# Sequestration of cucurbitacin analogs by New and Old World chrysomelid leaf beetles in the tribe Luperini

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## Summary

Two South American polyphagous leaf beetles, *Diabrotica speciosa* and *Cerotoma arcuata*, selectively accumulated the bitter tasting compound 23,24-dihydrocucurbitacin D in their body after ingesting root tissues of cucurbit plants. Similarly, three Asian Cucurbitaceae-feeding specialists in the genus *Aulacophora* were found to sequester the same compound. Cucurbitacin analogs were shown to deter feeding by a bird predator, indicating an allomonal role for these compounds in cucurbitacin-associated chrysomelid leaf beetles both of New and Old Worlds. The strong affinity to cucurbitacins, selective sequestration of the analogs and consequent protection from predators suggested an ecological adaptation mechanism developed in common among these two geographically isolated subtribes in the Luperini.

## Key words

sequestration, pharmacophagy, defense, allomone, kairomone, cucurbitacin, Cucurbitaceae, Coleoptera, Chrysomelidae, Luperini, *Diabrotica, Aulacophora*

## Introduction

A number of leaf beetles belonging to the tribe Luperini (Coleoptera: Chrysomelidae: Galerucinae) are associated with the plant family Cucurbitaceae. Cucurbitacins, a group of highly oxygenated tetracyclic triterpenes commonly found in the Cucurbitaceae have been shown to act as potent feeding stimulants for adults of these species (Chambliss & Jones 1966a, b; Shinha & Krishna 1969; DaCosta & Jones 1971; Metcalf *et al.* 1982; Nishida *et al.* 1986). Metcalf (1979, 1985, 1986) reviewed the ecological similarities between two closely related but geographically isolated subtribes, the Aulacophorina of the Old World and the Diabroticina of the New World both of which show strong affinity to cucurbitacins. It has been suggested that the powerful feeding response by adults of the polyphagous diabroticites to cucurbitacins may be a relic feature of their ancestral association with the Cucurbitaceae (Metcalf 1979). On the other hand, it has been suggested that the phagostimulant activity of cucurbitacins may be associated with chemical defense mechanism against predators by sequestering bitter principles in the bodies of insects (Howe *et al.* 1976; Gould & Massey 1984; Ferguson & Metcalf 1985; Nishida *et al.* 1986). Ferguson *et al.* (1985) have extensively studied the fate of cucurbitacin B in diabroticite adults. Although cucurbitacin B is common in the Cucurbitaceae, these plants contain a complex mixture of cucurbitacin derivatives in varying quantities (Lavie & Glotter 1971; Metcalf 1986). Chemical analysis of several cucurbitacin-associated leaf beetles revealed that both New and Old World species selectively accumulated cucurbitacin analogs which are not always the major constituents in the plants on which they feed. We have compared the chemical identity and contents of cucurbitacins in body tissues between the South and North American Diabroticina (*Diabrotica speciosa, Cerotoma arcuata* and *Acalymma vittatum)* and the Asian Aulacophorina (*Aulacophora femoralis, A. foveicollis* and *A. coffeae)* and examined the deterrent effect of cucurbitacins against sparrows.

## Materials and methods

### Instruments

Mass spectra (MS) were measured with a Hitachi M-80 mass spectrometer. Proton magnetic resonance (PMR) spectra were measured in $CDCL_3$ with a JEOL JNM FX 90Q spectrometer (90 MHz) using tetramethylsilane as an internal standard. Ultraviolet (UV) spectrum was recorded with a Shimadzu UV-360 recording spectrophotometer.

