Biochemistry of the Exocrine Secretion from Gypsy Moth Caterpillars (Lepidoptera: Lymantriidae)

JEFFREY R. ALDRICH, PAUL W. SCHAFFER, JAMES E. OLIVER, PRAPAI PUAPROOMCHAROEN, CHANG-JOO LEE, AND ROBERT K. VANDER MEER

Insect Chemical Ecology Laboratory, USDA-ARS Agricultural Research Center-West, Building 107, Room 301, Beltsville, MD 20705

ABSTRACT. Gypsy moth, Lymantria dispar L. caterpillars have paired dorsal abdominal glands on the 6th and the 7th segments and pairs of smaller glands on the 1st to 5th abdominal segments. Normally, material from these glands becomes sticky and is regularly dispersed onto setae by the caterpillars, but if larvae are held at saturated humidity for 3-4 days, droplets accumulate on the glands and remain fluid. The secretion is an aqueous mixture of low molecular weight molecules including the biogenic amine, d-amino-2-butryric acid, short-chain hydroxy acids, a-hydroxyisobutyric acid, and Krebs cycle acid (e.g., isocitric acid); plus higher molecular weight compounds (≥30,000 MW); 2-isobutyl-3-methoxyxypaetine, considered to be a warning odor in many aposematic insects, also occurs in the secretion in minute amounts (≤1 picogram per larva), yet is mainly responsible for the eschuate odor which is detectable from individual caterpillars. Natural secretion from L. dispar larvae was a feeding deterrent to foraging fire ants, Solenopsis geminata (F.), in a laboratory bioassay. All Lymantria species have dorsal abdominal glands; therefore, it is likely that secretion from these glands contributes to the irritating and allergenic properties associated with setae from tussock moth caterpillars.

KEY WORDS. Lymantria, Leucania salicis, tussock moths, gamma-amino-butyric acid, defense, allergy

"Possibly no other forest insect has been studied as thoroughly or has been the target of such intense containment, control, and eradication strategies as the gypsy moth, Lymantria dispar (L.) (McManus and McIntyre 1981). Indeed, research on gypsy moth has accelerated in the last decade, judging by the number of publications; nearly 800 citations involving L. dispar were listed on the AGRICOLA database from 1984 to the present. Nevertheless, until recently the chemical defenses of gypsy moths had not been thoroughly investigated, even though all Lymantria species are probably poisonous (Maschwitz and Klopf 1971).

Gypsy moth caterpillars are attacked by a variety of vertebrates and invertebrates (Campbell 1981, Leonard 1981). However, some birds are reluctant to take L. dispar larvae when hairless caterpillars are available (Whelan et al. 1989), and white-footed mice prefer nonhair caterpillars to gypsy moth caterpillars (Campbell 1981). Similarly, salicid and lycaenid spiders choose hairless caterpillars over gypsy moth larvae when given a choice (Bardwell and Averill 1996). The setae of neonate L. dispar larvae protect them from ant attack but, after feeding, 1st-instar larvae become vulnerable to ants because the hairs of engorged larvae are too widely separated for effective protection (Weseloh 1989). Thus, the setae of gypsy moth larvae apparently provide some protection against predators (and probably parasitoids).

One type of seta in 1st-instar L. dispar larvae has a balloon-like structure near the base that is thought to be a poison reservoir (Kawamoto and Kumaoka 1984). Shama et al. (1982) detected histamine by a radioenzymic assay in extracts from whole 1st-stage L. dispar caterpillars (17 ng per larva) and setae of 5th-stage larvae (80 ng per larva). They included micrographs of the swollen setae of 1st-instar gypsy moth larvae in their publication, implying that histamine is stored in them, and concluded that histamine, and possibly other reactive substances, contribute to the allergic reactions of people contacting gypsy moth setae (Beaucher and Farnham 1982). Although Shama et al. (1982) seem to have realized that the poison setae of L. dispar are only present in 1st-instar larvae, they made no mention of where the allergenic substances detected from the setae of 5th-stage larvae might be produced.
In fact, 2nd-stage and later gypsy moth caterpillars possess exocrine glands that may secrete chemical irritants (Trasher 1977). Two large, unpaired glands are located between the dorsal hair tufts on the 6th and 7th abdominal segments, and smaller paired glands occur dorsally on the 1st to 4th abdominal segments. Trasher (1977) referred to these glands as respiratory glands, and speculated that their secretion enhances the tactile stimulus of se- tae; noting that L. dispar larvae occasionally arch the anterior end of the body backward and rub the dorsal hair tufts in the glandular secretion.

