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Chemical Modification Produces Species-Specific Changes in Cucurbitacin Antifeedant Effect

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Supporting Information

ABSTRACT: Cucurbitacins are secondary metabolites that mediate insect plant interactions not only as allomones against generalists but also as kairomones for specialist herbivores. This study was undertaken to identify the potential of cucurbitacin derivatives as insect antifeedant agents. The antifeedant capacity against a Cucurbitaceae specialist [*Epilachna paenulata* (Coleoptera: Coccinellidae)] and a polyphagous insect [*Pseudaletia adultera* (Lepidoptera: Noctuidae)] was evaluated in preference tests in which the insects were given a choice between food plants either treated with the cucurbitacin derivatives or treated with the solvent. The activity was found not to be related to the basic cucurbitacin skeleton, as only 15 of the 28 tested cucurbitacin derivatives were active. Only one of the tested compounds was phagostimulant to the specialist insect (the hemissuccinate of 16-oxo-dihydrocucurbitacin B derivative), while all other active derivatives were deterrent against one of the insects (13 compounds) or both of them (3 compounds). Changes in ring A of the cucurbitacins, as well as in the side chain, modified the activity. As a general trend, when chemical modifications of the basic structure produced a change in activity, the response was opposite in both insects used as biodetectors, indicating that a selective variation in the activity may be achieved by chemical modifications of the cucurbitacin skeleton.

KEYWORDS: *anti-insect, deterrent, triterpenoid, antiherbivory, cucurbitacins, semisynthesis*

INTRODUCTION

Cucurbitacins are highly oxygenated tetracyclic triterpenes occurring mostly, but not exclusively, in plants of the family Cucurbitaceae.[1] Because cucurbitacins are highly toxic to many organisms including insects and vertebrates,[2] they are generally supposed to act as plant defense substances.[3,4] However, some herbivores have developed behavioral counter-adaptations to the production of cucurbitacins as induced plant defenses, avoiding these secondary metabolites produced as allomones (i.e., a substance used by individuals of one species in their own benefit that negatively affects individuals belonging to another species).[5,6] Furthermore, specialist herbivores may profit on cucurbitacin occurrence in their host plant in such a way that cucurbitacins, by being phagostimulants, become the token stimuli[7] that mediate host choice. Natural occurring cucurbitacins have been intensively investigated not only as kairomones[8] for beetles belonging to the family Chrysomelidae[8−12] but also as sequestered defense compounds for Diabroiticites.[11] They have also been studied in regard to their capacity as anti-insect agents, one mode of action being their ability to compete with ecdysteroids for their ligand binding site in the hormone receptors.[13,14] Cucurbitacin effects on insect feeding and oviposition[4,15] have been described for different insect orders;[16−20] and some attempts to develop cucurbitacin-containing lures to control leaf-beetles have been pursued in the past.[20−23] Cucurbitacins have been also intensively investigated for their cytotoxic and anti-inflammatory properties.[24,25] Most of these studies were performed with the natural occurring cucurbitacins, but more recently semisynthetic derivatives were also described.[26,27] Aiming to explore the biological properties of this class of compounds, we prepared a library of natural cucurbitacins and derivatives and describe herein their activity against two insects: a specialist on Cucurbitaceae [*Epilachna paenulata* Germar (Coleoptera: Coccinellidae)], and a polyphagous herbivore [*Pseudaletia adultera* Schaus (Lepidoptera: Noctuidae)] that prefers Poaceae species as host plants.[28]

MATERIALS AND METHODS

Cucurbutacins. The tested cucurbitacins and derivatives are shown in Figure 1. Compounds **1** (dihydrocucurbitacin B), **3** (cucurbitacin R), **4** (25-deacetyl-dihydrocucurbitacin B), **5** (cayaponoside A), and **6** (dihydrocucurbitacin B-2-*O*-glucoside) were isolated

