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Lucibufagins: Defensive steroids from the fireflies *Photinus ignitus* and *P. marginellus* (Coleoptera: Lampyridae)*

(arthropod defensive substances/antifeedants/bufadienolides/emetics/cardiotonics)

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ABSTRACT **Feeding tests with thrushes (*Hylocichla* spp.) led to the isolation of three novel steroid pyrones from fireflies (*Photinus ignitus* and *P. marginellus*) responsible, in part at least, for the unpalatability of these insects to the birds. The term *lucibufagin* is coined for these steroidal pyrones. The closest known relatives of lucibufagins are the familiar cardiotonic bufadienolides, found in certain toads and plants.**

It had previously been suspected that fireflies—the familiar luminescent beetles of the family Lampyridae—contain noxious substances that protect them against vertebrate predators. A diversity of lizards, birds, and mammals was said to reject these insects, but the defensive substances responsible, presumed to be present in the blood and tissues of the beetles, had not been characterized (2–4).

Pursuant to our finding that fireflies of the genus *Photinus* are rejected by birds (thrushes of the genus *Hylocichla*), we developed a bioassay with these predators by which the feeding deterrency of extracts of *Photinus*, and that of fractions of the extracts, could be evaluated. The procedure led to the isolation of three novel steroidal pyrones, deterrent to the birds, and present in the beetles at defensively effective concentrations. We here describe the isolation and partial characterization of these compounds, for which we propose the term *lucibufagins*. Steroidal pyrones, or *bufadienolides*, are of restricted occurrence in nature. They have been found in some well-known toad venoms as well as in a small group of plants (ref. 5, pp. 469–476). However, no examples have been reported from invertebrates.

MATERIALS AND METHODS

The two species of *Photinus* studied, *P. ignitus* and *P. marginellus*, are common in the northeastern United States. They were collected at night with nets in the environs of Ithaca, New York, and immediately brought to the laboratory for feeding tests or chemical extractions. Because the fireflies were taken in flight, they can be assumed to be virtually all males.

Thrushes were chosen for the feeding tests because they adapted well to captivity and were known to be broadly insectivorous in their habits. A single Swainson's thrush (*Hylocichla ustulata*, male) and five hermit thrushes (*H. guttata*, three females and two males) were used. All had been taken months beforehand in mist nets during the migratory season. They were caged singly in the laboratory and were "experienced" at the time of experimentation, having previously been offered palatable and unpalatable insects in connection with other studies. They were sexed at autopsy.

The initial tests with the single Swainson's thrush were intended to provide preliminary data on the food preferences of such a bird. These tests extended over a period of 23 days, during which the thrush was offered, in daily feeding sessions, an assortment of 6–29 live, field-collected arthropods. In each session the arthropods were offered one after the other, each for 2 min, and a record was made of whether the prey was eaten, or rejected after being "pecked" (which included instances in which the arthropod was picked up in the bill of the bird and then dropped), or ignored without being touched.

Over 500 individual prey items were presented to this bird, including several species of fireflies. The list comprised the following: thomisid spiders; phalangids; isopod Crustacea; Ephemeroptera; anisopteran and zygopteran Odonata; acridid and gryllid Orthoptera; Plecoptera; corixid, notonectid, gerrid, reduviid, phymatid, and pentatomid Hemiptera; corydalid and chrysopid Neuroptera; carabid, gyrinid, hydrophilid, silphid, scarabaeid, elaterid, lampyrid, cantharid, coccinelid, tenebrionid, meloid, and chrysomelid Coleoptera; bittacid and panorpid Mecoptera; limnephilid, hydropsychid, and other Trichoptera; tortricid, geometrid (adult and larvae), drepanid, ctenuchid, noctuid, hesperiid, pierid, and nymphalid Lepidoptera; tipulid, tabanid, asilid, syrphid, sciomyzid, muscid, tachinid, and other Diptera; tenthredinid (larvae and adult), formicid, and other Hymenoptera.

