

## Cucurbitacins as kairomones for diabroticite beetles

(terpenoids/coevolution/feeding stimulants)

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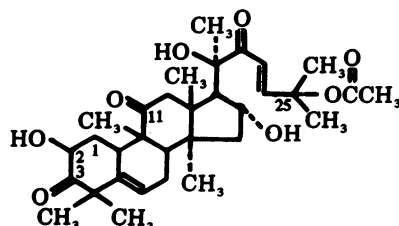
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**ABSTRACT** The characteristic bitter substances of the Cucurbitaceae act as kairomones for a large group of diabroticite beetles (Chrysomelidae, Galerucinae, Luperini), promoting host selection and compulsive feeding behavior. These beetles (e.g., *Diabrotica undecimpunctata howardi*) respond to as little as 1 ng of cucurbitacin (Cuc) B on thin-layer plates by arrest and compulsive feeding. Six species of diabroticite beetles were about 10 times more responsive to Cuc B than to Cuc E and less responsive to Cuc D, I, and L. Chloroform extracts of 18 species of *Cucurbita* were developed on thin-layer chromatograms and exposed to diabroticite beetles. The feeding patterns showed pronounced beetle responses to three general Cuc distribution patterns: Cuc B and D as in *Cucurbita andrea* and *C. eschscholiana*; Cuc E and I as in *C. okeechobeensis* and *C. martinii*; and Cuc E glycoside in *C. texana*. All the diabroticites responded in exactly the same feeding patterns. The results demonstrate a coevolutionary association between the Cucurbitaceae and the Luperini, during which the intensely bitter and toxic Cucs that arose to repel herbivores and protect the plants from attack became specific kairomone feeding stimulants for the beetles.

The coevolutionary association between the plants of the family Cucurbitaceae and diabroticite beetles of the tribe Luperini, family Chrysomelidae, provides a classic example of the role of secondary plant substances as kairomones, which promote host selection and feeding by phytophagous insects (1). The Cucurbitaceae contain some 900 species in about 100 genera, many familiar as the wild gourds, squash, cucumbers, and melons of *Cucurbita*, *Cucumis*, *Citrullus*, *Marah*, *Echinocystis*, *Lagenaria*, *Sicyos*, *Echallium*, and *Bryonia*. At least 100 species in 30 genera have been shown to contain a group of about 20 oxygenated tetracyclic triterpenes—the cucurbitacins (Cucs) (Fig. 1) that are responsible for the characteristic bitter taste of most wild Cucurbitaceae (2, 3). Current thinking holds that the Cucs were selected by evolutionary processes to protect the Cucurbitaceae against attack by herbivores, both invertebrate and vertebrate. The Cucs are the most intensely bitter substances yet characterized; a taste panel in our laboratory has repeatedly detected Cuc B (in water) at dilutions as low as 1 ppb and Cuc E glycoside at 10 ppb. [Brucine alkaloid has been described (4) as the bitterest substance known to man with a limit of detection of about 1 ppm.] The Cucs are not only extremely bitter but also highly toxic, and there have been instances of severe poisoning and death in sheep and cattle that consumed bitter fruits from *Cucumis* and *Cucurbita* (5). Intraperitoneal median lethal dose (LD<sub>50</sub>) values for pure Cucs determined by David and Vallance (6) are: Cuc A, mouse 1.2 mg/kg of body weight, rat 2; Cuc B, mouse, 1.0; Cuc C, mouse, 6.8.

Plants of the Cucurbitaceae are eaten by a large number of beetles of the family Chrysomelidae, subfamily Galerucinae, tribe Luperini. Host-plant records are remarkably scarce;



Cucurbitacin B  
E 1,2 C=C  
D 25-OH  
I 1,2 C=C; 25-OH  
L 1,2 C=C; 25-OH, 23,24H,H  
R 23,24H; 25-OH

FIG. 1. Chemical structures of Cucs active as kairomones for diabroticite beetles.