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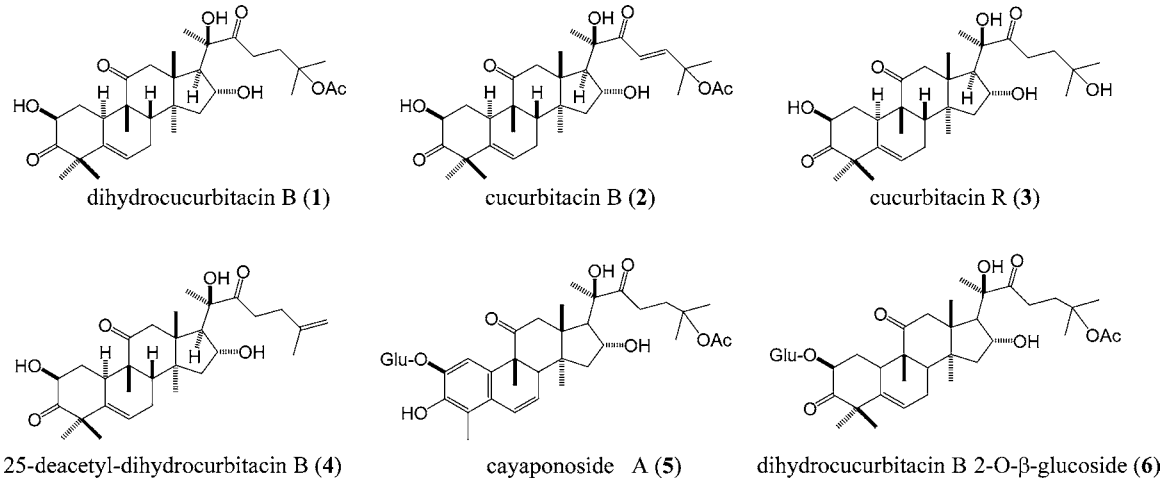

Figure 1. Natural cucurbitacins tested for their antifeedant capacity.