Since the initiation of the current study, results of the 1st rigorous chemical investigations of the dorsal abdominal glands and the balloon hairs of Lymantria caterpillars have been published (Deml and Dettner 1995a, b). These authors reported the presence of, among other constituents, nicotine, 2-pyrrolidinone, and N-methyl-2-pyrrolidinone in both types of glandular secretions. Nicotine was the abundant component found from the balloon hairs of 1st-instar larvae, whereas the predominant component of the dorsal abdominal gland secretion was 2-pyrrolidinone with nicotine being at its lowest concentration in the secretion from last instar larvae. However, these authors pointed out that 2-pyrrolidinone could actually be a decomposition product of y-aminobutyric acid (GABA). Therefore, J.E.O. devised a procedure to convert GABA into a derivat suitable for gas chromatographic analysis while leaving pyrrolidinone undervatized.

Here we report the results of our investigation of the chemistry and biological activity of gypsy moth caterpillar dorsal abdominal gland secretion, and provide evidence that GABA is indeed the naturally produced constituent in this secretion.

Materials and Methods

Insects and Sample Collection. Gypsy moth caterpillars were from laboratory colonies reared on artificial diet under standard conditions (Bell et al. 1981) at the USDA Forest Service Laboratory (Hamden, CT), the Beneficial Insects Introduction Research Laboratory (Newark, DE), or Insect Neurobiology and Hormone Laboratory (Beltsville, MD). We found that if larvae are held at saturated humidity for 3-4 d, droplets of secretion accumulate on the glands and remained fluid. Therefore, late-stage larvae were held for 3-4 d with ample food (either diet or leaves) in sealed terraria having a false bottom with standing water underneath. Thereafter, the larvae were removed, and dorsal abdominal gland secretion was collected under a dissecting microscope in micropipettes. Secretion was expelled into 50-100 μl of CH2Cl2, methanol, or high-pressure liquid chromatography (HPLC)-grade water. Most samples included only material from the 6th and 7th dorsal abdominal glands, but some samples of the anterior paired dorsal abdominal glands were collected separately.

Material was pooled (20-130 larvae per sample), and stored in a refrigerator or freezer until analysis.

One collection of satin moth caterpillars, L. ciliata salicis (L.) (Lymantriidae), was made 12 June 1993 near Milford, CT, from Populus grandidentata Michaux (Salicaceae). These caterpillars have a single dorsal abdominal gland on the 7th abdominal segment, and a comparable appearing dorsal abdominal gland on the 1st abdominal segment (PWS, unpublished data). Secretion was collected from 45 anterior and 91 posterior dorsal abdominal glands of satin moth caterpillars and combined for analysis of alkylmethoxyxypyraneines.

Chemical Analysis. Samples were analyzed by gas chromatography (GC), and GC-mass spectrometry (GC-MS). Most samples were collected in methanol and treated with diazomethane by standard procedures to convert acids suspected of being present in the material to methyl esters for improved GC and GC-MS. Initial GC analyses were performed on a DB-1 column (0.25 μm film, 30 m by 0.25 mm i.d.; J&W, Folsom, CA) in a Varian 3500 GC, with helium as carrier (50 cm/s), a temperature program from 50°C for 2 min to 235°C at 15°C/min, with a flame ionization detector. Data were recorded using the Varian GC-Star Workstation software on a Gateway 2000 386/25 computer. Electron impact-mass spectra (EI-MS) for most compounds were obtained using a Hewlett Packard 5971 GC-MS instrument at 70 eV, with an HP-5 column (0.11 μm film; 25 m by 0.2 mm i.d.), programmed from 50°C for 2 min to 250°C at 15°C/min. Samples processed to look for the presence of alkylmethoxyxypyraneines were analyzed on a Finnigan INCOS XL GC-MS instrument at 70 eV, with a 60-m DB-1 column, helium as carrier (50 cm/s), a temperature program from 50°C for 2 min to 230°C at 5°C/min, operated in the EI mode (L. dispar extract) or selected ion monitoring mode (SIM; monitoring m/z 124, 137, 138, 151, and 152; Moore et al. 1990) (L. salicis extract).

