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IRIDOID GLYCOSIDES IN THE BUTTERFLY *EUPHYDRYAS CYNTHIA* (LEPIDOPTERA, NYMPHALIDAE)

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Key Word Index—*Plantago lanceolata*; Plantaginaceae; *Euphydryas cynthia*; Nymphalidae; iridoids; aucubin; catalpol; 6-*O*-glucopyranosylaucubin; unpalatability.

Abstract—Larvae and pupae of the alpine butterfly *Euphydryas cynthia* contain three iridoid glucosides: two of them, aucubin and catalpol, are very common in the Plantaginaceae, but the third, 6-*O*-glucopyranosylaucubin, has hitherto only been isolated from *Odontites verna* and *Verbascum sinuatum* (Scrophulariaceae). The amounts of the three iridoids found in the insects were 0.53, 0.31 and 1.48% dry wt, respectively. Preliminary feeding experiments with an insectivorous bird indicated some unpalatability in the insect, which probably stems from the iridoid content.

INTRODUCTION

The development of insect chemical defence through the uptake and storage of secondary substances from their food plants is a widespread phenomenon (see examples from Lepidoptera [1–6]). This functional flux of material between organisms is in a wider sense a case of allelopathy, i.e. noxious chemical effects between organisms [7].

Species of the butterfly genus *Euphydryas* are found in the Northern hemisphere with some remarkable adaptations to montane areas and to high latitudes [8]. Members of this genus have been studied for their larval food plant preference and their presumed subsequent unpalatability for predators [9]. Many of the preferred food plants of the larvae of these butterflies—such as the Scrophulariaceae [10] and Plantaginaceae [10, 11]—contain iridoid glycosides. In many organisms, iridoids are recognized as major herbivore deterrents [12]. In experiments with North American *Euphydryas* species, Bowers [6, 13–16] observed that unpalatability of the butterflies was strongly correlated with the iridoid glycoside content of the larval diet.

We also considered the palatability question following observations on the larvae of the European alpine species *Euphydryas cynthia* Hb. in southern Switzerland and in Tyrol, Austria. With the comparatively dense population of larvae in the upper Rhone valley area near Crans-sur-Sierre in Switzerland, we observed a peculiar behaviour. In the habitat of an open, short-grass and herb biotope (still with patches of snow in June at 2200 m sea level), the larvae demonstrated thermo-regulatory behaviour: they aggregated in clumps of five or more in cloud shade and spread out on the food plant (*Plantago alpina* L.) in sunshine (for larval basking, see ref. [17]). The black larvae with yellow rings were visible, particularly when grouped together, and we thought they led a risky life with all the birds around breeding and searching for food.

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Since iridoid glycosides were suspected to be the food plant-derived protective chemicals in *Euphydryas* species, we checked for iridoids in the insects and here report our findings. The description of the larval aggregation and thermal regulation behaviour (including interspecies larval associations with the similarly patterned *Zygaena exulans* Hock. in Tyrol) will be described later. Incidentally, *Z. exulans* larvae were also refused by the birds, as described below. The protective chemicals in the genus *Zygaena* are known to be cyanogenic glycosides [18, 19]. Notably, both these glycosides are bitter to the human tongue and might also be so to birds.

RESULTS

Some larvae of *E. cynthia* were fed with *Plantago lanceolata* to pupation. Since the number of specimens in the final experiments with the Tyrol collection was rather small, only some were available for a feeding (repellency) test with birds. We offered living final instar larvae to tame, caged, hungry stonechats (*Saxicola torquata*). The first bird took a larva, released it, wiped its beak, took it again, beat it to death, and after some minutes, left it alone. Dead larvae were probed by several stonechats, but after up to 5 min eventually always refused them. A young tame stonechat, used to hand-feeding by a keeper, was given a larva into its gaping, wide open beak. It swallowed it instantaneously without any emetic or ill after effects. Although these observations are anecdotal, they indicate a certain degree of unpalatability of the *E. cynthia* larvae, a supposition which is in agreement with the literature [13–16].