Wilcox (7) lists only 29 records for 1528 species of Luperini (1.90% of described species), of which 21 host plants were Cucurbitaceae (72%). A detailed literature search has disclosed the following numbers of species in the various genera of Luperini feeding on Cucurbitaceae or associated with Cucs, together with percentage of total described species: *Acalymma*, 14 (20.9%); *Agetocera*, 2 (12.5%); *Aulacophora*, 20 (11.4%); *Diabrotica*, 9 (2.7%); *Lamprocopa*, 1 (10%); *Paranaptacaba*, 2 (3.5%); *Parietia*, 5 (13.5%). Thus, more than 80% of the host-plant records for the Luperini portray a relationship between the Cucurbitaceae and these beetles that is widely distributed among a number of genera in both the Old World (Aulacophorina) and New World (Diabroticina).

Contardi (8) first described the compulsive feeding of *Diabrotica speciosa* on the wild, bitter squash *Cucurbita andrea* in Argentina. This behavior has been identified in the spotted cucumber beetle *D. undecimpunctata howardi* (9), the banded cucumber beetle *D. balteata* (10), the striped cucumber beetle *Acalymma vittata* (10), and the western corn rootworm *D. virgifera* (11). In the Old World, *Aulacophora foveicollis* and *A. atripennis* show specific host preference for bitter Cucurbitaceae (12).

This paper describes the response of a number of species of North American diabroticites to the Cucs.

### MATERIALS AND METHODS

The Cucs in the leaves, fruits, blossoms, and roots of 18 species of *Cucurbita* and in related genera were identified after chloroform extraction and concentration by thin-layer chromatography (TLC) on silica gel 254-F (E. Merck) using, as solvent system, ether/hexane/methanol, 70:30:5 (vol/vol), and chloroform/methanol, 95:5 (vol/vol). Areas containing the Cucs were detected by the quenching of fluorescence under UV

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Abbreviations: TLC, thin-layer chromatography; LR, limit of response; Cuc, cucurbitacin; LD<sub>50</sub>, median lethal dose.

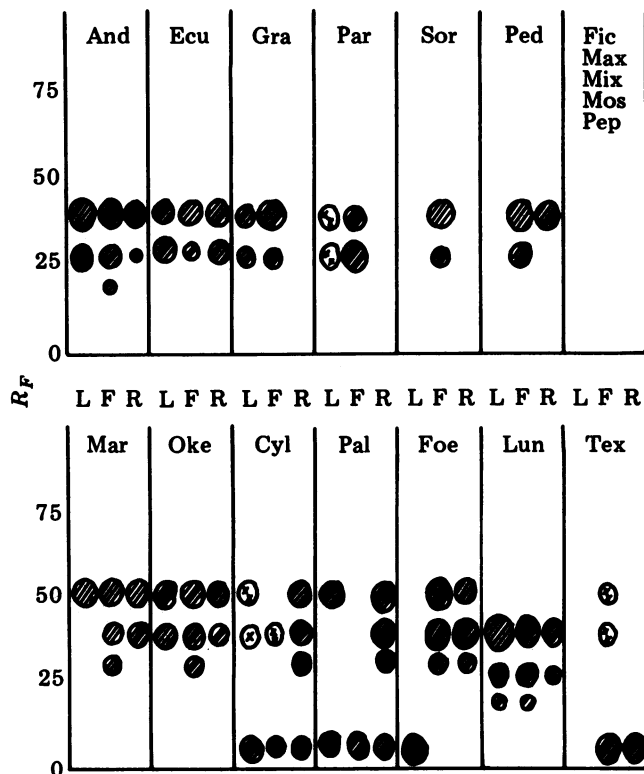


FIG. 2. Diagrams of TLC plates from chloroform extracts of the leaves (L), fruits (F), and roots (R) of *Cucurbita* spp., showing areas eaten from silica gel plates by *D. undecimpunctata* and *D. virgifera*. *Cucurbita* species are: *andreaana* (And), *cylindrata* (Cyl), *ecuadorensis* (Ecu), *ficifolia* (Fic), *foetidissima* (Foe), *gracilior* (Gra), *lun-delliana* (Lun), *martinezii* (Mar), *maxima* (Max), *mixta* (Mix), *moschata* (Mos), *okechobeensis* (Oke), *palmata* (Pal), *palmeri* (Par), *pedatifolia* (Ped), *pepo* (Pep), *sororia* (Sor), and *texana* (Tex).

