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IRIDOID GLYCOSIDES OF *AUREOLARIA FLAVA* AND THEIR SEQUESTRATION BY *EUPHYDRYAS PHAETON* BUTTERFLIES

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Key Word Index—*Aureolaria*; Scrophulariaceae; *Euphydryas*; Nymphalidae; sequestration; unpalatability; chemical defense; iridoid glycoside; aucubin; catalpol.

Abstract—Four iridoid glycosides were isolated and identified from *Aureolaria flava* (Scrophulariaceae): aucubin, gardoside methyl ester, 8-epiloganin, and 8-*O*-acetylharpagide. The iridoid glycoside content of *Euphydryas phaeton* (Nymphalidae), a principal herbivore of *A. flava*, was quantitatively determined by gas chromatography. In general, only aucubin was found in the butterflies, and amounts of this compound were low and variable among individuals.

INTRODUCTION

The genus *Aureolaria* Rafinesque (Scrophulariaceae) contains 11 species distributed in eastern North America and northern Mexico [1]. There has been little investigation of the secondary chemistry of the members of this genus: *Aureolaria virginica* (L.) was surveyed for flavonoids [2] and two species, *A. virginica* and *A. grandiflora* var. *pulchra* (Pennell), were reported, on the basis of paper chromatography, to contain the iridoid glycoside aucubin [3]. All species of *Aureolaria* are hemiparasitic upon the roots of oak (*Quercus*) [1]. *Aureolaria flava* Pennell, the subject of this paper, is widely distributed throughout the eastern and Great Lakes regions of the United States and has never been surveyed for iridoid glycosides.

In the Ozark region of the central United States, *Euphydryas phaeton* (Drury) (Nymphalidae) utilizes species of *Aureolaria*, and especially *A. flava*, as its primary host plants [4]. Populations of *E. phaeton* in this area are morphologically distinct from those of the northeast, and have been designated as a separate subspecies: *E. phaeton ozarkae* Masters. From their host plants, *E. phaeton* and other members of the genus *Euphydryas* sequester iridoid glycosides [5–7], compounds which render them unpalatable to birds [8, 9]. This paper describes the isolation and structural determination of the iridoid glycosides from *A. flava*. In addition, the iridoid glycosides sequestered by *E. phaeton* butterflies which were fed as larvae on *A. flava* were identified and quantified.

RESULTS

Aureolaria flava was found to contain four iridoid glycosides, with a total iridoid content of at least 5.2% of the dry weight. The relative iridoid content of the plant was estimated from the four MPLC fractions collected: fraction 1 [gardoside methyl ester (**1**), and 8-epiloganin (**2**), 1:1 mixture], 21%; fraction 2 (8-*O*-acetylharpagide, **3**), 28%; fraction 3 (aucubin, **4**), 60%.

^{1}H NMR spectroscopy was of primary importance in the identification of these compounds. The ^{1}H NMR spectrum of fraction 1 revealed two singlets at δ7.39 and 7.43, which corresponded to the H-3 protons of two separate iridoids. Therefore both of the iridoids were substituted at the C-4 position. Twin peaks of 3H each at 3.67 and 3.68 ppm represented carbomethoxy substituents at C-4 for both compounds. A broad 2H singlet at δ5.34 represented the two C-10 protons of gardoside methyl ester **1**. The exocyclic methylene group of this molecule puts H-5 and H-9 into similar chemical environments resulting in a broad 2H singlet at δ3.06. A broad triplet at δ4.42 for H-7 and a doublet at δ5.44 for H-1 provided sufficient evidence for the presence of **1**. For component **2**, the characteristic signals at δ2.63 and 2.95 for H-9 and H-5 respectively, suggested the absence of electron withdrawing groups at C-8. The 3H doublet at δ0.90 was consistent with a methyl group at C-8 and the orientation was confirmed by its chemical shift when compared to that of the epimer loganin **2a** (C-10 at δ1.06). Compounds **1** and **2** were identical with authentic samples by TLC and ^{1}H NMR spectroscopy.