### Sampling of insects

Adults of *Diabrotica speciosa* Germer (225 insects) and *Cerotoma arcuata* Oliv. (160 insects) were collected from a bean field in Goiânia, Goiàs, Brazil (February 1985), and allowed to feed on either the slices of roots of *Cerato-santhes hilariana* Cogn. or leaves of *Cucurbita moschata* Poiret for one day and then on cowpea leaves for two days. The insects were placed in ethanol and stored in a refrigerator. Adults of *Acalymma vittatum* Fabr. (16 insects) were collected from a cucumber garden in Geneva, New York, USA (August 1988) and immediately placed in ethanol. Adults of *Aulacophora foveicollis* Lucas (40 insects) and *A. coffeae* Hornst. (14 insects) found feeding on pumpkin leaves in Serdang, Selangor, Malaysia (March 1986) were placed in ethanol. *A. femoralis* Motschulsky (20 adults) were collected from a vegeta-

ble garden in Sakyo-ku, Kyoto, Japan (September 1985) fed on pumpkin leaves (*Cucurbita pepo* L.) for about 2 months in an outdoor screen cage. After the insects stopped feeding to begin hibernation the culture was given only water for 3 weeks and then extracted with ethanol.

### Isolation of cucurbitacins from leaf beetles

Insects in ethanol were finely ground and extracted successively with mixtures of ethanol, chloroform and acetone and then filtered (Toyo filter paper No. 2). The extracts were combined, concentrated under reduced pressure (20 mmHg, 35 °C), dissolved in ethyl acetate (0.2–0.5 ml/insect) and washed twice with saturated sodium chloride. The organic layer was dried over anhydrous sodium sulfate overnight, and the solvent removed *in vacuo* to yield an oily material.

The ethyl acetate layer from *C. arcuata* (85 mg/160 adults) was chromatographed on a short silica gel column (3 g of Wako gel C-200, 50 mm × 12 mm i.d.) and eluted with benzene (10 ml), 10% ethyl acetate in benzene (10 ml) 30% ethyl acetate in benzene (10 ml), 50% ethyl acetate in benzene (10 ml) and ethyl acetate (20 ml). The last ethyl acetate eluate (5.8 mg) was chromatographed by HPLC on Nucleosil 100-5 (300 mm × 8 mm i.d.) eluted with 60% ethyl acetate in hexane at 3 ml/min and monitored with a refractive index detector (Knauer differential refractometer). Compound **1** was isolated at a retention time (*Rt*) of 14.0 min (yield: 2.8 mg).

A similar procedure used for the fractionation of the *D. speciosa* extract (ethyl acetate layer: 78 mg) yielding 1.3 mg of compound **1** (*Rt* = 14.0 min) and 0.9 mg of compound **2** (13.6 min) (see Fig. 1). From 40 insects *A. foveicollis* and 14 insects *A. coffeae* 0.10 mg and 0.12 mg of compound **1** was respectively isolated. In the case of *A. femoralis,* the silica gel column chromatographic fractions eluted with 50% ethyl acetate in benzene (10 ml) and ethyl acetate (20 ml) were combined, and subjected to HPLC (Fig. 3). Cucurbitacin analogs **1**, **3**, **4** and **5** were isolated at *Rt* = 14.2 min (yield: 0.10 mg), 13.1 min (0.04 mg), 5.8 min (0.015 mg) and 6.4 min (0.02 mg), respectively

### Feeding deterrent bioassay of cucurbitacins to sparrows

Feeding deterrency of cucurbitacins was examined using Japanese tree sparrow, *Passer montanus saturatus* Stejneger. Polished rice grains were treated with chloroform solution of cucurbitacins (1–5 μg/μl) to coat the grain (2 μg or 10 μg/grain). Two square plates (16 × 16 $cm^2$) were placed next to each other in an outdoor bird-feeding arena (Kyoto, Japan), and 30 treated grains were placed in one plate and 30 untreated grain (solvent blank) were placed in the other plate. The deterrent effect was evaluated from average number of grains remaining from an initial number of 30 grains after both plates were almost evenly visited by sparrows (at least 3 trials for each compound).

## Results

### Sequestration of cucurbitacins by *Diabrotica*

Adults of *D. speciosa* and *C. arcuata* aggregating on the cucurbit plants were arrested for long period until their bodies were extraordinarily swollen (Contardi 1939; Nishida *et al.* 1986). The insects which had been fed on roots of *Ceratosanthes hilariana* had an extremely bitter taste to human, while the adults raised on cowpea leaves did not taste bitter. An ethanolic extract of *D. speciosa* fed on leaves of *Cucurbita moschata* during adult stage also tasted moderately bitter. The bitter principles were separated from the whole body extracts of the beetles fed on the roots of *C. hilariana* by means of solvent extraction, column chromatography and HPLC. Figure 1 shows liquid chromatograms of the bitter fractions obtained from *D. speciosa* (upper) and *C. arcuata* (lower). Both species contained compound **1** as the major bitter factor. This compound was identified as 23,24-dihydrocucurbitacin D (Fig. 2) from its *Rt*-value on HPLC, PMR and mass spectra ($M^+ - H_2O$: m/z 500), compared with its authentic sample isolated from *C. hilariana* roots. The spectral data are shown below:

Compound **1** (23,24-dihydrocucurbitacin D). MS (20 eV) *m/z*(%): 500($M^+ - H_2O$, 3.8), 482($M^+ - 2H_2O$, 24), 403(100), 386(42), 385(78), 369(24), 343(24), 325(13), 237(15), 219(11), 189(25), 187(17), 166(20), 149(26), 142(93), 115(29), 113(53), 87(31), 86(32), 85(34), 84(51), 83(42), 70(26), 59(25), 43(37). PMR δ: 5.79(1H, multiplet), 4.3(2H, multiplet), 3.23(1H, broad doublet, $J$ = 15 Hz), 1.43(3H, singlet), 1.37(3H, singlet), 1.35(3H, singlet), 1.28(3H, singlet), 1.25(3H, singlet), 1.22(3H, singlet), 1.08(3H, singlet), 0.98(3H, singlet).

Compound **2** in *D. speciosa* showed a molecular ion at *m/z* 402 in its mass spectrum. The PMR spectrum of **2** showed signals typical of the tetracyclic structure of cucurbitacin, but lacking signals corresponding to the side chain (from C-22 to C-27). Compound **2** isolated from *C. hilariana* roots was characterized as hexanorcucurbitacin D (Fig. 2) by

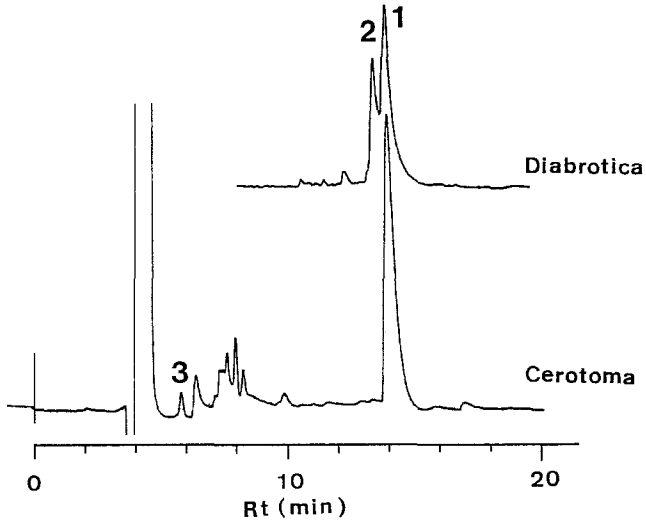


**Fig. 1** Liquid chromatograms (HPLC) of "bitter" fractions from *Diabrotica speciosa* (upper) and *Cerotoma arcuata* (lower) fed with the roots of *Ceratosanthes hilariana*