The tests with the hermit thrushes were of two types. One type, designed to determine how these birds rate fireflies relative to a provenly palatable insect, involved presenting these birds with *Photinus* and with larvae of the beetle *Tenebrio molitor* (these larvae, called mealworms, have been marketed for years as laboratory food for insectivorous birds, amphibians, and other animals). Each of the five thrushes was tested in a series of daily feeding sessions, in which it was given individual live *Photinus* and mealworms, one at a time, each in a glass dish. Sequence of presentation was such that each series of three consecutive items contained two mealworms and one randomly placed *Photinus*. Each item was left with a bird until it was eaten, or for a maximum of 3 min. Fifteen to 16 items per session were offered. Bird responses were scored as follows: *eaten* (E, if the bird swallowed the item after pecking it no more than three times); *eaten with hesitation* (EH, if the bird swallowed the item after pecking it more than three times); *rejected* (R, if the bird ignored the item after pecking it one or more times); *not touched* (NT, if the bird failed to make contact with the item during the 3 min of presentation). If one or more items were not touched at the end of a session, they were not tallied, because the negative response might have been due to satiation of the bird. *Photinus ignitus* and *P. marginellus* were tested in separate sessions, for a total of 15 sessions with *P. ignitus* (3 sessions with each of 5 birds) and 12 sessions with *P. marginellus*

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(3 sessions with each of 4 birds). Sessions with any one bird and species of *Photinus* were held on consecutive days, except for weekend breaks.

The second type of test with the hermit thrushes was comparable to the preceding, except that only mealworms were used. These were of two kinds: *treated* mealworms, to which a topical dosage of firefly extract, extract fraction, or lucibufagin had been added, and *control* mealworms, which were devoid of such addition. The protocol of item presentation was the same as in the *Photinus* tests, except that treated mealworms were used instead of fireflies (15–16 items per session were again offered, except as noted below). Firefly extract, extract fractions, or lucibufagins were applied to the treated mealworms in methylene chloride solution at dosages measured out volumetrically with a micromanipulator-operated microsyringe. Control mealworms received the equivalent volume of methylene chloride alone. Presentation of mealworms to the birds was delayed for some seconds to allow for solvent evaporation. Four of the same birds used in the *Photinus* tests were employed in these assays (two males, two females). The tests with the firefly extracts and extract fractions were used in conjunction with the initial chemical procedures and provided the information that led to the isolation of the lucibufagins. As a matter of routine, extracts and extract fractions were tested on at least two birds and in several sessions per bird. The tests with the three purified lucibufagins provided a check on the antifeedant activity of these compounds. Due to limitation in their quantity, they were each tested at only one dosage (25 μg), in four single sessions with separate birds (6–15 items per session).

Nuclear magnetic resonance spectra were obtained on a Varian Associates XL-100-15 spectrometer operating at 100 MHz for protons and at 25.2 MHz for ^{13}C. The ^{13}C NMR spectra were measured in $CDCl_3$ solution or in 40% (vol/vol) CD_3OD in $CDCl_3$ solution, at a probe temperature of 35° with an internal ^2H lock. Chemical shifts δ are reported in ppm downfield from internal tetramethylsilane as zero. Infrared spectra were determined using $CHCl_3$ solutions with a Perkin-Elmer model 237 spectrophotometer. The ultraviolet spectra were recorded on a Cary 14 spectrophotometer in methanol solution.

RESULTS

The preliminary tests with the Swainson's thrush showed distinctly that fireflies are rated as undesirable by this bird. The more than 500 arthropods offered to the animal included representatives of over 100 species. Of those that were offered in greater quantity than three specimens (more than 50 species), only eight were consistently rejected or left untouched. Three of these were fireflies: *Photinus marginellus* (1 rejected, 2 untouched), *Photuris versicolor* group[§] (1 rejected, 3 untouched), and *Lucidota atra* (1 rejected, 4 untouched). The other species thus avoided included one sciomyzid fly, and four beetles belonging to families known to be chemically protected: *Epicauta pennsylvanica* (Meloidae), *Chauliognathus pennsylvanicus* (Cantharidae), *Coccinella transversalis* (Coccinellidae), and *Silpha americana* (Silphidae) (6–9).