radiation at 254 nm, with a limit of detection of 0.25  $\mu\text{g}$ . Average  $R_f$  values for pure Cucs in the two solvent mixtures, respectively, were: Cuc E, 0.49, 0.80; Cuc B, 0.38, 0.77; Cuc I, 0.36, 0.72; Cuc L, 0.28, 0.59; Cuc D, 0.26, 0.70; and Cuc E glycoside 0.02, 0.27. More specific identification of Cuc-containing areas was obtained by spraying with 5% ferric chloride in ethanol (wt/vol), which produced violet spots with Cucs containing the diosphenol grouping ( $\alpha$ -hydroxy unsaturated ketone)—i.e., Cucs E, I, J, K, and L (13)—or spraying with vanillin/phosphoric acid reagent (14). For further identification and quantification, the Cucs were eluted from TLC plates for

mass spectrometry (15) or for UV absorption spectrometry in absolute methanol at 210 nm (limit of detection 1 ppm).

Chemically pure Cucs D, E, I, L, and R were supplied by D. Lavie (Rehovot, Israel) and Cucs B and E glycoside were obtained by preparative TLC from chloroform extracts of *C. andreana* and *C. texana*, respectively.

## RESULTS

**Diabroticite Response to Pure Cucs.** It was discovered that the several species of beetles studied could be used as sensitive detectors of the Cucs on TLC plates, separated from *Cucurbita* extracts. Approximately 100 beetles were placed in a transparent plastic container and provided with 20% sucrose solution on cotton. Plates of silica gel on polyvinyl acetate (Eastman Chromatosorb) were placed on the bottom of the container and exposed to the beetles at 26°C for 4 days. The beetles searched at random over the plates but were clearly arrested in areas containing Cucs, where they began to chew at the silica gel surface. Eventually the silica gel was eaten completely away from areas conforming exactly to quenched spots observed under UV light. Such plates were routinely prepared with 20  $\mu\text{l}$  of standard (wt/vol) chloroform extracts of *Cucurbita* spp. concentrated 10:1, and they provided a permanent record (Fig. 2). *D. undecimpunctata howardi* and *D. undecimpunctata undecimpunctata* were the most sensitive indicator species evaluated, consistently detecting as little as 1–3 ng of pure Cuc B that had been pipetted onto the TLC plates from reagent acetone solution. The beetle-feeding assay was, therefore, at least 1000-fold more sensitive than the UV spectrophotometric assay.

Microgram quantities of the Cucs were detected and fed upon by all six species of diabroticites studied, and Cuc B consistently evoked the lowest limit of response (LR), with levels 0.1–0.3 those of Cuc E (Table 1). Cuc D was clearly less attractive than Cuc B, and Cuc I was less attractive than Cuc E. These results are somewhat at variance with those of Chambliss and Jones (9), who reported that *D. u. howardi* exposed to Cucs on filter paper fed heavily upon Cuc B and Cuc E glycoside (elaterinide), less on Cucs E and D, and not on Cuc I. In our experiments the beetles consistently fed on both Cuc I and Cuc D as expected because Cuc I and Cuc D are the 25-desacetoxy derivatives of Cuc E and Cuc B. Cuc L differs from Cuc I in the saturation of the double bond between carbons 23 and 24, and this change had little effect on LR values.