A dorsal abdominal gland sample collected from 127 gypsy moth caterpillars (CT colony) was processed, concentrated, and injected in toto to look for alkylmethoxyxypyraneines. Empore extraction disks (octadecyl, 1-cm o.d., 3M, St. Paul, MN) were used to prepare samples for analysis of alkylmethoxyxypyraneines. An extraction disk was placed in a 1-ml glass-fritted funnel, washed with 0.5-ml aliquots of diethyl ether, methanol, and water (HPLC-grade MeOH and H2O). Solvents were filtered through the disk by applying a slight positive pressure above the liquid layer. The dorsal abdominal gland sample collected in water was added to the funnel, and filtered through the disk by positive pressure. Organic compounds adsorbed on the disk were eluted with ≈500 μl of freshly distilled ether. Extracts were dried over sodium sulfate, and concentrated to 1-2 μl for injection into the GC-MS.
To determine whether 2-pyrrolidinone was an artifact or a true natural product in the dorsal abdominal gland secretion of gypsy moth caterpillars, a derivitization procedure was developed for GABA that would prevent its cyclization and not affect 2-pyrrolidinone (J.E.O. and J.R.A., unpublished data). Five microliters of a L. dispar dorsal abdominal gland extract in 80% ethanol (≈20 larval equivalents; MD colony) was injected into a small conical vial and most of the solvent was removed with a gentle stream of nitrogen. A solution of pentfluorohexenaldehyde in acetonitrile (1 mg/μl) was added, the vial was capped tightly, and heated at 80°C for 25 min. The solvent was then removed with nitrogen, and ≈2 drops of ethereal diazomethane was added which in turn was evaporated after a few minutes. Ethyl acetate (100 μl) was added for GC analysis. The peak area representing the methyl ester of 4-(pentfluorohexenyl)hexanoic acid was compared with those produced by known amounts of GABA derivatized under identical conditions.

Most identifications were verified by comparisons to the known commercial standards: α-hydroxyisobutyric acid, β-hydroxyisovaleric acid, α-hydroxyisovaleric acid, phosphoric acid, N,N-di-methyl-γ-aminobutyric acid, succinic acid, 2-pyrrolidinone, 1-methyl-2-pyrrolidinone, 8-hydroxyquinoline, cis-aconitic acid, citric acid, 4-hydroxyquinoline-2-carboxylic acid, 2-isopropyl-3-methoxyprazine, 2-sec-butyl-3-methoxyprazine, 2-isobutyl-3-methoxyprazine (Aldrich, Milwaukee, WI); γ-aminobutyric acid, isocitric acid (Sigma, St. Louis, MO).

A single sample of dorsal abdominal gland secretion from 59 gypsy moth larvae (MD colony) was analyzed by HPLC under conditions designed for isolation of compounds up to ≈30,000 molecular weight (MW), using UV detection at 210 and 254 nm: chromatographic system No. 2, but with a TSK SW-2000 column (Tosohaas, Montgomeryville, PA) (Masler et al. 1994).

Olfactometer Bioassay. The Y-tube olfactometer that was used has been described elsewhere (Vander Meer et al. 1996). Compressed air (breathing air quality) was passed into the choice arms at a regulated 0.2 liters/min for a total flow of 0.4 liters/min. A test sample composed of 5 μl of gypsy moth caterpillar posterior dorsal abdominal gland secretion (≈5 larval equivalents per 5 μl methanol; DE colony) and a solvent blank (5 μl methanol) were applied to filter paper strips (0.3 by 2.5 cm, Whatman No. 1), air dried for 1–2 min, then each was placed into one of the choice arms. Worker ants were introduced into the entrance stem and the initial choice of the first 20 workers was recorded. After the 1st choice test the olfactometer was rinsed with acetone, dried, and set up as before. The sample was restested with workers from the same colony, but with the sample and control in reverse positions. The procedure eliminated bias inherent in the apparatus. A complete replicate was the sum of the results from the 2 tests. The bioassay was replicated 6 times, and the data were analyzed by a chi-square test.