Hermit thrushes are closely related to Swainson's thrushes, so it came as no surprise that they too discriminated against fireflies. The results were decisive with both species of *Photinus* (Table 1). Whereas not a single mealworm was left uneaten, all fireflies except one *P. marginellus* were rejected or left untouched, and this single exception was regurgitated shortly after being swallowed. Most of the *Photinus* that were rejected by the birds (that is, discarded *after* contact) represented individuals offered to them early in the experimental sequence; fireflies presented later tended to be left untouched, indicating that the birds had learned to discriminate visually against these insects. Also worthy of note is that 10 of the 30 fireflies that were rejected by the birds survived the pecking without noticeable injury.

Table 1. Fate of two species of *Photinus* offered to thrushes (*Hylocichla guttata*) in conjunction with palatable mealworms

	H. guttata (3 ♀♀, 2 ♂♂)		*H. guttata* (2 ♀♀, 2 ♂♂)	
Fate	*P. ignitus* (n = 75), %	Mealworms (n = 151), %	*P. marginellus* (n = 60), %	Mealworms (n = 123), %
E	—	99	—	98
EH	—	1	2*	2
R	23†	—	22‡	—
NT	77	—	76	—

Data from 5 and 4 birds are lumped. Numbers are percentages of total number of prey items shown in parentheses; E, eaten; EH, eaten with hesitation; R, rejected; NT, not touched (further details in text).

* The single firefly in this category was regurgitated after 1 min.

† Seventeen fireflies, which fared as follows: 5 killed; 3 injured; 6 seemingly uninjured; 3 not checked for injury.

‡ Thirteen fireflies, which fared as follows: 3 killed; 6 injured; 4 seemingly uninjured.

The chemical extraction procedures used for *Photinus* were the same for both species. Whole fireflies were suspended in methylene chloride at 0° for periods of 24–72 hr. The extract was decanted and concentrated *in vacuo* to give an oily residue, which was washed with hexane and filtered. The bioassay revealed that the hexane-soluble material was essentially inactive, and it was therefore discarded. The hexane-insoluble material was found to be active by the bioassay and accordingly it was subjected to further fractionation. Preparative thin-layer chromatography [silica gel, 20% (vol/vol) CH_3OH in CH_2Cl_2] and bioassay of the resulting fractions revealed that the majority of the bioactivity was contained in a band of UV-absorbing material(s). A second use of preparative thin-layer chromatography (silica gel, 3:3:4, by volume $CHCl_3/(CH_3)_2CO/C_6H_{14}$) resolved the UV-absorbing material into three components, designated as lucibufagins *A*, *B*, and *C*, in order of increasing polarity.

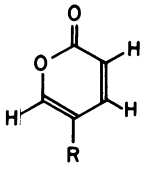
Specimens of *Photinus marginellus* afforded *ca* 50 μg of lucibufagins per animal by this procedure, consisting of about equal amounts of components *A* and *C*, and a trace of component *B*. *Photinus ignitus* contained approximately the same amounts of total lucibufagins, although in this species *B* appeared to be the predominant member of the series.

The key structural features of all three isolated distasteful compounds were readily discerned from spectral data. Because the compounds proved to be closely related to each other, we will present the results obtained with compound *A* (the least polar material), for which the data are most complete.

The low-field portion of the ^1H NMR spectrum of *A* shows a set of three interacting protons at δ 7.74 (1, doublet of doublets, J = 9.2 Hz), 7.42 (1, doublet, J = 2 Hz) and 6.30 (1, doublet, J = 9 Hz); a small additional coupling ($J \cong 1$ Hz) between the two higher field protons is incompletely resolved. Both the observed chemical shifts and the coupling constants suggest a monosubstituted α-pyrone moiety (10) and correspond par-

§ The specific designation *Photuris versicolor*, as currently used, may include several sibling species (J. E. Lloyd, personal communication).

ticularly well to values reported for 5-substituted α-pyrones (I) (11).