The ^{1}H NMR spectrum of **3** (Table 1) showed characteristic doublets at δ4.91 and 6.39 for H-4 and H-3 respectively. The multiplicity indicated that C-4 was unsubstituted. Hydroxy substitution at the C-5 position was indicated by the singlet at δ2.88 for H-9, and the absence of long range coupling to H-3. The methyl at C-8 appeared at δ1.34. The relatively unshielded position of this methyl signal (normally at δ1.22–1.25) in conjunction with the presence of an acetate methyl signal at δ2.0 suggested the placement of the OAc group at C-8. ^{1}H NMR decoupling experiments revealed a doublet at 3.76 ppm indicating a hydroxyl group at C-6 or C-7. A

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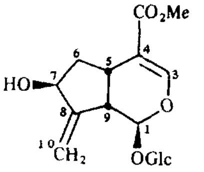

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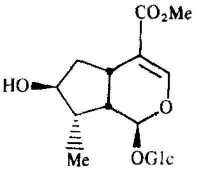

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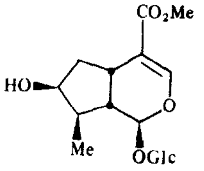

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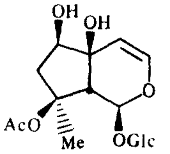

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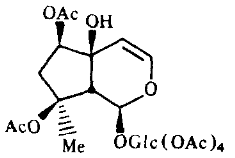

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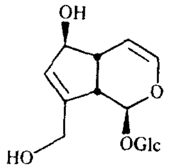

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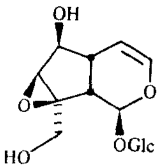

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closely spaced pair of doublets at 2.0 ppm showed geminal coupling of 15.3 Hz and was assigned to the two H-7 protons. The upfield doublet (1.94 ppm) was further split into a doublet of doublets by the neighbouring C-6 proton. The alternative structure, with a hydroxyl at C-7 instead of C-6, would place the β-proton at C-6 between two neighboring β-hydroxyls This would cause the β-proton at C-6 to be shielded relative to the β-proton at C-7 of **3**, which is between a β-hydroxyl and a β-OAc group. The placement of the acetate group at C-8 was confirmed by a ^{13}C NMR spectral comparison with literature values for **3** and for compound **3a** (harpagide hexaacetate) [10, 11] (Table 1)

Table 1 ^{13}C NMR chemical shifts for iridoids **3**–**3a** (values given in ppm, relative to TMS = 0.00 ppm)

C	**3** (CD$_3$OD)	**3*** (CD$_3$OD)	**3a**† (CDCl$_3$)
1	94.9	94.5	94.1
3	143.7	143.7	141.7
4	107.3	106.8	107.3
5	73.3	73.2	71.5
6	78.0	78.0	77.7
7	46.3	46.0	43.6
8	88.4	88.5	86.1
9	56.0	55.5	54.8
10	22.1	22.5	22.2
1′	99.9	99.8	96.5
2′	74.8	74.5	71.2
3′	78.2	77.6	72.1
4′	72.1	71.6	68.7
5′	77.7	77.6	72.1
6′	63.2	62.8	61.9

*Literature values, refer to ref [11]

†Reference 20

The major iridoid of the plant, aucubin (**4**), is common among the Scrophulariaceae and was easily identified by its ^{1}H NMR spectrum. Compound **4** was identical with an authentic sample by TLC, GC, and ^{13}C NMR spectrum.