Phagostimulant Bioassay. Details of the fire ant phagostimulant bioassay have been published (Vander Meer et al. 1988). Laboratory colonies of Solenopsis invicta Buren were reared from queens collected near Gainesville, Fl., using standard procedures, and each colony used for bioassays had at least 50,000 workers. Four 50-μl drops consisting of water, 1% sucrose solution, sucrose solution plus 5 μl of methanol, and sucrose solution plus 5 μl of gypsy moth caterpillar posterior dorsal abdominal gland secretion (≈5 larval equivalents/5 μl methanol; DE colony) were applied to filter paper squares, and randomly placed in the foraging arena of a laboratory fire ant colony. The filter paper squares were treated with silicone (Vander Meer et al. 1988) so that the drops formed beads on the paper rather than being adsorbed. Ten counts of the number of ants feeding on the drops were recorded at 3-min intervals. The results for the 10 time periods were added, and the total was used to calculate the ranking. The treatments were ranked by setting the water response at zero and the sucrose response at 100; this nullified much of the natural inter- and intracolony variation, and allowed comparison of results between tests. The ranking was calculated as

\[(\text{#ants at the treatment}) - (\text{#ants at water}) \times 100/(\text{#ants at sucrose}) - (\text{#ants at water})\]

The experiment was replicated 3 times using different colonies each time, and the mean and standard errors were calculated for each treatment.

Results

Chemistry. Gypsy moth caterpillars held at saturated humidity for 3–4 d accumulated up to 0.2 μl of fluid on each posterior dorsal abdominal gland. Secretion on the anterior paired dorsal abdominal glands was usually obvious when viewed under the microscope, as well, but much less material occurred on these glands. The appearance of the secretion ranged from clear to dark brown. The secretion of larvae that was resampled after only 2 d was mostly clear, suggesting that darkening of the exudate occurs with time by formation of melanin in the presence of a tyrosinase (Deml and Dettner 1994). Secretion collected from satum moth caterpillars appeared similar to L. dispar secretion. Both satum and gypsy moth caterpillar anterior and posterior dorsal abdominal gland secretions have distinctive odors that J.R.A. associated with the odor of the reflexively released blood of ladybird beetles (Coccinellidae). This odor was detectable from the secretion of single caterpillars.

Dorsal abdominal gland secretion was insoluble in CH₂Cl₂, completely soluble in water, and partially soluble in methanol. When secretion was expelled into methanol, a cloudy white precipitate formed which was assumed to indicate the presence of high
MW material such as peptides or proteins. HPLC analysis of *L. dispar* dorsal abdominal gland secretion from 50 larvae (resampled after 2 d) showed 3 broad peaks of UV-absorbing material: one peak corresponded to standards ranging from 10,000-30,000 MW, a 2nd peak corresponded to 3,000-5,000 MW compounds, and a 3rd peak contained components of 1,500 MW. The first 2 peaks were estimated to represent 25-50 µg of material, at least some of which is proteinaceous.

A typical reconstructed ion chromatogram of a methanolic extract of the posterior dorsal abdominal gland secretion from 35 late stage gypsy moth caterpillars (DE colony) is shown in Fig. 1. Reported compounds correspond to the derivatized natural products. A series of low MW hydroxy acids was identified (compounds 1-5): α-hydroxyisobutyric acid (1), β-hydroxybutyric acid (3), and α-hydroxyisovaleric acid (5) were verified by comparisons to methyl esters of synthetic standards; β-hydroxypropionic acid (2*), and α-hydroxyisovaleric acid (4*) are tentative identifications based on matches to spectra in the proprietary Hewlett-Packard computerized mass spectral library. Free phosphoric acid was detected (6), along with the Krebs cycle acids, succinic (8), cis-aconitic (12), citric (13), and isocitric (14), all verified by comparison to standards. N,N-Dimethyl-γ-amino-butyric acid (7) was detected and verified by comparison to the authentic standard. Compound 11 was identified as 8-hydroxyquinoline by comparison to the authentic standard. Based on the mass spectrum of derivatized 15, this compound appears to be a hydroxyquinoline with a carboxylic acid moiety: m/z (%): 59 (5), 63 (16), 89 (15), 115 (25), 143 (100), 171 (25), 203 (72). The structure of 15 remains speculative because the only commercially available hydroxyquinoline carboxylic acid, kynurenic acid (4-hydroxy-2-quinoline carboxylic acid), was eluted at a position as a possible structure for the insect-derived material.