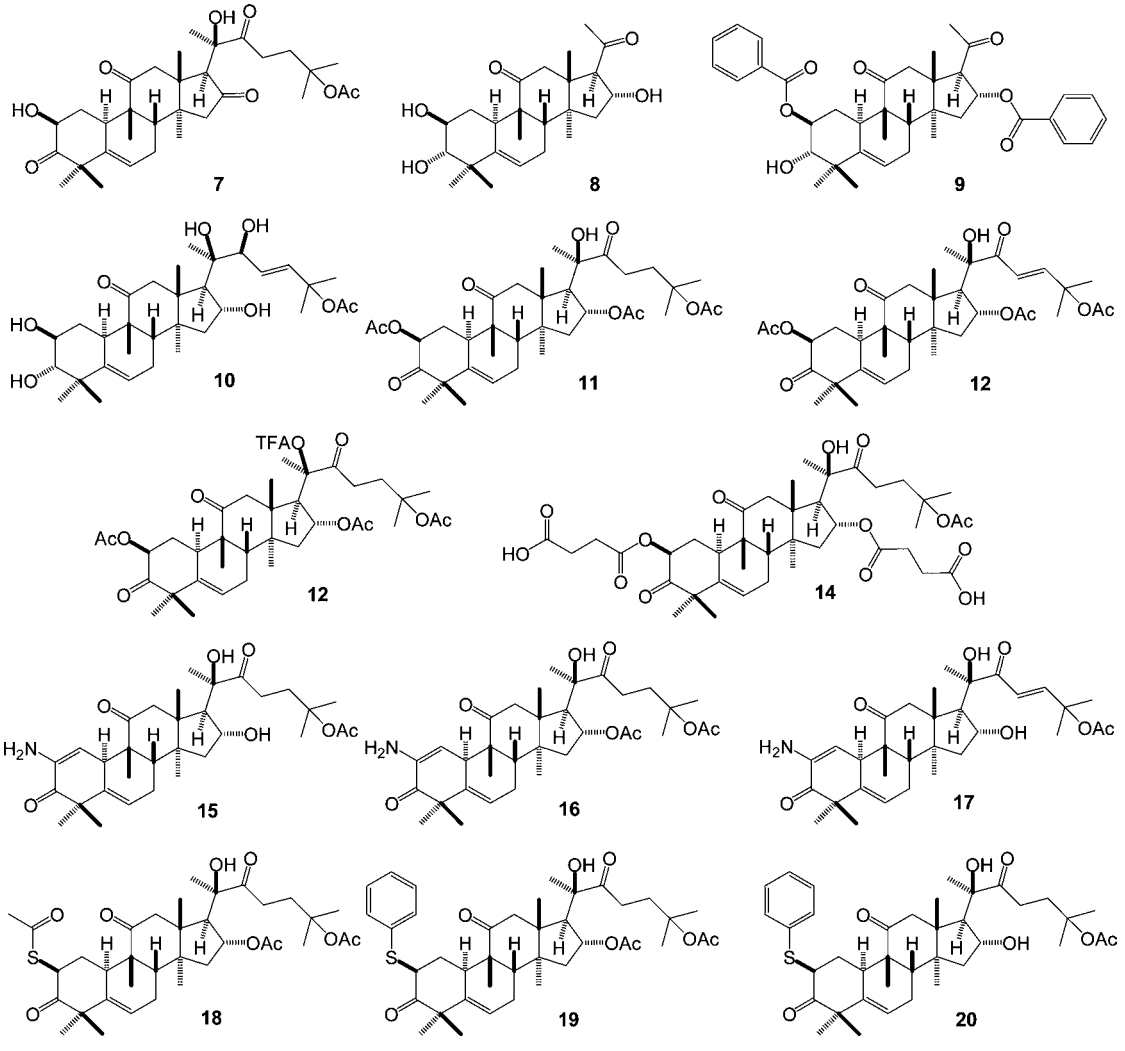

Figure 2. Semisynthetic cucurbitacins[25] tested for their antifeedant capacity.

from the roots of *Wilbrandia ebracteata* Cogn. (Cucurbitaceae), as previously described.[29,30] Compound **2** (cucurbitacin B) was isolated from the fruits of *Luffa operculata* (L.) Cogn. (Cucurbitaceae).[26] Compounds **7**−**20** (Figure 2) were synthesized from dihydrocucurbitacin B or cucurbitacin B as previously described,[26,27] and the synthesis of the compounds **21**−**28** is described here for the first time (Schemes 1 and 2) and within the Supporting Information.

Insects. *Epilachna paenulata*. Germar (Coleoptera: Coccinellidae). A laboratory colony was maintained on squash (*Cucurbita maxima* Duchesne, Cucurbitaceae) under controlled conditions of temperature (20 ± 2 °C) and photophase (14L:10D). The colony was initiated with individuals collected on squash plants in organic farms near Montevideo, and new field-collected individuals have been added every year.[31]