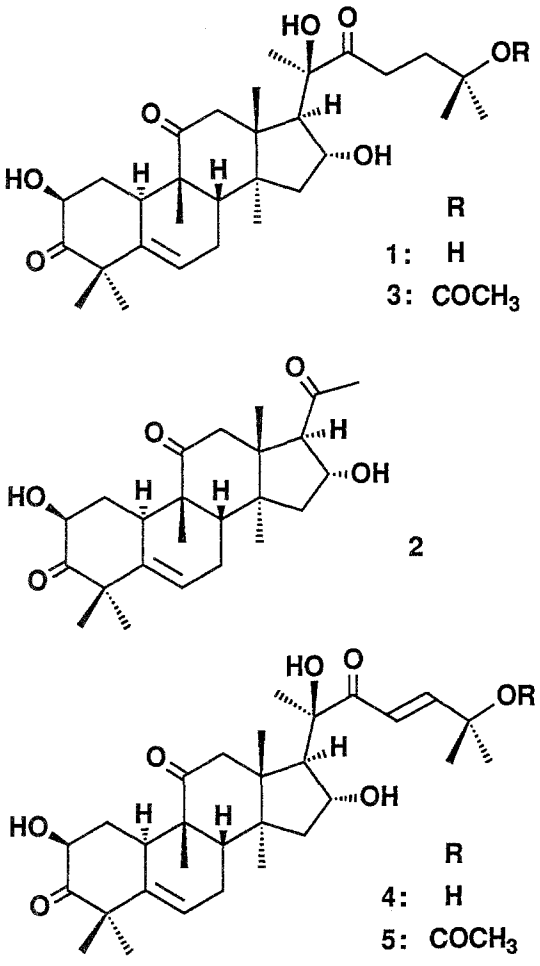


**Fig. 2** Structures of cucurbitacin analogs sequestered by various leaf beetles. 1 23,24-Dihydrocucurbitacin D, 2 Hexanorcucurbitacin D, 3 23,24-Dihydrocucurbitacin B, 4 Cucurbitacin D, 5 Cucurbitacin B

comparison of its PMR and $^{13}$C-NMR spectra with those of reported data (Doskotch & Hufford 1970; Velde & Lavie 1983).

Compound **2** (hexanorcucurbitacin D). MS (70 eV) *m/z*(%): 402($M^+$, 31), 387($M^+ - CH_3$, 46), 384($M^+ - H_2O$, 40), 369($M^+ - CH_3 - H_2O$, 56), 359($M^+ - COCH_3$, 17), 341(14), 237(14), 231(17), 207(24), 189(56), 187(43), 177(26), 175(57), 174(26), 173(42), 166(30), 161(36), 159(44), 148(50), 145(37), 137(43), 136(44), 135(58), 133(38), 125(46), 123(28), 121(32), 119(60), 111(55), 109(47), 107(49), 105(65), 95(45), 93(52), 91(64), 87(40), 83(62), 79(43), 67(41), 55(57), 43($COCH_3$, 100), 41(91). PMR δ: 5.80(1H, multiplet), 4.98(1H, broad triplet, $J=7$ Hz), 4.44(1H, double doublet, $J=6$ and 13 Hz), 3.42(1H, broad doublet, $J=15$ Hz), 3.18(1H, doublet, $J=7$ Hz), 2.53(1H, doublet, $J=15$ Hz), 2.19(3H, singlet), 1.41(3H, singlet), 1.37(3H, singlet), 1.31(3H, singlet), 1.08(3H, singlet), 0.69(3H, singlet).

A small quantity of 23,24-dihydrocucurbitacin B (**3**) was found to be present in the body extract of *C. arcuata* (Fig. 1). Compounds **1** and **3** were also detected from adults of *D. speciosa* which had been compulsively fed on leaves of *C. moschata* (contents of **1**: 2μg/insect; **3**: 0.5μg/insect).

**Table 1** Cucurbitacin contents (μg/insect)[1] in the body tissues of cucurbitacin associated leaf beetles

| Insect species | Cucurbitacin analogs[2] 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| *Diabrotica speciosa*[3] | 6 | 4 | 0 | — | 0 |
| *Cerotoma arcuata*[3] | 18 | 0 | 0.5 | 0 | 0 |
| *Acalymma vittatum* | 0 | 0 | 0 | — | 0 |
| *Aulacophora femoralis* | 5 | — | 1 | 2 | 1 |
| *A. foveicollis* | 3 | 0 | 0 | — | — |
| *A. coffeae* | 9 | 0 | 0 | 0 | — |

[1] Contents of compounds with undetectable amounts (<0.1 μg/insect) were given by the value 0. The presence of compounds with the sign "—" could not be verified due to interferences by unidentified chemicals on the HPLC analysis

[2] Compounds 1, 2, 3, 4 and 5 represent 23,24-dihydrocucurbitacin D, hexanorcucurbitacin D, 23,24-dihydrocucurbitacin B, cucurbitacins D and B, respectively

[3] Fed on *Ceratosanthes hilariana* roots

The striped cucumber beetle, *A. vittatum*, captured on cucumber plants in the US did not contain compound **1** in a significant quantity, although the presence of an unidentified cucurbitacin analog was suggested in an ethyl acetate eluate of the silica gel column. Table 1 summarizes the contents of cucurbitacin analogs found in these insects.