GC analysis of *E. phaeton ozarkae* butterflies reared on *A. flava* (Table 2) revealed that they contained primarily aucubin. Out of five male butterflies examined, four contained only aucubin (**4**) while the fifth contained 0.19 mg aucubin of a total 0.22 mg iridoids. The remainder consisted of 0.033 mg of catalpol (**5**). The average weight of *E. phaeton ozarkae* tested was 42.3 mg with an average of 0.44% of dry weight being iridoid glycosides. Analyses of *E phaeton phaeton* butterflies yielded similar results (Table 2). Out of nine male *E. phaeton phaeton* butterflies reared on *A. flava*, eight contained only aucubin (**4**). The ninth contained 0.029 mg aucubin and 0.024 mg catalpol (**5**) out of 0.53 mg total iridoids. The average weight of the *E phaeton phaeton* tested was 53.7 mg with an average of 0.10% of dry weight being iridoid glycosides

DISCUSSION

The array of iridoids found in *A. flava* is not unusual for members of the Scrophulariaceae [12], which contain

Table 2 Iridoid sequestration from *A flava* by *E. phaeton**

	Avg dry weight (mg)	Avg. total iridoids (mg)	Avg. % total iridoids	Avg total aucubin (mg)	Avg % aucubin	Avg total catalpol (mg)	Avg. % catalpol
E phaeton ozarkae	42.3	0.19	0.44	0.18	0.42	0.007	0.017
N = 5	(12.7)	(0.11)	(0.20)	(0.11)	(0.19)	(0.015)	(0.038)
E phaeton phaeton	53.7	0.050	0.096	0.047	0.091	0.003	0.005
N = 9	(7.3)	(0.017)	(0.043)	(0.018)	(0.045)	(0.008)	(0.016)

*Numbers in parentheses are standard deviations iridoids derived from 8-epiloganin (**2**), rather than loganin (**2a**) [13]. Although *A. flava* is a hemiparasite on *Quercus* species and plant secondary compounds may be transferred from host to parasite [14], oaks do not contain iridoid glycosides [15]. Thus the iridoid glycosides in *A. flava* are synthesized by *A. flava* itself. The total amount of iridoid glycosides found in *A. flava*, 5.2% dry weight, is relatively high for the concentration of these compounds reported in other plant species [5, 16, 17].

Of the four iridoid glycosides found in *A. flava* (aucubin **4**, gardoside methyl ester **1**, 8-epiloganin **2**, 8-*O*-acetylharpagide **3**), only aucubin was found in the butterflies. These results agree with others showing that only aucubin, catalpol, and macfadienoside were sequestered by *Euphydryas* butterflies, regardless of the other iridoids present in the hostplant [5, 6, 16] (Bowers, Fixman, L'Empereur, Seewald, unpublished data). The catalpol found in the *E. phaeton* butterflies analysed in the present study was probably due to early instar larval feeding on *C. glabra*, because no catalpol was found in *A. flava*. The amount of aucubin in *E. phaeton* butterflies, however, was relatively low. Although aucubin was 3.10% dry weight of the plant, the butterflies only contained a mean of 0.21% dry weight aucubin. This suggests that these insects are generally inefficient at sequestering aucubin through to the adult stage.

Interestingly, the data suggest that *E. phaeton ozarkae* individuals are more efficient at sequestering aucubin than are *E. phaeton phaeton* individuals (Table 2); their aucubin content was significantly higher than that of *E. phaeton phaeton* (T-test, df = 12, $P < 0.001$). Quantification of the iridoid glycoside content of another *Euphydryas* species, *E. anicia* (Doubleday and Hewitson) showed that *E. anicia* were relatively efficient at sequestering catalpol, some individuals containing as much as 9% dry weight [6, 16].

Euphydryas phaeton butterflies reared on *A. flava* were unpalatable but not emetic to blue-jays [8]. In contrast, *E. phaeton* butterflies reared on *Chelone glabra* (Scrophulariaceae), the primary hostplant of *E. phaeton phaeton* were both unpalatable and emetic [8]. These latter butterflies contained primarily catalpol, and little if any aucubin [6]. These results corroborate those suggesting the primary importance of catalpol in the unpalatability of *Euphydryas* [6, 16]. *Euphydryas phaeton ozarkae* butterflies, whose primary host plant in nature is *A. flava*, and which thus contain aucubin (as shown here), were observed to have a 'stronger and faster flight' than *E. phaeton phaeton* [4]. In addition, *E. phaeton ozarkae* appears to be less warningly coloured than *E. phaeton phaeton* [4]. These observations, coupled with our data, suggest that *E. p. ozarkae* individuals, by using a host plant, *A. flava*, which contains aucubin, and thereby being less unpalatable than butterflies fed on a host plant species containing catalpol, *C. glabra*, may have evolved faster flight and less warning coloration than *E. p. phaeton* individuals because they are less chemically protected. The larvae of these two subspecies, however, have not diverged in coloration or behaviour: larvae of both *E. p. phaeton* and *E. p. ozarkae* are gregarious and warningly coloured bright orange and black. Determination of the iridoid glycoside content of the larvae, as well as a more complete quantitative study of ingestion, metabolism, sequestration and elimination of iridoids in these insects is clearly needed and currently underway.