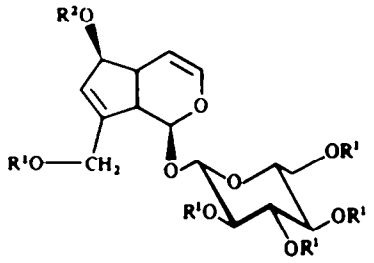
Crude methanol extracts of eight final instar larvae and eight pupae of *E. cynthia* showed a positive Trim and Hill reaction [20] and the same three iridoids in approximately equal amounts on TLC. We therefore combined all the extracts for further work-up. Column chromatography on silica gel and neutral Al_2O_3 yielded two iridoid-containing fractions, each of which was divided into two

portions: one part was methylated by a modified Hakomori method [21, 22] to analyse the products by GC/MS, and the other part was rechromatographed by HPLC to identify the isolated iridoids by comparison with authenthic samples (TLC, HPLC, IR)

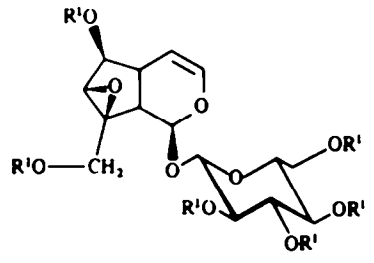
After methylation of the first fraction, preparative TLC of the reaction products afforded two iridoid permethyl ethers, identified as 6,10,2',3',4',6'-hexa-*O*-methylaucubin (**1b**) and 6,10,2',3',4',6'-hexa-*O*-methylcatalpol (**2b**) by GC/MS analysis and by comparison with authentic material [23].

The methylation product of the second fraction showed a mass spectrum very similar to that of the aucubin permethyl ether. However, the higher molecular weight (*m/z* 634) and the fragment at *m/z* 398 (**5**) revealed the presence of two hexosyl moieties in **3b**, which were eliminated successively (*m/z* 525/*m/z* 289; *m/z* 416/*m/z* 307; *m/z* 459/*m/z* 241) [23]. The absence of fragment **4** at *m/z* 194 and the peak at *m/z* 162 in the mass spectrum of **3b** showed that the aglycon contains only one *O*-methyl group. Furthermore, the fragmentation of two tetra-*O*-methylhexosyl moieties produced ions observed at *m/z* 267, 253 and 235, indicating a diglucosylated iridoid.

The ^{1}H NMR and ^{13}C NMR data of **3a** showed significant differences from those of 10-*O*-glucosylaucubin [24] but they were identical with the data published for 6-*O*-glucosylaucubin [25, 26] isolated from *Odontites verna*. Compound **3a** was finally identified as 6-*O*-glucosylaucubin by comparison with an authentic sample (IR, TLC). Working up 1280 mg of dry weight of the insects, we found the following iridoid amounts: 0.53% aucubin, 0.31% catalpol and 1.48% 6-*O*-glucopyranosylaucubin.



1a $R^1 = R^2 = H$
1b $R^1 = R^2 = Me$
3a $R^1 = H$, $R^2 = Glc$
3b $R^1 = Me$, $R^2 = Glc(Me)_4$

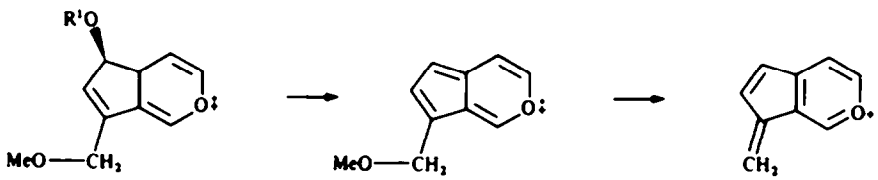


2a $R^1 = H$
2b $R^1 = Me$

DISCUSSION

Members of the butterfly genus *Euphydryas* appear to be well protected from predatory birds [13–16]. The claim that this protection is due to iridoid glycosides which the larvae obtain and sequester from their food plants, has a high degree of probability, although a stick insect, *Anisomorpha*, was found to have the capacity to synthesize an iridoid (dolichodial) [27]. *Euphydryas cynthia* butterflies, however, have to our knowledge never before been chemically analysed. Our finding of three iridoid glycosides in the larvae and pupae—adding up to 2.32% of the dry weight of the insects—is therefore confirmation of the earlier claim, albeit now with another species than that examined earlier.