### Sequestration of cucurbitacins by Aulacophorina

Extracts of adults of the Japanese cucurbit leaf beetle, *A. femoralis* which stopped feeding for diapause were analyzed by HPLC (Fig. 3). Besides the major component, compound **1**, at least 3 other analogs (**3**, **4** and **5**) were found in the chromatogram. Compound **4** was unequivocally identified as cucurbitacin D (Fig. 2) from its mass fragmentation pattern (Audier & Das 1966) and the PMR spectrum. The presence of cucurbitacin B (**5**) was confirmed by its specific UV absorption ($\lambda_{max}$ 228 nm), and the mass spectrum. The total cucurbitacin content was as high as 10 μg (Table 1). Two Malaysian pumpkin leaf beetles, *A. foveicollis* and *A. coffeae*, were also analyzed under the same condition, and the major bitter component was identified as compound **1** (Table 1). Followings are the spectrometric data of compounds **3**, **4** and **5** isolated from *A. femoralis:*

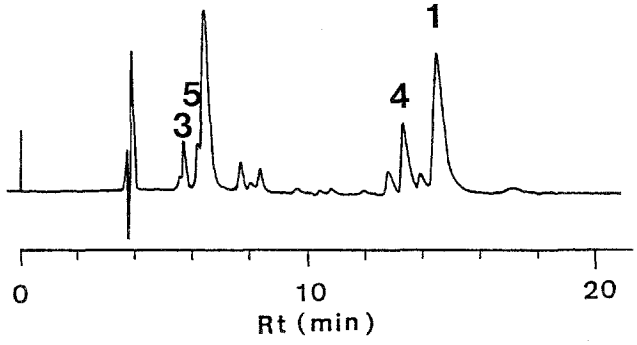


**Fig. 3** Liquid chromatogram (HPLC) of a "bitter" fraction from the overwintering adults of *Aulacophora femoralis*

Compound **3** (23,24-dihydrocucurbitacin B). MS (70 eV) *m/z*(%): 500($M^+ - CH_3COOH$, 0.2), 403(26), 385(18), 286(33), 115(16), 113(19), 105(16), 97(17), 96(19), 87(30), 70(31), 69(26), 55(28), 43(100), 41(51).

Compound **4** (cucurbitacin D). MS (70 eV) *m/z*(%): 498($M^+ - H_2O$, 1), 403(5), 385(3), 368(5), 236(3), 113(28), 112(29), 97(38), 96(97), 87(33), 81(36), 71(42), 69(48), 57(58), 55(52), 43(100), 41(88). PMR δ: 7.14(1H, doublet, $J$=15 Hz), 6.70(1H, doublet, $J$=15 Hz), 5.80(1H, multiplet), 4.4(2H, multiplet), 1.40(3H, singlet), 1.36(3H, singlet), 1.30(3H, singlet), 1.28(3H, singlet), 1.08(3H, singlet), 0.98(3H, singlet).

Compound **5** (cucurbitacin B). MS (70 eV) *m/z*(%): 498($M^+ - CH_3COOH$, 0.3), 385(11), 113(26), 96(60), 69(29), 55(33), 43(100), 41(68). UV $\lambda_{max}$: 228 nm (in ethanol).

### Deterrent effect of cucurbitacins against sparrows

Table 2 shows the average number of grains remaining after 30 grains were exposure to sparrow feeding treated with cucurbitacin analogs **1**, **2**, **3**, **4** and **5**. All analogs tested showed deterrent effects at some dose. However, cucurbitacin B (**5**) was the most potent since it was consistently rejected at a dose of 2 μg.

