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# Cucurbitane-type compounds from *Hemsleya carnosiflora* antagonize ecdysteroid action in the *Drosophila melanogaster* $B_{II}$ cell line

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**Abstract.** The ecdysteroid agonist and antagonist activities of 3 cucurbitanes, 2 cucurbitane glycosides and 2 cucurbitacins isolated from *Hemsleya carnosiflora* (Cucurbitaceae) have been determined in the *Drosophila melanogaster* $B_{II}$ bioassay. Carnosiflogenins A and C and carnosiflosides II and VI possess antagonistic activity. Carnosiflogenin A was also found to induce the formation of spindle-shaped cells with high frequency in both the agonist and antagonist assays. At $10^{-3}$ M, carnosiflogenins B and C were cytotoxic. 23,24-Dihydrocucurbitacin F and 25-acetoxy-23,24-dihydrocucurbitacin F are also antagonistic at high concentrations. The concentration dependencies of the antagonistic activities of these two cucurbitacins, carnosiflosides II and VI and carnosiflogenin C are presented. The biological and ecological significance of these results are discussed in relationship to the concentrations present in the rhizomes of *H. carnosiflora*.
**Key words.** Antagonist; Cucurbitaceae; cucurbitane; cucurbitacin; ecdysteroid; *Hemsleya carnosiflora*; receptor; steroid hormone.

It is believed that many plant secondary compounds serve to deter phytophagous invertebrates, and there is evidence that some of them affect insect growth and development if they are incorporated into the diet. However, the modes of action of most of these compounds are largely unknown. Since ecdysteroids are essential to the normal development of insects, and probably of other invertebrates too (for reviews see ref. 1), it seems plausible that some of the compounds elaborated by plants may interfere with the action of these steroid hormones resulting in developmental disruption. Plants are known to produce analogues of insect juvenile hormones, and some juvenile hormone analogues have been developed as commercial insecticides (reviewed in ref. 2). It has been known for 30 years that some plants contain phytoecdysteroids, which act as ecdysteroid agonists in non-adapted insect species (reviewed in ref. 3). We have recently initiated a search for new plant chemicals which interact with the insect ecdysteroid receptor, either as agonists or antagonists [4]. Several cucurbitacins (Dinan et al., unpublished materials) and withanolides [5] have been identified as ecdysteroid antagonists. In view of this, it was desirable to test structurally related triterpenoids, of which many have already been isolated from plants, to determine if they also possess activity. Here we report on the biological activities of several cucurbitane-type compounds isolated from *Hemsleya carnosiflora*, and demonstrate that several of them possess antagonistic activity at relatively high concentrations. The biological and evolutionary significance of these findings will be discussed.

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## Materials and methods

**Compounds.** Cucurbitacins and carnosiflosides were isolated from *Hemsleya carnosiflora* and the carnosiflogenins generated from the carnosiflosides by enzymic hydrolysis as described previously [6]. Purity was verified by hplc (Excelpak SIL-C18 column, 15 cm × 4.6 mm i.d, eluted isocratically at 1.5 $cm^3$/min with acetonitrile/water [3:7 v/v] for the carnosiflosides and at 1 $cm^3$/min with acetonitrile/water [4:1 v/v] or methanol/water [4:1 v/v] for the carnosiflogenins and cucurbitacins).

**Bioassay.** Compounds were prepared as stock solutions ($10^{-2}$ M) in methanol and serially diluted to give solutions of concentrations from $10^{-3}$ M to $10^{-7}$ M. The compounds were tested for their ecdysteroid agonistic and antagonistic activities in the *Drosophila melanogaster* $B_{II}$ bioassay [7, 8]. Briefly, aliquots (20 $mm^3$) of each solution were dispensed in quadruplicate into 96-well microtitre plates. For the antagonist assay, $5 \times 10^{-7}$ M 20-hydroxyecdysone in methanol (20 $mm^3$) was also added. Solvent was allowed to evaporate in a laminar-flow cabinet and then 200 $mm^3$ $B_{II}$ cell suspension in Schneider's medium was added. Plates were incubated under sterile conditions for 7 days at 25 °C.