EXPERIMENTAL

Plant material. *A flava* material was collected in June 1986, from a population in Leverett, Franklin Co., Massachusetts. It was dried at 50° for 4 days and stored at room temp Voucher specimens have been deposited at the Herbaria of Harvard University, Cambridge, MA

Butterfly material. Butterflies were obtained from two sources *E. phaeton ozarkae* were the first generation originating from stock collected at Greensfelder Park, St. Louis, Missouri, where the primary host plant is *Aureolaria flava* (J Sullivan, P. Koenig, personal communication) *E phaeton phaeton* were collected as eggs from a population in Leverett' Franklin Co., Massachusetts, where the host plant is *Chelone glabra* (Scrophulariaceae). Larvae of adult butterflies of both subspecies used for analysis had been reared in the laboratory as post-diapause larvae on *A. flava*. Diapausing larvae weigh 5–12 mg, and GC analyses of diapausing larvae of *Euphydryas* have shown that iridoid amounts are low, and in some cases undetectable (Bowers and Collinge, unpublished data) However, catalpol found in the butterflies analysed for this paper was probably from prediapause larvae feeding on *C glabra*. There does not appear to be metabolism from aucubin to catalpol, or more of the butterflies would likely have contained catalpol. Butterflies were frozen, then dried for at least 24 hr at 50° before being extracted.

General chemical and analytical methods. TLC was conducted on silica gel with CH_2Cl_2–EtOH developing solvents or on C_{18} silica gel eluting with H_2O–MeOH. For visualization, plates were sprayed with a solution of 90 ml 50% EtOH, 10 ml conc. H_2SO_4 and 0.5 ml *p*-anisaldehyde, followed by heating at about 110° for 3–5 min.

GC analyses of iridoid glycosides were performed on the trimethylsilylated derivatives using a gas chromatograph equipped with a split/splitless injector, FID detector and a 3392A Hewlett Packard integrator. The method was essentially that of ref. [16]. The column was a (DB-1) capillary (J&W Scientific), 30 m (length), 0.32 mm (i d.) with a film thickness of 0.10 μm. The injector was used in the split mode, split ratio of 30:1, and at a temp. of 275°. Carrier gas was high grade He (99.996%) with a column head pressure of 12 psi and a linear velocity of 30 cm/sec (1.44 ml/min). The detector temperature was 320° with a H_2 flow of 38 ml/min, air flow of 200 ml/min, and a He (make up gas) flow of 40 ml/min. Samples were run under the following program: initial column temperature 200° for 1.00 min, which was then raised to 260° at a rate of 20°/min and held for 8.00 min (aucubin, $R_t = 7.50$ min, catalpol, $R_t = 8.49$ min).

Extraction of butterflies for GC analysis. Individual butterflies were weighed, placed in screw cap test tubes, crushed, and 3–5 ml of MeOH added. The samples were extracted for *ca* 4 hr, filtered through cotton and the filtrate evapd to dryness. The dry residue was triturated with 2 ml H_2O and 3 ml Et_2O and both triturates combined in screw cap test tubes. To each tube was added 1.00 ml of internal standard, phenyl-β-D-glucose (0.50 mg/ml) Samples were mixed by shaking and placed in a centrifuge to aid in separating the layers. The Et_2O layer was removed and the aqueous layer washed two more times with 3 ml Et_2O. After the final Et_2O wash the aqueous layer was evapd to dryness, and the dry residue removed with two washings of 0.5 ml MeOH. The MeOH rinses were combined together in a 0.5 dram screw cap vial. A 10% aliquot was placed into a small glass sample tube (closed end 0.5 mm), and evapd to dryness. To this sample tube was added 0.10 ml of the silylation reagent (TRI-SIL 'Z', Pierce Chemical Company) and the closed tube was heated at 70–80° for 15 min. After the solution was cooled, one μl was injected onto the gas chromatograph. The remaining MeOH solution was also evapd to dryness and stored under refrigeration.