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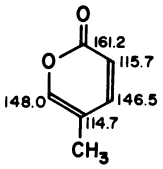
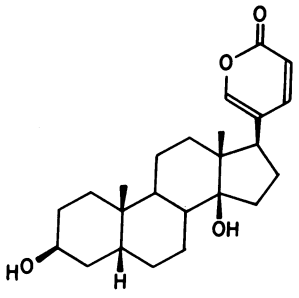


II

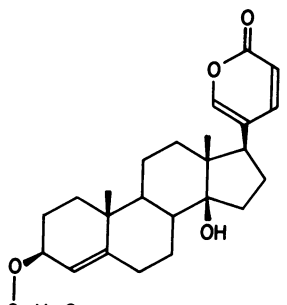
This assignment is strongly supported by the ^{13}C NMR spectrum of *A*, which shows a set of five absorptions at δ 161.9, 150.2, 146.4, 120.5, and 115.7, in good agreement with expectations based on the resonances reported for the ring carbon atoms of 5-methyl-2-pyrone (see formula II) (12). The ultraviolet spectrum of *A* shows a maximum at 300 nm, again characteristic of the α-pyrone chromophore (13). Finally, in the infrared spectrum of *A*, absorptions at 1635 and 1535 cm^{-1} may be assigned to pyrone double bonds (14); strong, overlapping carbonyl bands between 1700 and 1750 cm^{-1} suggest the presence in *A* of two carbonyl groups in addition to that of the pyrone nucleus.

The nature of the R group in partial formula I can be deduced from its ^1H and ^{13}C NMR spectra. Two prominent three-proton singlets (δ 1.20 and 0.96) in the ^1H NMR spectrum of *A* suggest the angular methyl groups of a steroid. A third three-proton singlet (δ 2.10) corresponds to the methyl group of an acetate ester. The ^{13}C NMR spectrum of *A* corroborates these conclusions. Twenty-six absorptions are observed; as discussed earlier, five of these are accounted for by the five carbon atoms of the pyrone ring, and two additional resonances may be assigned to the acetate moiety. This leaves nineteen signals, attributable to a steroid nucleus (15). Thus, the firefly defensive compounds are steroidal pyrones.

Further examination of the spectral evidence made it clear that these compounds are members of the well-known class of cardiotonic agents, the bufadienolides, typical representatives of which are the toad-derived toxin bufalin (III) (ref 5, p. 469) and scillarin (IV) from white squill (ref. 5, p. 472). However, the lucibufagins appear to be new members of this class of compounds. A detailed account of their chemistry will be presented elsewhere.



III



IV

All three lucibufagins proved to be active in hermit thrush assays (Table 2). Although the dosage at which each compound was tested (25 μg) was equivalent to only about half the total lucibufagin per *Photinus*, treated mealworms were significantly less acceptable (eaten or eaten with hesitation) than the controls ($\chi^2 > 10.6$, one degree of freedom; $P < 0.005$ for all three controls). A total of 52% of all treated mealworms was rejected or left untouched, while only 7% of all controls went similarly uneaten. In connection with the earlier finding that the single bird that ingested a *Photinus* later vomited the beetle, it should be noted that one of the birds that ingested treated mealworms also vomited during that feeding session.

Table 2. Fate of lucibufagin-treated and control mealworms offered to thrushes

	Lucibufagin					
	A		*B*		*C*	
	T	C	T	C	T	C
	(*n* = 17),	(*n* = 34),	(*n* = 12),	(*n* = 25),	(*n* = 19),	(*n* = 37),
Fate	%	%	%	%	%	%
E	12	41	17	68	58*	95
EH	29	44	—	24	16	5
R	53	15	83	8	16	—
NT	6	—	—	—	10	—

Mealworms were treated either by addition of 25 μg of lucibufagin (T) or untreated (C) and were fed to birds (*Hylocichla guttata*, 2 females, 2 males). Data from 4 birds are lumped; numbers are percentages of total number of mealworms shown in parentheses. Other conventions as in Table 1.

* One of the birds, which ate 2 of the 11 mealworms in this category, vomited twice during the feeding session.