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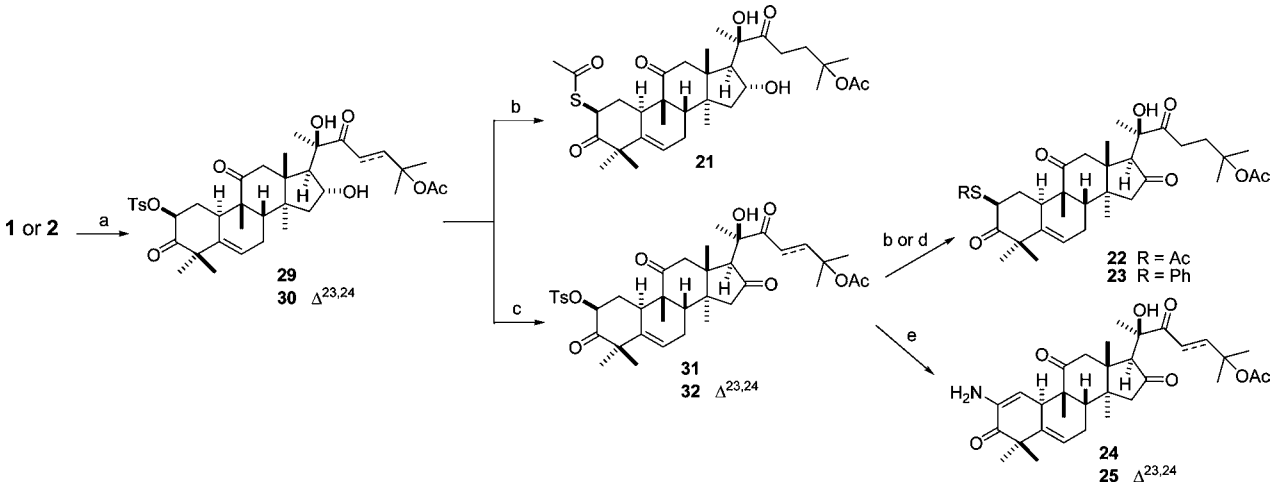
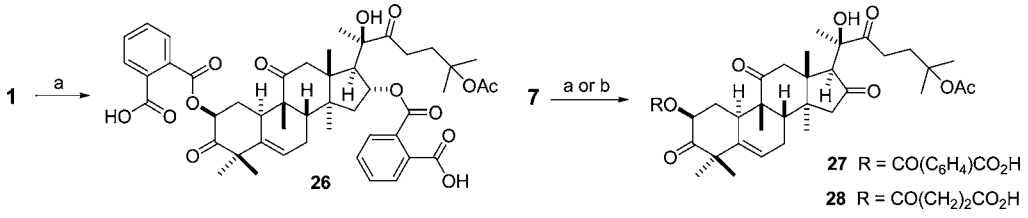
Scheme 1. Synthesis of the compounds 21−25[a]



[a]Reagents and conditions: (a) 4-toluenesulfonyl chloride, DABCO, CH_2Cl_2, 0 °C; (b) CH_3COSK, acetone; (c) PCC, $BaCO_3$, CH_2Cl_2; (d) C_6H_5SH, THF, NaH; (e) NaN_3, DMF, 70 °C.

Scheme 2. Synthesis of the compounds 26−28[a]



[a]Reagents and conditions: (a) phthalic anhydride, Py, DMAP, CH_2Cl_2; (b) succinic anhydride, Py, CH_2Cl_2; DMAP.

Pseudaletia adultera Schaus (Lepidoptera: Noctuidae) were reared on an artificial diet[32] at 24 ± 1 °C, > 70% relative humidity, with a photoperiod of 16L:8D in a growth chamber. The colony was initiated with adults collected in light-traps placed on wheat crops.

Tests of Preference. The compounds were evaluated in choice-bioassays in Petri dishes (9 cm diameter) completely lined at the bottom with a 0.3 mm width layer of agar (2%) to avoid leaf desiccation. Insects were offered four leaf pieces (1 cm^2) of the appropriate host plant [*C. maxima* for *E. paenulata* and *Hordeum vulgare* L. (Poaceae) for *P. adultera*]. Two of the leaf pieces (treatment: *T*) were coated with 100 μg of the substance (10 μL of a 1% MeOH solution), and the other two (control: *C*) were treated with 10 μL of MeOH. For *E. paenulata*, 3−4 day old adults were tested individually (10 replicates per substance). In the case of *P. adultera*, fourth-instar larvae were used (7−10 replicates per extract). Tests with both insects were run for 180 min or until 75% of one of the options was consumed. To measure food intake, a visual score of the consumed area (in one-eighth intervals) was assigned for all leaf pieces within the plate, and a feeding preference index (PI) was determined for each replicate using the formula PI = $(C - T)/(C + T)$, where *C* and *T* are the consumed amounts of the control and treatment leaves respectively.[33,34] (In this manner, PI is greater than 0 when compounds are deterrent[35] and lower than 0 when phagostimulation occurs.)