**Table 2** Deterrent effects of cucurbitacins against sparrows in the rice grain feeding test

| Sample name | (Dose) | Sample* | Control* |
|---|---|---|---|
| 23,24-Dihydrocucurbitacin D [1] | (10 μg) | 13.4±10.3 | 0 |
| Hexanorcucurbitacin D [2] | (10 μg) | 10.5± 7.7 | 0 |
| 23,24-Dihydrocucurbitacin B [3] | (10 μg) | 21.8± 6.6 | 0 |
| Cucurbitacin D [4] | (2 μg) | 14.2±11.1 | 0 |
| Cucurbitacin B [5] | (2 μg) | 28.6± 2.3 | 0 |

* Average number of grain remaining on the tray

## Discussion

Cucurbitacins are known, on the one hand, to play a decisive role as protective semiochemicals of the Cucurbitaceae against the attacks by herbivores, and on the other hand, to act as phagostimulants for many chrysomelid leaf beetles in the tribe Luperini (Chambliss & Jones 1966a, b; DaCosta & Jones 1971; Metcalf 1986). In addition to anti-feedant activities cucurbitacins show strong toxicity and other biological activities for various animals (David & Vallance 1955; Metcalf 1986). We have demonstrated here the chemical identity of cucurbitacins selectively sequestered by both Diabroticina and Aulacophorina species, and shown the allomonal activity of these cucurbitacin analogs against a bird predator.

Although cucurbitacin B (**5**) was the most predominant constituent of *Ceratosanthes hilariana* roots, *D. speciosa* and *C. arcuata* appeared to sequester a relatively minor components, 23,24-dihydrocucurbitacin D (**1**) and/or hexanorcucurbitacin D (**2**) in the body tissues [The contents of cucurbitacins per 100 g of the fresh root were as follows: Cucurbitacins B, 110 mg; D, 28 mg; E, 7 mg; L, 20 mg; G + H, 23 mg; 23,24-dihydrocucurbitacin B, 8 mg; 23,24-dihydrocucurbitacin D, 24 mg; hexanorcucurbitacin D, 0.2 mg (R. Nishida, unpubl.)]. Dihydro-derivative **1** was also detected from *D. speciosa* fed with *Cucurbita moschata* leaves. Our preliminary feeding experiment using pure cucurbitacins implied that both *D. speciosa* and *C. arcuata* did not incorporate cucurbitacin B in a significant quantity in spite of its potent phagostimulant activity. According to feeding experiments by Ferguson *et al.* (1985) several North American diabroticites were shown to excrete the bulk of $^{14}C$-labeled cucurbitacin B, while a small portion of cucurbitacin B was permanently incorporated as a conjugate. The profile of free cucurbitacins in those North American species has not been clarified. It is uncertain whether the insects directly accumulate the analogs from the plants or convert the major analogs such as cucurbitacin B to its hydrolyzed-hydrogenated form (**1**) or cleaved form (**2**) metabolically.

Similar to the polyphagous diabroticites, the Cucurbitaceae-feeding specialists of the Old World, *A. femoralis, A. foveicollis* and *A. coffeae,* were strongly stimulated to feed on the filter paper impregnated with cucurbitacin B and other analogs (Shinha & Krishna 1969, 1970; R. Nishida, unpubl.). All of these *Aulacophora* species were also shown to sequester 23,24-dihydrocucurbitacin D as a major component. Since the latter two tropical species were extracted immediately after the insects were captured in a pumpkin field, the extracts should contain cucurbitacins in the undigested leaf matters remaining in the intestine. In the case of *A. femoralis,* however, the adults were analyzed in the overwintering stage and several analogs including cucurbitacin B were detected. The profile of cucurbitacins in the *A. femoralis* body seemed to vary, probably depending upon the availability of the food plants in their natural habitat. Leaves of cultivated cucurbit plants frequently contain very low concentration of cucurbitacins even when the roots have very high concentration (Rhodes *et al.* 1980; Metcalf *et al.* 1982). Since *Aulacophora* larvae feed exclusively on the roots of the host cucurbits, a large portion of cucurbitacin may have been acquired during the larval stage. It should be also noted that all of these three *Aulacophora* adults display characteristic trench-cutting behavior and usually eat inside of the circles. We have not yet determined whether this behavior is associated with induction of cucurbitacins or contrarily avoidance of excess cucurbitacin intake (Carroll & Hoffman 1980; Tallamy 1985).