**Cucs as Arrestants and Feeding Stimulants.** As indicated by the data of Table 1, the Cucs arrest the searching behavior of the diabroticite beetles and produce a compulsive feeding behavior at very low dosages, even on a dry silica gel surface.

Table 1. Feeding response of diabroticite beetles to Cucs

Beetle	LR, $\mu\text{g}$					
	Cuc B	Cuc E	Cuc D	Cuc I	Cuc L	Cuc E glycoside
<i>D. undecimpunctata howardi</i> Barber (spotted cucumber beetle)	0.001	0.01	0.03	0.1	0.01	0.05
<i>D. undecimpunctata undecimpunctata</i> Mannerheim (western spotted cucumber beetle)	0.003	0.03				
<i>D. virgifera</i> LeConte (western corn rootworm)	0.03	0.3	0.1	0.3	1.0	0.05
<i>D. longicornis</i> Say (northern corn rootworm)	0.1	0.3				5
<i>D. cristata</i> Harris	0.1	1.0				50
<i>A. vittata</i> (Fabricius) (striped cucumber beetle)	0.3	10				50

It appears that the beetles are even more responsive to Cucs in plant tissues and feed on bitter *Cucurbita* fruit until only the waxy skin remains. Beetles are most readily attracted to and feed on leaves and fruit that are bruised or wounded. This suggests that the relatively nonvolatile Cucs codistill with water vapor from the plant and can attract the beetles from distances up to at least several meters. The beetles make mass attacks on cotyledons or homogenized fruits of bitter *Cucurbita* (e.g., *andreana*, *okeechobeensis*, or *texana*) from which the distinctive bitter flavor of the Cucs is perceived by humans at distances up to about 1 m.

The role of Cucs as feeding stimulants was demonstrated conclusively by painting microgram quantities of Cuc B on soybean leaves that are not normally eaten by the beetles. *D. undecimpunctata*, *D. virgifera*, and *D. longicornis* fed heavily upon the areas where the Cuc was applied. In field experiments we found that these beetles would eat a wide variety of weeds if they were treated with Cuc extracts. This feeding stimulation appears to be highly specific for the tribe Luperini of the Chrysomelidae; the bean leaf beetle *Ceratoma trifurcata* clearly was repelled by Cuc B and refused to feed on its normal host, the pinto bean, when Cuc B in reagent acetone was painted on both leaf surfaces at a level of  $\approx 1.6 \mu\text{g}/\text{cm}^2$ . Control leaves treated with acetone alone were almost totally eaten.

In contrast to the extreme bitterness and the high toxicity of Cuc B to laboratory animals (LD<sub>50</sub> values of about 1 mg/kg), groups of 25 *D. undecimpunctata* or *D. virgifera* adults completely consumed 1 mg of Cuc B in 72 hr without any perceptible ill effects. *D. undecimpunctata* and *D. virgifera* weigh about 20 and 10 mg, respectively, so that the LD<sub>50</sub> for the beetles is  $\gg 2000$  mg/kg. These data are consistent with the closeness of the evolutionary association between diabroticites and Cucurbitaceae and indicate that the beetles must have developed specific detoxication mechanisms for these normally poisonous substances.

**Localization of Cuc Receptors.** Both male and female *D. undecimpunctata* and *D. virgifera* responded to the Cucs as feeding stimulants and fed heavily upon 30  $\mu\text{g}$  quantities of Cuc B applied to silica-gel plates. To determine the location of the Cuc receptors, surgical experiments were performed on two groups of 20 *D. undecimpunctata*: in group *a*, both antennae were removed, and in group *b* both maxillary palpi were removed. These groups of beetles were exposed to 30  $\mu\text{g}$  of Cuc B on silica gel and compared with untreated controls (group *c*). After 24 hr, group *c* had eaten the entire treated area, group *a* had eaten about 50% of the treated area, and group *b* did not feed. This demonstrates that the Cuc receptors are located on the maxillary palpi.