Statistical Procedures. Bioassay data were analyzed by Wilcoxon Rank Tests. The activity on insect feeding was evaluated on the basis of the percentage of consumed leaf treated with solvent (control) compared to consumed leaf treated with tested substance (treatment).[36] When the results from two samples were compared, a Mann−Whitney Test on PI was run.[36]

RESULTS AND DISCUSSION

The tested cucurbitacins were selected from previous studies,[26,27,30] and eight new compounds described herein (Schemes 1 and 2) were obtained in order to explore the importance of substituents at C-2, together with some modifications at C-16. In this way, five new derivatives (**21**−**25**) were prepared by nucleophilic substitution[25] using the tosylate as leaving group, as shown in Scheme 1. Compounds **26** and **27** (Scheme 2) were synthesized by esterification of compounds **1** and **7**, respectively, using phthalic anhydride, and compound **28** was obtained by esterification of **1**, using succinic anhydride. Spectroscopic data of these new compounds are available in the Supporting Information.

These 28 natural and semisynthetic cucurbitacins were tested for their antifeedant activity against a specialist on Cucurbitaceae (*E. paenulata*) and a polyphagous species (*P. adultera* larvae). All active compounds were deterrents with the exception of **28**, the hemissuccinate ester of 16-oxo-dihydrocucurbitacin B that was phagostimulant to *E. paenulata* (Table 1). The natural occurring cucurbitacins were in general more active as deterrents than the modified compounds. In particular, the natural cucurbitacins cucurbitacin B (**2**) and cucurbitacin R (**3**) were inactive against both species, and dihydrocucurbitacin B (**1**) and the glycoside **5** were very active against *E. paenulata* and inactive against *P. adultera*, while the reverse was true for **4**, a natural cucurbitacin with a terminal vinyl group, and **6**, the 2-*O*-glycoside of dihydrocucurbitacin B, which were active against *P. adultera* and inactive against *E. paenulata*. The effect of glycosylation is not clear, although it should be considered that only two glycosylated compounds

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Table 1. Preference Indexes (PI as Means ± Standard Error) of *E. paenulata* and *P. adultera*

cucurbitacin	*P. adultera* larvae	*E. paenulata* adults
1	0.1 ± 0.2	0.7 ± 0.2*[a]
2	0.2 ± 0.2	0.4 ± 0.3
3	−0.2 ± 0.2	0.3 ± 0.2
4	0.2 ± 0.1*[a]	0.2 ± 0.3
5	0.0 ± 0.2	0.8 ± 0.2*[a]
6	0.91 ± 0.04*[a]	0.2 ± 0.3
7	0.3 ± 0.1*[a]	0.3 ± 0.3
8	0.1 ± 0.2	NT
9	−0.1 ± 0.2	0.1 ± 0.4
10	0.65 ± 0.06*[a]	0.1 ± 0.4
11	0.2 ± 0.2	0.3 ± 0.4
12	0.1 ± 0.2	0.2 ± 0.3
13	−0.1 ± 0.1	0.2 ± 0.4
14	0.19 ± 0.09*[a]	0.2 ± 0.2
15	0.0 ± 0.1	0.6 ± 0.3
16	0.32 ± 0.09*[a]	0.7 ± 0.2**[b]
17	0.0 ± 0.2	0.2 ± 0.3*[a]
18	0.09 ± 0.09	0.4 ± 0.3
19	0.6 ± 0.1*[a]	0.1 ± 0.2
20	−0.01 ± 0.08	0.5 ± 0.1*[a]
21	0.0 ± 0.1	0.4 ± 0.3
22	−0.1 ± 0.2	0.1 ± 0.3
23	0.0 ± 0.1	−0.1 ± 0.3
24	0.3 ± 0.2	0.3 ± 0.3
25	0.6 ± 0.1*[a]	0.0 ± 0.2
26	0.3 ± 0.1*[a]	0.85 ± 0.08*[a]
27	06 ± 0.1*[a]	0.2 ± 0.3
28	0.8 ± 0.1*[a]	−0.6 ± 0.3*,**[a,c]

[a]*$P < 0.05$ (2-tailed). [b]**$P < 0.05$ (1-tailed) by Wilcoxon Rank tests. NT: not tested. [c]Phagostimulant.

were tested. Compound **6** was inactive against *E. paenulata* but on the other hand was very active against *P. adultera*. The other C-2 glycoside, **5**, was almost as active as compound **1** against *E. paenulata*, but in this case, ring A is aromatic, so it is unclear which feature, or a combination thereof, is responsible for the bioactivity.