It is interesting that the two rather common iridoids **1a** and **2a** are known from the food plant genus *Plantago* on which we found the larvae. The third iridoid, however, has hitherto only been found in *Odontites verna* and *Verbascum sinuatum* [25, 26], plants not known in the high Alps where the insects live. Unless the larvae feed on a still unknown plant which contains **3a**, it may be assumed that iridoid **3a** is formed from **1a** or **2a** in the insect, unless synthesized *de novo*. With respect to the larval food plant of *E. cynthia*, contrary to earlier reports of a variety of species (including *Viola*, grasses, etc.), an experienced breeder could feed them only with *Plantago* [28]. The question as to where the iridoids are stored in the insect has not yet been answered but they may be present in the haemolymph which must taste bitter when a bird picks and squeezes the larva. The black-yellow larval pattern would then serve as *aide memoire* [29] for the bird to avoid such



4 $R^1 = Me$ (*m/z* 194)
5 R^1 = 2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl (*m/z* 398)
6 (*m/z* 162)
7 (*m/z* 131)

an unpleasant experience in the future. Incidentally, one of the North American *Euphydryas* species is mimicked by a palatable butterfly which then also enjoys a protected status [16].

EXPERIMENTAL

Animal material. All the specimens of *Euphydryas cynthia* were collected as final instar larvae in Tyrol, Austria (North of the Brenner Pass above the tree line at *ca* 2000 m see level) where they were seen feeding on *Plantago alpina* L. Possibly, they also fed on other plants although we did not see this. In Seewiesen, these larvae were offered *Plantago lanceolata* L., which they readily accepted. They pupated normally and the emerging imagines were perfect. Living larvae and pupae (which were ready to emerge) were mailed to Freiburg for the chemical analysis.

Analytical methods. CC: silica gel (Merck, 40 μm), CH_2Cl_2 MeOH H_2O (70:30:3), neutral Al_2O_3 (Woelm), 50% EtOH; TLC: silica gel 60, CH_2Cl_2 MeOH H_2O (70:30:3–90:10:1). Spray reagent for iridoids: 3% vanillin and 1% H_2SO_4 in 100 ml EtOH followed by heating at 110° for 5 10 min. HPLC: μ-Bondapak C_{18} (9.4 mm × 250 mm), 5% MeOH, flow rate 0.675 ml/min. GC/MS: OV-17 (3%), column (1.2 m × 2 mm i.d.), temp. of injector 270°, column 240°, source 130° and injector 290°, column 280°, source 170°, He 30 ml/min, 70 and 30 eV.

Isolation of iridoidic fractions. The lyophilized material of eight larvae (770 mg) and eight pupae (510 mg) was separately extracted by refluxing for 30 min once with 100 ml 96% EtOH and twice with 80 ml 70% EtOH. The dried extracts of the larvae (500 mg) and those of the pupae (350 mg) were combined and separated successively by CC on silica gel and Al_2O_3. We obtained two fractions, A (16 mg) and B (24 mg), which were used for further purification by HPLC and for methylation.

Methylation. 5 mg of fraction A and 4 mg of fraction B were methylated using $NaCH_2SOMe$ and MeI in DMSO [21 23]. Pure iridoid permethyl ether was obtained by TLC on prep. silica gel with CH_2Cl_2–MeOH–H_2O (90:10:1).

Aucubin (**1a**, 1.8 mg). Identification by comparison with an authentic sample (TLC, HPLC, IR) and by GC/MS of 6,10,2',3',4',6'-hexa-*O*-methylaucubin (**1b**, 2.0 mg). IR ν_{max}^{KBr} cm^{-1}: 2979, 2927, 2833, 1654; GC/EIMS (column 240°, source 130°), 70 eV, *m/z* (rel. int.): 430 (0.18), 398 (0.56), 370 (0.12), 321 (0.12), 255 (2.46), 226 (0.56), 219 (6.69), 218 (16.03), 195 (2.73), 194 (4.15), 187 (81.24), 180 (7.04), 163 (11.58), 162 (9.19), 155 (19.26), 147 (4.69), 145 (12.73), 143 (5.74), 131 (52.95), 127 (18.55), 115 (10.19), 111 (93.61), 101 (88.01), 89 (39.41), 88 (21.14), 45 (100.0).