2-Pyrrolidinone and 1-methyl-2-pyrrolidinone were found in both derivatized and underderivatized methanolic extracts by GC-MS, and verified by comparisons to authentic standards, but it was suspected that pyrrolidinones could be artifacts formed by cyclization (Kato et al. 1992). We found that brief heating of GABA with pentfluorobenzylaldehyde resulted in formation of a Schiff base between the amino and aldehyde functions, and that subsequent treatment with diazomethane converted the carboxylic acid to its methyl ester. This compound behaved well on any of several GC columns, and it would be expected to offer enhanced response to electron capture detection should the extra sensitivity be necessary. When this method was applied to an aliquot of dorsal abdominal gland secretion, a strong peak for the GABA derivative was observed representing 0.3-0.4 µg GABA per larva, although the efficiency of our collection procedure is unknown. No peak for pyrrolidinone was observed, indicating that GABA was responsible for the pyrrolidinone peak in our extracts, and presumably those prepared by Deenl and Dettner (1995a, b). By analogy, we assume that N-methyl-α-amino-butyric acid (9) (standard not obtained) is the true natural product secreted by the insect, but this supposition remains to be proven.

The analysis for alkylmethoxyazines in gypsy moth dorsal abdominal gland secretion showed a compound eluting at 19 min 52 s, which produced the EI-MS shown in Fig. 2B. Under our GC-MS conditions, standards (1 ng/µl hexane) of the 3 alkylmethoxyazines commonly found in insects, 2-isopropyl-3-methoxyazine, 2-isobutyl-3-methoxyazine, and 3-isobutyl-3-methoxyazine, eluted at 17 min, 19 min 33 s, and 19 min 47 s, respectively. Thus, the gypsy moth dorsal abdominal gland product eluted within 5 s of the 2-isobutyl-3-methoxyazine standard. Given that reduced concentration slightly decreases retention time, and that the natural product in question produces an MS (with only 3,648 ions) having a good match to that of the standard (Fig. 2A), the identity of the unknown is tentatively established as 2-isobutyl-3-methoxyazine. The efficiency of our extraction procedure for alkylmethoxyazines was not determined; however, based on the ion counts of the MS-MS from the standard and natural product, we estimate that gypsy moths produce on the order of 1 picogram of 2-isobutyl-3-methoxyazine per larva.

The selected ion monitoring analysis of the satin moth caterpillar dorsal abdominal gland extract indicated the presence of 2-isopropyl-3-methoxyazine based on detection of the characteristic ions for this compound (m/z 124, 137, and 152), and a retention time of the natural product within 3 sec of the standard.

**Bioactivity.** The results of the olfactometer bio-assay indicated that the gypsy moth caterpillar dorsal abdominal gland secretion was neither attractive nor repellent to fire ants (mean ± SEM = 52.1% ± 4.9; P > 0.5). However, the dorsal abdominal gland secretion was a strong feeding deterrent to foraging fire ants (Table 1).

**Discussion**

Our results, and those of Deenl and Dettner (1995a, b), leave no doubt that the medical abdominal protuberances of gypsy moth caterpillars are, in fact, exocrine glands whose secretion contributes to the defensive capability of the larvae. All Lymantriidae possess homologous structures, often brightly colored red, orange, or yellow (P.W.S., unpublished data). We believe the periodic "back-arching" behavior of *L. dispar* larvae probably serves to spread secretion from the dorsal abdominal glands onto the setae (Traxler 1977).

The derivatization procedure developed for GABA and applied to analysis of the gypsy moth larval exudate showed that, in fact, GABA is the true natural product in the secretion rather than...
Fig. 1. Reconstructed ion chromatogram of a diazomethane-treated methanic extract of secretion from dorsal abdominal glands of 35 *L. dispar* caterpillars (reported compounds correspond to the underivatized natural products; asterisks indicate inferred identifications as explained in text).
Fig. 2. Electron impact mass spectra of (A) authentic 2-isobutyl-3-methoxypyrine and (B) the natural product isolated from the dorsal abdominal gland secretion of L. dispar caterpillars (M⁺ = molecular ion)
2-pyrrolidinone, Denil and Dettner (1995a) reported various phenolics in the larval \textit{L. dispar} secretion which we did not detect, but it is possible that the caterpillars sequester these substances from oak leaves (Denil and Dettner 1996) and therefore, were lacking in our extracts of caterpillars reared on artificial diet. However, we detected short-chain hydroxy acids as their methyl-ester derivatives in the dorsal abdominal gland secretion of gypsy moth caterpillars that may have gone undetected in the derivatized samples of Denil and Dettner (1995a). Although the short-chain hydroxy acids are reminiscent of eversible (ozometal) gland secretions from swallowtail caterpillars (Lepidoptera: Papilionidae) (e.g., Honda 1980), overall the secretion is decidedly venom like. Indeed, GABA is a known neurotransmitter in vertebrates and invertebrates (Piek 1985) and occurs in bee and spider venoms along with peptides and proteins (Geren and Odell 1984, Shipolini 1984). Citric and phosphoric acids, plus GABA and other low molecular weight compounds, were identified in the venom of 	extit{Atrax robustus} (Duffield et al. 1979). Some saturniid moth caterpillars eject an irritant defensive spray that includes GABA, pyrazine, various small molecules analogous to those in the larval \textit{L. dispar} dorsal abdominal gland secretion, and proteins (Denil and Dettner 1993, 1994).