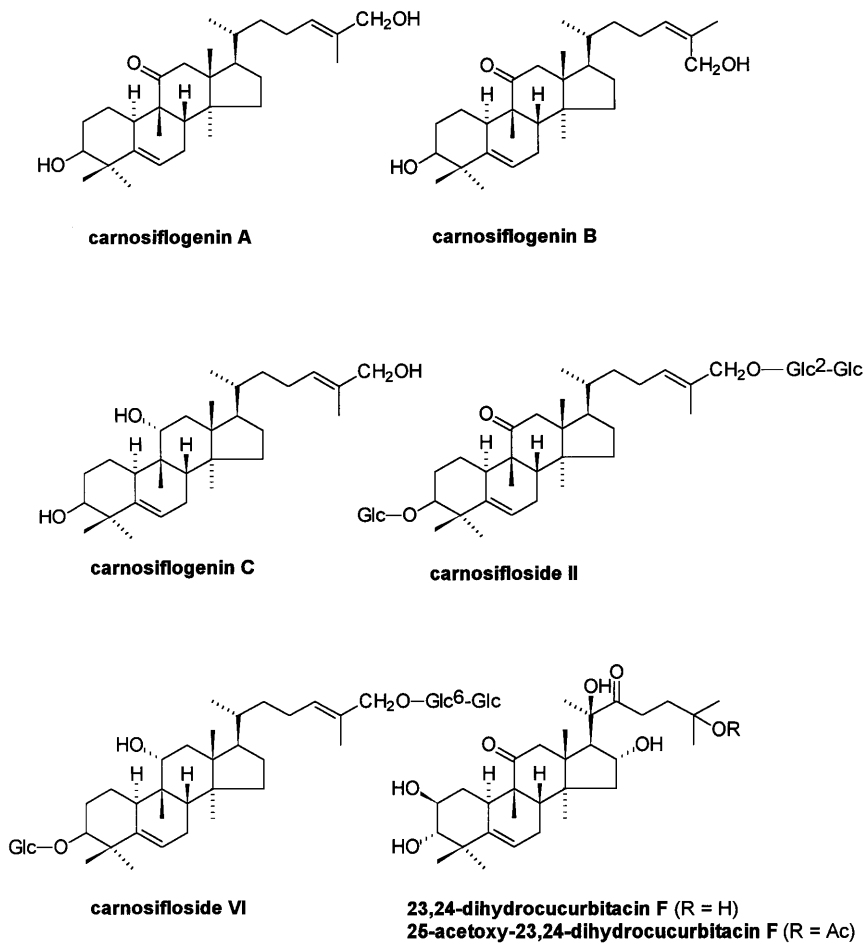


Figure 1. Structures of the compounds investigated.

The absorbance of each well was measured at 405 nm with an Anthos htII microplate reader and cell size, distribution and morphology were observed in situ with an inverted microscope.

## Results and discussion

The structures of the tested compounds are presented in flgure 1. Initial results for the agonist and antagonist versions of the $B_{II}$ bioassay are summarised in table 1. None of the compounds showed the specific responses (cell clumping, reduced cell density and increased cell size) associated with agonistic activity, although carnosiflogenin A did change the morphology of many of the cells and increased the proportion of spindle-shaped cells. However, this morphology was also seen in the antagonist assay, and this is not characteristic of the specific ecdysteroid-induced responses in this cell line. In addition to the induction of spindle-shaped cells, carnosiflogenin A at $10^{-4}$ M also inhibited the response induced by 20E, i.e. it was as an ecdysteroid antagonist. Carnosiflogenin C and carnosiflosides II and VI also demonstrated distinct antagonistic activity at $10^{-4}$ M, as revealed by higher densities of smaller, unclumped cells relative to 20E-treated controls. Carnosiflogenins B and C were cytotoxic at $10^{-3}$ M (causing cell fragmentation), while this was not apparent with the two carnosiflosides. Carnosiflogenin B initially appeared to have a weak antagonistic activity at $10^{-4}$ M, but it was not possible to confirm this with a second sample of the compound. More extensive concentration dependency curves were determined for carnosiflogenin C and carnosiflosides II and VI (fig. 2). All three show significant antagonistic activity at $10^{-4}$ M, but carnosiflogenin C becomes cytotoxic at higher concentrations (reflected in the drop in $A_{405}$). The carnosiflosides show a gradual increase in antagonistic activity with increasing concentration. This is in contrast to the sharper sigmoidal curves generally seen with active cucurbitacins (fig. 2; and as demonstrated by carnosiflogenin C between $2.5 \times 10^{-5}$ and $1 \times 10^{-4}$ M). The extended activity concentration range and the reduced cytotoxicity of the carnosiflosides may be a result of the higher solubility of these compounds and/or the gradual hydrolysis of the glucose units by enzymes present in the $B_{II}$ cells or Schneider's medium to release the aglycone as the active component. This aspect deserves further examination once more of the carnosiflosides are avail-