**Cuc Receptor.** The information in Table 1 makes it possible to speculate about the nature of the Cuc receptor located on the maxillary palpi of the beetles. The LR values for six species of diabroticites were consistently the lowest for Cuc B and ranged from about 0.1 to 0.3 those of Cuc E. Therefore, Cuc B has the maximum complementarity to the Cuc receptor and is likely to be the primitive Cuc to which the sensory receptor of a diabroticite became tuned evolutionally. The greater prevalence of Cuc B in the Cucurbitaceae and the enzymatic formation of Cuc E from Cuc B also suggest that Cuc B is the primitive form. Cuc B also has LR values about 0.1 those of the C25 desacetoxy derivative Cuc D, and the same relative difference was found between Cuc E and its C25 desacetoxy derivative Cuc I, in the response of *D. undecimpunctata* (Table 1). Therefore the acetoxy C=O must be involved in complete binding of the Cucs to the receptor. Cuc E differs from Cuc B only in having a double bond adjacent to the hydroxy group in ring A. A similar difference exists between Cuc D and Cuc I. The decreased

affinity of Cucs E and I for the receptor may be the result of the consequent planarity of C1—C2—C3 ring A (Fig. 1) in Cuc E and Cuc I, which substantially changes the orientation of the three close O atoms (C3=O, C2—OH, C11=O) from a staggered pattern in the cyclohexyl moiety of Cuc B to a planar pattern in the aromatized Cuc E. It seems likely that the depolarization of the nerve associated with the Cuc receptor results from allosteric changes brought about through interactions of free paired electrons on the several oxygen atoms of the Cuc molecule.

**Feeding Response of Diabroticites to *Cucurbita* spp.** Standard extracts were made from leaves, fruits, and roots of 18 species of *Cucurbita* grown in the field at Urbana, IL. The plant material was thoroughly homogenized in water and extracted with 10 vol of chloroform, filtered, and concentrated to represent 10 g of plant material (wet weight) per ml of chloroform. These standard plant extracts were applied in 20- $\mu\text{l}$  quantities to the origin of TLC plates, which were developed in ether/hexane/methanol. After development, the plates were exposed to groups of about 100 *D. u. howardi* and *D. virgifera* for several days until areas containing the attractive Cucs were eaten away. The patterns of feeding by the two *Diabrotica* species were virtually identical, although *D. u. howardi* demonstrated greater sensitivity.

Four distinct types of beetle-feeding responses were observed (Fig. 2). Groupings based on the nature of the Cucs present agree reasonably well with evolutionary groupings based on numerical taxonomy (16), cross compatibilities (17), and isozyme analysis (18). Group 1 included the Cuc B- and D-forming species: (a) *C. andreana* and *C. ecuadorensis*, and (b) *C. gracilior*, *C. palmeri*, and *C. sororia*. Group 2 included the Cuc E- and I-forming species: (a) *C. martinexii* and *C. okeechobeensis*, and (b) *C. cylindrata* and *C. palmata*. Four species were apparently distinctive: *C. pedatifolia* containing Cucs B and D, *C. foetidissima* containing Cucs E and I, *C. texana* containing Cuc E glycoside, and *C. lundelliana* appearing to contain Cucs B and D rather than Cucs E and I as found in its nearest relatives *C. martinexii* and *C. okeechobeensis*. The remaining five domesticated (nonbitter) species, *C. ficiifolia*, *C. maxima*, *C. mixta*, *C. moschata*, and *C. pepo*, showed no discernible beetle feeding. The quantitative determinations of the Cuc contents of these 18 species of *Cucurbita* will be described elsewhere.