Catalpol (**2a**, 0.9 mg). Identification by comparison with an authentic sample (TLC, HPLC, IR) and by GC/MS of 6,10,2',3',4',6'-hexa-*O*-methylcatalpol (**2b**, 1.0 mg). IR ν_{max}^{KBr} cm^{-1}: 2927, 2883, 2849, 1652; GC/EIMS (column 240°, source 130°), 70 eV, *m/z* (rel. int.): 446 (0.07), 429 (0.05), 414 (0.23), 271 (1.99), 242 (0.33), 239 (0.18), 219 (8.71), 218 (19.08), 211 (2.23), 207 (0.57), 187 (58.95), 155 (19.77), 157 (7.79), 145 (10.26), 143 (6.41), 131 (6.49), 127 (19.89), 115 (14.93), 111 (73.80), 101 (78.10), 89 (30.80), 88 (20.79), 45 (100.0).

6-O-β-D-Glucosylaucubin (**3a**, 9.0 mg). ^1H NMR (250.1 MHz, D_2O): δ6.32 (1H, *dd*, $J_{3,4}$ = 6, $J_{3,5}$ = 2 Hz, H-3), 5.98 (1H, *br s*, H-7), 5.25 (1H, *d*, $J_{1,9}$ = 6 Hz, H-1), 5.21 (1H, *dd*, $J_{3,4}$ = 6 Hz, $J_{4,5}$ = 3 Hz, H-4), 4.81 (1H, *d*, $J_{1',2'}$ = 7 Hz, 1'-H), 4.71 (1H, *br s*, H-6), 4.62 (1H, $J_{1',2'}$ = 7 Hz, H-1"), 4.38 (1H, *d*, $J_{10A,10B}$ = 14 Hz, H-10A), 4.32 (1H, $J_{10B,10A}$ = 14 Hz, H-10B), 4.0–3.2 (12H, *m*, sugar protons), 3.15 (1H, *ddd*, $J_{9,5}$ = 8, $J_{9,1}$ = 6, $J_{9,7}$ = 1 Hz, H-9), 3.03 (1H, *m*, H-5). ^{13}C NMR (62.83 MHz, D_2O): δ147.08 (C-8), 138.47 (C-3), 125.27 (C-7), 104.70 (C-4), 101.54 (C-1"), 98.24 (C-1'), 95.54 (C-1), 89.73 (C-6), 76.64 (C-3"), 76.41 (C-5"), 76.26 (C-3'), 76.11 (C-5'), 73.72 (C-2"), 73.29 (C-2'), 70.24 (C-4"), 70.18 (C-4'), 61.57 (C-6"), 61.50 (C-6'), 60.45 (C-10), 47.87 (C-9), 42.32 (C-5). Identification by comparison with an authentic sample (TLC, HPLC, IR) and by GC/MS of 6-*O*-β-D-glucosyl-10,2',3',4',6',2",3",4",6"-nona-*O*-methylaucubin (**3b**, 1.5 mg). IR ν_{max}^{KBr} cm^{-1}: 3047, 2908, 2825, 1653; GC/EIMS (column 280°, source 170°), 30 eV, *m/z* (rel. int.): 634 (0.02), 602 (0.02), 570 (0.02), 557 (0.01), 525 (0.02), 471 (0.06), 459 (0.19), 416 (0.05), 415 (0.04), 398 (0.43), 384 (0.16), 370 (0.07), 339 (0.04), 307 (0.07), 289 (0.04), 275 (0.19), 267 (0.04), 253 (0.04), 241 (0.07), 235 (0.87), 219 (3.61), 218 (7.81), 191 (0.95), 187 (70.94), 163 (6.12), 162 (10.52), 155 (13.65), 147 (6.47), 145 (10.37), 143 (4.93), 131 (44.66), 127 (12.47), 115 (8.54), 111 (68.18), 101 (100.0), 89 (25.83), 88 (42.07).

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