Extraction of plant material. Dried and ground leaves of *A. flava* (20 g) were stirred in 400 ml MeOH overnight, filtered, and the solution evapd to dryness. The residue was taken up in H_2O and extracted × 3 with Et_2O. The layers were separated and the aq. portion evapd, leaving a mixture of predominantly sugars and iridoid glucosides (4.40 g)

Isolation of compounds from Aureolaria flava 490 mg of concd extract was subjected to MPLC over silica gel [column: 300 × 25 mm (o d.)] elution beginning with $CHCl_3$–MeOH (4:1) and continuing with increasing amounts of MeOH. TLC examination of the fractions collected and combined revealed three iridoid containing portions

Structural determinations. The first portion (14 mg), yielded a mixture of compounds **1** and **2**. Reversed phase TLC showed this isolate to be *ca* 1:1 mixture The second (32 mg) and third (69 mg) portions afforded compounds **3** and **4**, respectively

Gardoside methyl ester (**1**) Amorphous solid. TLC and ^{1}H NMR were identical with a previous isolate [18] ^{1}H NMR (D_2O, HDO 4.73 ppm) δ1.8–2.2 (2H, *m*, H-6), 3.06 (2H, *br s*, H-5, 9), 3.15–3.50 (4H, *m*, H-2′, 3′, 4′, 5′), 3.66 (3H, *s*, OMe), 3.70–3.90 (2H, H-6′), 4.42 (1H, *br t*, H-7), 4.70 (1H, *d*, 8.1, H-1′), 5.34 (2H, *br s*, H-10), 5.44 (1H, *d*, 3.5, H-1), 7.43 (1H, *s*, H-3)

8-*Epiloganin* (**2**) Amorphous solid TLC and ^{1}H NMR were identical with a previous isolate [18]. ^{1}H NMR (D_2O, HDO 4.73 ppm), δ0.90 (3H, *d*, 7.0, H-10, 1.8–2.2 (3H, *m*, H-6, 8), 2.63 (1H, *br s*, H-9), 2.95 (1H, *m*, H-5), 3.15–3.50 (4H, *m*, H-2′, 3′, 4′, 5′), 3.67 (3H, *s*, OMe), 3.7–3.90 (2H, H-6′), 4.70 (1H, *d*, 8.0, H-1′), 5.50 (1H, *d*, 3.0, H-1), 7.39 (1H, *s*, H-3).

8-*O-Acetylharpagide* (**3**) Amorphous solid TLC and ^{1}H NMR spectrum were identical with a standard sample from K Ogawa ^{1}H NMR (D_2O, HDO 4.73 ppm): δ1.34 (3H, *s*, C-10), 1.94 (1H, *dd*, 0.94, 15.3, H-7α), 2.18 (1H, *d*, 15.3, H-7β), 2.88 (1H, *s*, H-9), 3.25–3.45 (4H, *m*, H-2′, 3′, 4′, 5′), 3.65 to 3.91 (2H, H6′), 3.72 (1H, *d*, 0.94, H-6), 4.60 (1H, *d*, 8.0, H-1′), 4.91 (1H, *d*, 6.1, H-4), 6.08 (1H, *s*, H-1), 6.39 (1H, *d*, 6.1, H-3) ^{13}C NMR see Table 1

Aucubin (**4**). Amorphous solid. GC, TLC, ^{1}H NMR and ^{13}C NMR were identical with a previous isolate [19]

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