The data suggest that the three lucibufagins might differ in antifeedant potency (compounds *A* and *B* may be more active than compound *C*), but due to the inadequate supply of pure lucibufagins this could not be substantiated by further testing.

DISCUSSION

Defensive substances of insects are widespread and of considerable chemical diversity (16), yet the lucibufagins represent a truly novel addition to the list. Nothing was previously known about the defensive chemistry of fireflies, and steroidal pyrones were known from no other insect source. There are, however, other defensive steroidal agents found in insects. Cardenolides have been isolated from certain danaid butterflies, grasshoppers, chrysomelid beetles, and other insects (17–21), and a diversity of steroids is secreted by dytiscid beetles (22). It is of interest that the vertebrate counterpart of the lucibufagins, the bufadienolides from toads, also serve as defensive agents. These compounds must certainly have an antifeedant role in toads, but they may even serve in this capacity *vis-à-vis* herbivores in plants. All in all, the defensive roles that steroids play in nature are worthy of increased investigation.

Our finding that fireflies are unacceptable to thrushes are in line with previous claims of the unacceptability of these insects to some birds (4). Particularly noteworthy are the experiments of Jones (23), who, in line with our results with the single Swainson's thrush, found fireflies to be rated as among the least desirable of insects, together with, among others, cantharid, meloid, coccinellid, and silphid beetles, in tests in which assortments of insects were offered in open trays to birds at a natural feeding site. The unacceptability of both species of *Photinus* to our hermit thrushes was virtually absolute. As subjects for bioassay the birds were therefore ideal, and their eventual discrimination against lucibufagin-treated mealworms provided proof that these substances are responsible, in some measure at least, for the deterrent quality of fireflies.

A definitive elucidation of the mode of action of lucibufagins will need to await further experimentation when additional material becomes available. It is clear from our tests that these substances are capable of immediate deterrency, that is, of causing a food item to be rejected before it is actually swallowed. But swallowing of such an item did occasionally occur (witness the fate of one *Photinus* and several lucibufagin-

treated mealworms), and under such circumstances the lucibufagins may take action through some sort of systemic effect. Vomiting may be the natural concomitant of such an effect, and the fact that it occurred after ingestion of both a *Photinus* and a treated mealworm offers support to this view. It is interesting in this connection that the cardenolides from insects such as monarch butterflies are also emetic to birds (24). Cardenolides are cardiotonic to vertebrates (ref. 5, p. 465), as are bufadienolides (ref. 5, p. 469), and the lucibufagins may well share with these compounds an overall similarity in physiological action. Also in this context it should be noted that in the bioassays with the pure lucibufagins (Table 2) even some of the controls (untreated mealworms) were rejected or eaten with hesitation. While it is possible that the birds were merely generalizing on the basis of the distastefulness of the treated mealworms and had become "wary" of *all* mealworms, it is equally possible that their appetite or general "attitude" toward feeding had been affected by "nausea" or some other ill effect induced by the ingested lucibufagins. Whatever the expalantion, we know from experience with assays of nonsteroidal substances that *Hylocichla* do not necessarily develop a discriminatory tendency toward untreated mealworms simply because the treated items are also mealworms. The fact that lucibufagins may have cardiotonic or other physiological action raises still another point: their possible use as medicinal agents should obviously be investigated.

Nothing definitive can be said about the biosynthetic origin of the lucibufagins. *De novo* synthesis by the fireflies themselves seems unlikely, because the only available evidence indicates that insects are incapable of carrying out steroid biosynthesis from nonsteroidal precursors (25). The most reasonable hypothesis, therefore, is that the beetles produce the lucibufagins from ingested cholesterol, a process already demonstrated to account for bufadienolide synthesis in toads (26). Other defensive steroids of insects need not be produced by such endogenous transformation. The cardienolides of monarch butterflies, for example, appear to be sequestered as such from the milkweed plants upon which their larvae feed (27, 28). Because surprisingly little is known about the food of *Photinus*, a comparable dietary origin of their lucibufagins cannot definitively be ruled out. But it seems improbable, at any rate, that fireflies come upon their defenses by feasting on toads.

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