Variations of the side chain of dihydrocucurbitacin B (**1**) [unsaturation at C-23 (**2**), deacetylation at C-25-OH (**3**), and unsaturation at C-25−C-26 (**4**)] led to a loss of activity against the specialist *E. paenulata*, suggesting that the side chain of **1** should remain intact. However, in the case of the polyphagous *P. adultera* the presence of the $\Delta^{25,26}$ bond in **4** led to a weak increase of deterrence. In the same direction, a side chain shortening (compounds **8** and **9**) of the very active diol derivative of cucurbitacin B (**10**) led to a complete loss of activity against *P. adultera*. The diesterification of C-2 and C-16 hydroxyls of dihydrocucurbitacin B (**1**) gave results that were variable depending on the acylating group. The diacetate **11**, the dihemisuccinate **14**, and the derivatives with an acetate at C-16 and a thiophenyl group (**19**) or a thioacetate at C-2 (**18**) lost all the deterrent activity against *E. paenulata* exhibited by the parent compound **1**. Interestingly, **19** was considerably more active than parent compound **1** against *P. adultera*. A similar trend was observed with cucurbitacin B (**2**): the diacetate **12** seems to be less active than the parent compound. On the other hand, the diphtalate of dihydrocucurbitacin B (**26**) was more active than **1** not only against *E. paenulata* but also against *P. adultera*.

Changes in the oxidation pattern of the main skeleton also have produced activity shifts. Oxidation of C-16 OH in dihydrocucurbitacin B (**1**) yielded compound **7**, which lost all activity against *E. paenulata* but showed increased deterrence against *P. adultera*. On the other hand, the reduction of the carbonyls at C-3 and C-22 gave compounds with different species-related activities. Reduction of both carbonyls in cucurbitacin B (**2**) yielded compound **10**, which was inactive against *E. paenulata* but seemingly more active than the parent compound against *P. adultera*.

The combined effect of both transformations, acylation and oxidation, can be observed in compounds **27** and **28**, which are acyl derivatives of **7** (dihydrocucurbitacin B oxidized at C-16). As in the case of the acyl derivatives of dihydrocucurbitacin B (**1**), acylation of **7** yielded derivatives with variable activities. The introduction of a phthalate group at C-2 (**27**) in compound **7** decreased the activity ($p < 0.05$, Mann−Whitney test) against *E. paenulata*. In the case of the dihemisuccinate, **9**, a loss of activity against *E. paenulata* was observed when compared to its parent compound dihydrocucurbitacin B (**1**), but at the same time an increase in its effect against *P. adultera* was obtained. Compound **28** (the hemisuccinate of **7**) not only showed an increase in its deterrent effect against *P. adultera* but also the degree of change toward *E. paenulata* was such that the effect reverted to phagostimulation. Quite surprisingly, **28** was the only tested compound that exhibited a phagostimulant effect on *E. paenulata*, being at the same time deterrent against *P. adultera*.

The derivatives with an enaminone on ring A produced some interesting results. The enaminone of **1** (compound **15**) and its C-16 acetyl derivative, **16**, had almost identical activities as the parent compound against *E. paenulata*. Compound **16** was active against *P. adultera*, whereas its parent compound was not. In particular, compound **16** has an acetate group, which in other derivatives was detrimental. For instance, the dihydrocucurbitacin B diacetylated derivative, **11**, had lower bioactivity against *E. paenulata* than dihydrocucurbitacin B (**1**); however, an enaminone group at ring A, **16**, instead of an acetate at C-2, **11**, gives a product with almost the same activity as **1**. Oxidation of the C-16-OH group gives the enaminone **25** with reduced bioactivity, suggesting that the enaminone by itself does not guarantee the deterrent effect, which instead arises from a combination of an α-hydroxyketone or an enaminone at ring A and a hydroxyl or acetyl group at C-16. When the enaminones derived from cucurbitacin B (**2**) were tested, the results showed a different pattern. In fact, the enaminone **25** was inactive against *E. paenulata* while considerably active against *P. adultera*.