**Evolutionary Aspects of Cuc Receptors.** There were no significant qualitative differences in the feeding of *D. u. howardi* and *D. virgifera* upon the TLC patterns formed by the chloroform extracts of the leaves, fruits, and roots of 18 species of *Cucurbita*. To evaluate other diabroticite species, fruit extracts of *C. andreana* containing the Cuc B-D series, *C. okeechobeensis* containing the Cuc E-I series, and *C. texana* containing Cuc E glycoside were chromatographed on silica gel, and the plates were exposed to *D. longicornis*, *D. cristata*, and *A. vittata*. Each species of diabroticite consumed the Cuc B and D spots of *C. andreana*, the Cuc E and I spots of *C. okeechobeensis*, and the Cuc E glycoside spot of *C. texana*, exactly as shown in Fig. 2. Thus, there is no evidence of any change in the spectrum of sensitivity to the Cucs of the various species of *Cucurbita* over the evolutionary period of  $>7$  megayears BP encompassed during the evolutionary divergence of these five diabroticite species (1). This is remarkable in view of the substantial differences in their present host preferences. *D. u. howardi* is perhaps the most generalized and feeds predominately on Cucurbitaceae, but the larvae also feed on the roots of corn and the adults also feed on corn silks and pollen. *D. virgifera* and *D. longicornis* originally were described from adults collected from the blossoms of *C. foetidissima*, but the

larvae apparently develop only on the roots of grasses and are severe pests of corn, where the adults feed on silks and pollen. *D. cristata* apparently develops only on the roots of grasses such as *Andropogon*, but adults have been collected occasionally from blossoms of *Cucurbita*. *A. vittata* is a notorious pest of Cucurbitaceae and is not associated with grasses. The demonstration that a functional Cuc receptor is present in all of these species suggests not only that they originally coevolved with the Cucurbitaceae but also that the host transfer to the Gramineae must have been relatively recent (19). The data in Table I indicating the relative sensitivity of the receptors to the Cucs shows evidence of evolutionary drift of the individual species away from a primitive association with plants producing Cucs. Thus, the order of response is: *D. u. howardi* = *D. u. undecimpunctata* > *D. virgifera* > *D. longicornis* > *D. cristata* = *A. vittata*.

An interesting aspect of the role of the Cuc receptor in host selection was demonstrated with *Iberis umbellata* or "candy tuft." This ornamental, a Cruciferae, represents one of the few genera outside the Cucurbitaceae to contain substantial amounts of Cucs (20). *I. umbellata* plants interspersed with *Cucurbita* varieties were massively attacked by *D. u. howardi* and *D. virgifera* and were killed within 2 weeks. Chloroform extracts of leaves and stems were chromatographed as previously described and the silica gel plates were exposed to adult beetles. Both species fed compulsively upon Cucs E and I, which were identified by  $R_F$  and by violet coloration with alcoholic  $FeCl_3$ .

The Aulacophorina are a large group of Luperini that are the Old World counterparts of the Diabroticina (21). Many of the Aulacophorina feed on the Cucurbitaceae (12, 22), and *A. foveicollis* has been shown to be a compulsive feeder on pure Cuc E (23, 24). Therefore, it seems likely that a primitive ancestor of both the New World Diabroticina and the Old World Aulacophorina developed the original coevolutionary association with a primitive Cucurbitaceae during a period when continental land bridges were present (1). During this association, the extremely bitter and toxic Cucs—arising evolutionally to repel herbivores and to protect the plants from attack—have become the specific kairomone feeding stimulants for these Luperini beetles. The original association between *Cucurbita* and diabroticites developed in Central or South America, an area where *Cucurbita* originated and where diabroticites attained maximal diversity (1).

The demonstration that an active Cuc receptor still exists in species such as *D. cristata*, *D. longicornis*, and *D. virgifera* that are presently herbivores of Gramineae shows not only that the original association of these species was with the Cucurbitaceae but also that the host transfer to Gramineae may have been relatively recent. It is evident that olfactory and gustatory receptors for specific secondary plant chemicals evolved in the Insecta millions of years ago and that their depolarization led

to behavioral patterns that have remained fixed over widespread species divergence and host plant alterations (25). Study of the evolution of these receptors is an important aspect of understanding insect behavior and ecology.

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