **1997**, *40*, 2102. (b) Alali, F. Q.; Faakeh, W.; Bennett, G. A.; McLaughlin, J. L. *J. Econ. Entomol.* **1998**, *91*, 641−649.
(9) (a) Gu, Z.-M.; Zhao, G.-X.; Oberlies, N. H.; Zeng, L.; McLaughlin, J. L. *Recent Adv. Phytochem.* **1995**, *29*, 249−310. (b) Meyer, B. N.; Ferringni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31−34. (c) McLaughlin, J. L. In *Methods in Plant Biochemistry*; Hostettsman, K., Ed.; Academic Press: London, 1991; Vol. 6; pp 1−31.
(10) Ratnayake, S.; Rupprecht, J. K.; Potter, W. M.; McLaughlin, J. L. *J. Econ. Entomol.* **1992**, *85*, 2353−2356.
(11) Gu, Z.-M.; Zhou, D.; Wu, J.; Shi, G.; Zeng, L.; McLaughlin, J. L. *J. Nat. Prod.* **1997**, *60*, 242−248.
(12) McLaughlin, J. L.; Zeng, L.; Oberlies, N. H.; Alfonso, D.; Johnson, H. A.; Cummings, B. A. In *Phytochemical Pest Control Agents*; Hedin, P. A., Hollingworth, R. M., Masler, E. P., Miyamoto, J., Thompson, D. G., Eds.; Symp. Ser. No. 658; American Chemical Society: Washington D.C., 1997; pp 117−130.

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