The gypsy moth dorsal abdominal gland secretion proved to be neutral in olfactometer bioassays, but inclusion of the secretion in highly phagostimulatory sugar solutions caused total avoidance by foraging fire ants. These results are indicative of feeding deterrent activity rather than repellency. In bioassays involving lyosol and salticid spiders, Barwell and Averill (1996) found that no 2nd-instar gypsy moth caterpillars were killed by spiders in the presence of alternate prey larvae lacking dense setae (a sally, a geometrid, and a tortricid species). Moreover, the presence of a chemical deterrent, in addition to the physical protection afforded by the dense setae, was implicated by observations that spiders that contacted \textit{L. dispar} larvae with their tarsi often immediately backed away without attacking.

As early as 1940, arctic species were reported to smell like coccinellids (references in Detter 1939). Now there is convincing chemical data that alkylmethoxypyrazines are "communal" warning odors of poisonous plants and animals worldwide, equivalent to the color red as an alerting signal (Moore et al. 1990). One or more of the 3 common alkylmethoxypyrazines was detected by selected ion monitoring in 60% of the 45 aposematic species surveyed. Mimetic insects and toxic plants also produce these pyrazines. Although live adult female moth larvae, \textit{Lymnauthria monacha} (L.), "smell unmistakably of pyrazines" (Moore et al. 1990), the compounds were not found in the male moths available for testing. Therefore, our identifications of 2-isobutyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine in gypsy and satin moth caterpillars, respectively, are the first verifications that \textit{Lymnauthria} also express these ubiquitous warning odors. The existence of alkylmethoxypyrazines in the dorsal abdominal gland secretions of gypsy and satin moth caterpillars is further compelling evidence that these exudates are defensive. Although the influence of diet on the composition of the dorsal abdominal gland secretion was not rigorously tested here, the secretion from gypsy moth larval fed leaves appeared and smelled the same as that of larvae reared on artificial diet.

In the laboratory, domestic chickens quickly learned to avoid water containing 0.1% quinine and 0.0000025% 2-isobutyl-3-methoxypyrazine, and they could detect the pyrazine by olfaction (Guilford et al. 1987). Furthermore, this aversion was a conditioned response because, once familiar, the pyrazine itself was not aversive. Similarly, pyrazine odors potentiated associative learning in laboratory rats (Kay et al. 1989). Thus, minute amounts of alkylmethoxypyrazines in lymantriid exudates alert vertebrate predators to potential poisoning but what, if any, costs might be associated with this type of chemical advertisement? One obvious possibility, in fact the original motivation for this investigation, is that specific parasitoids (Leonard 1981) exploit the odor from gypsy moth caterpillar dorsal abdominal gland secretion as a host-finding kairomone. Identification of 2-isobutyl-3-methoxypyrazine and volatile hydroxy acids in \textit{L. dispar} dorsal abdominal gland secretion offers a realistic opportunity to test this kairomonal hypothesis.

Acknowledgments

We thank Miriam Riedesel for bringing to our attention that the odor from coccinellid blood is caused by pyrazines. We acknowledge E. P. Masner (USDA-ARS, Insect Neurobiology and Hormone Laboratory, Beltsville, MD) performing the HPLC analyses reported herein. Konrad Dettner (University of Bayreuth, Germany) generously provided his latest reprints and preprints dealing with lymantriid venomics chemistry. We are also grateful to Sue Barth, Jennifer Graf, and Ben Barros for valuable technical assistance.
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