As a whole, these results were unexpected in that phagostimulation by natural cucurbitacins toward *E. paenulata* was not detected as predictable if these chemicals were token stimuli.[7] The deterrent effect on this Cucurbitaceae specialist may be due to a dose-related effect. To clarify this issue, more studies will be carried out. However, it is also possible that deterrence is an ecological significant effect as it has been previously documented for other *Epilachna* species which, as a matter of fact, perform a trenching behavior to avoid cucurbitacins produced after plant damage as induced defenses.[5,6] On the other hand, deterrence against the polyphagous *P. adultera* was found as expected. Finally, an important finding from these results is the trend by which when chemical modifications correlated to changes in activity, those changes were opposite for both insects, that is, a chemical more

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active against the specialist becomes less active against the generalist insect in its capacity to deter feeding.

Considering the compounds here investigated, the most active were, in general, the natural cucurbitacins, but some of the semisynthetic derivatives were as active as the parent compounds. Another general trend arises from the fact that more active compounds were found against the polyphagous insect than against the specialist (12 vs 7) (Table 1). This trend is opposite to that most generally observed, where specialists are usually more sensitive than generalists to plant chemicals not usually found in their normal diet due to some inability for specialists to physiologically adapt to a different chemical profile from the one they usually encounter.[37] However, opposite patterns have also been described previously.[34,38]

The results show that the cucurbitane skeleton by itself does not ensure activity because almost half of the tested substances were inactive. Another general observation is that the activity is very different against both tested species and that it was deterrent at the tested concentration in all cases with the exception of cucurbitacin **28**. The activity is also markedly influenced by the structure modifications, as it was in our previous study on cytotoxic activity,[27] although a pattern of correlations between both activities was not found. However, some general trends could be observed by comparison of structurally related compounds in this series, which can be useful for the design of a more active compound.

The side chain structure of dihydrocucurbitacin B (**1**) is a common feature in most of the active compounds, not only among the natural substances but also in the semisynthetic derivatives as well. In this regard, an additional conclusion is that the observed activity is a combination of all the structural features of the side chain because a single modification on any of them produces a loss of activity, indicating that the side chain of **1** should remain intact in the design of a more active derivative.

Among the semisynthetic derivatives, only a few could match dihydrocucurbitacin B in terms of bioactivity against *E. paenulata*. The modifications performed on rings A and D of the cucurbitane skeleton gave results that in some cases were more difficult to rationalize. Diesterification at C-2 and C-16 gave interesting results: only the diphthalate **26** was active against both insects, while the remaining diester derivatives show a tendency by which this chemical modification decreases the activity against *E. paenulata* and at the same time increases the activity against *P. adultera*. At the same time, oxidation at C-16 gave the same trend in selectivity (**7**, **27**, **28**), including compound **28**, which has an hemisuccinate group at C-2 and drastically changed the sense of activity, becoming a phagostimulant for *E. paenulata*. This kind of modification in activity illustrates the potential of chemical modification to produce compounds with selectivity, a fundamental goal when developing pest control agents. The fact that compound **28** was considerably more active than the dihydrocucurbitacin B dihemisuccinate, **14**, suggests that the observed activity may arise by a combination of structural features at C-2 and C-16. These results also suggest that it would be worth to test the effect of substituents with greater lipophilicity at C-2 and C-16.

The effect of an enaminone on ring A (instead of the α-hydroxy-ketone) is also species-dependent. In the case of dihydrocucurbitacin B, the enaminones were active against *E. paenulata* but considerably less active or definitely inactive against *P. adultera*. However, the enaminone obtained from cucurbitacin B with further oxidation at C-16 (**27**) was very active against *P. adultera* while inactive against the specialist insect. Once again, these seemingly contrasting changes in activity are indeed a required feature when seeking for potential pest control agents with selectivity. These results stimulate the preparation of additional derivatives in order to explore further changes in the structure of ring A, as well as to investigate the effect of glycosylation on the insect feeding activity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Preparation of compounds **21**−**28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

PI, feeding preference index

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