Howe *et al.* (1976) suggested a possible defense mechanism in cucurbitacin-associated diabroticite beetles against birds or other insect-feeding vertebrates. Ferguson & Metcalf (1985) have clearly shown the defensive effect against a praying mantis, *Tenodera aridifolia sinensis* Saussure, using diabroticite beetles which fed on bitter squashes. Our results indicated the effectiveness of cucurbitacins to a vertebrate predator. Although cucurbitacin B was found to be most effective to sparrows, most of the leaf beetle species analyzed here sequestered 23,24-dihydrocucurbitacin D with moderate deterrent activity. The selective accumulation of the dihydro-derivative may be due to a more comprehensive defense effectiveness against a wide range of enemies, or due to its physiological properties including chemical stability in the insect tissue. Gould & Massey (1984) tested bitter-tasting dia-

broticites against mice, quails and toads, but no significant rejecting responses were induced from those vertebrates. Clearly, there are differences in the acceptability of cucurbitacins among animals and further work is needed to understand the predatory spectrum created by these compounds in the diet of insects.

The Luperini comprises about 1000 species of the Diabroticina (New World) and about 500 species of the Aulacophorina (Old World) (Smith 1966). The remarkably similar affinity of these two subtribes to the cucurbitaceous plants suggested that the coevolutionary association between the Luperini and the Cucurbitaceae must have occurred during a geological period when continental land bridges were still present (Metcalf 1979, 1986). The genus *Aulacophora* seems to have evolved in Indonesia and to have radiated into Southeast Asia, extending north to the continent and south to Australia (Metcalf 1985). Some of the species such as *A. nigripennis* Motschulsky and *A. bicolor* (Weber) appear to feed primarily on wild Cucurbitaceae (*e.g. Trichosanthes* spp.) and occasionally invade cultivated plants (in Japan), whereas *A. femoralis* and *A. foveicollis* are more strongly associated with cultivated cucurbits. Since the genus *Cucurbita* is indigenous to the New World and was not known in the Old World prior to Columbus' voyage (Whitaker & Bemis 1975), this association must have occurred as the result of man's activity in rather recent time. The Diabroticina of the New World is almost entirely neotropical in distribution, and a number of the serious pest diabroticites in South and North America also extended in recent times in association with the development of agro-ecosystem (Smith 1966). Some of the species such as *Acalymma blandulum* (LeConte) are intimately associated with wild *Cucurbita* and do not move over to cultivated *Cucurbita* (Smith 1966). The striped cucumber beetle, *A. vittatum,* is a specialist on cultivated cucurbits. In contrast, a number of diabroticites such as *Cerotoma arcuata, Diabrotica speciosa, D. undecimpunctata howardi* Barber and *D. balteata* LeConte exhibit wide range of host preference and show strong affinity to cucurbitacins (Chambliss & Jones 1966a; Metcalf *et al.* 1982; Nishida *et al.* 1986). The western corn rootworm, *D. virgifera virgifera* LeConte, is essentially monophagous on corn (Branson & Ortman 1970) and yet stimulated to feed on cucurbitacins (Howe *et al.* 1976; Metcalf *et al.* 1982). Since these species are attracted to cucurbit plants for the purpose of acquisition of the bitter defense substances, they are regarded as the pharmacophagous species (Boppré 1984; Nishida & Fukami 1990).

The strong affinity to cucurbitacins by the diabroticites even after shifting their hosts to non-cucurbitaceous plants seems to be the remnant of a host-finding behavioral pattern, which has been effectively retained in the gene pool of the subtribes under the very similar ecological pressure. It is thus conceivable that the pharmacophagous species in the New World have been more recently derived from the oligophagous cucurbitaceae-feeders. Although a number of Asian species in the tribe Luperini are polyphagous, the pharmacophagous species associated with cucurbitacins is not known so far from the Old World. A similar relationship between Old and New World insects can be seen in the association of danaine and ithomiine butterflies (Nymphalidae) with pyrrolizidine alkaloids, which also involves pharmacophagous interactions with plants (Boppré 1978, 1990; Brown 1984; Trigo & Brown 1990; Nishida *et al.* 1991). Comparative studies on the roles plant semiochemicals play in the ecological adaptation of insects may provide some understanding of the evolutionary pathway between Old and New World insect species.

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