Table 1. Activities of the compounds in the agonist and antagonist versions of the $B_{II}$ bioassay.

| Compound | Bioassay | | | | | |
|---|---|---|---|---|---|---|
| | agonist | | | antagonist | | |
| | $10^{-5}$ M | $10^{-4}$ M | $10^{-3}$ M | $10^{-5}$ M | $10^{-4}$ M | $10^{-3}$ M |
| Carnosiflogenin A | - | S | nt | (Ant) | Ant/S | nt |
| Carnosiflogenin B | - | - | C | - | Ant? | C |
| Carnosiflogenin C | - | - | C | - | Ant | C |
| Carnosifloside II | - | - | - | - | Ant | Ant |
| Carnosifloside VI | - | - | - | - | Ant | Ant |
| Dihydrocucurbitacin F | - | - | - | - | Ant | Ant |
| 25-Acetoxydihydrocuc. F | - | - | C | - | Ant | C |

- = no activity; Ant = antagonistic; Ant? = antagonistic with one sample, but not with a second; (Ant) = weakly antagonistic; C = cytotoxic; S = induction of spindle-shaped cells; nt = not tested.

able. It should be noted that it is cucurbitane glycosides which occur naturally in *H. carnosiflora*. Thus, glycosylation of the cucurbitanes in the plant together with enzymic hydrolysis in the insect after digestion may be an effective way of enhancing the effective concentration range and specificity of these compounds. The concentrations of carnosiflosides II and VI required to give a 50% reversal of the reduction in $A_{405}$ brought about by $5 \times 10^{-8}$ M 20E are $3.4 \times 10^{-4}$ M and $1.2 \times 10^{-4}$ M, respectively. Carnosiflogenin C becomes cytotoxic before it brings about a 50% response. The amount of carnosiflogenin A available was not sufficient to carry out a similar experiment with this compound, but the initial data (table 1) indicate that it is slightly more potent than carnosiflogenin C.

The site of antagonism of the cucurbitanes is not known at present. However, it has been shown that the ecdysteroid antagonists isolated from *Iberis umbellata*, cucurbitacins B and D, interact with the ligand binding site on the ecdysteroid receptor (Dinan et al., unpublished materials). Thus, it seems probable that the cucurbitanes from *H. carnosiflora* also interact with this site.

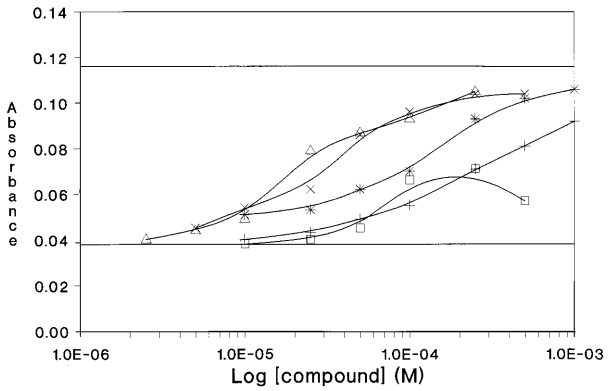


Figure 2. Concentration dependency of the active compounds: $B_{II}$ cells were grown in the presence of 23,24-dihydrocucurbitacin F (×), 25-acetoxy-23,24-dihydrocucurbitacin F (△), carnosifogenin C (□), carnosifloside II (+) or carnosifloside VI (∗) together with $5 \times 10^{-8}$ M 20-hydroxyecdysone for 7 days. The absorbance at 405 nm was determined and compared to the absorbance of untreated cells (upper horizontal line) and 20-hydroxyecdysone-treated cells (lower horizontal line).

The activities of the active cucurbitanes from *H. carnosiflora* are low in comparison to those found with cucurbitacins B and D which show activity in the micromolar range i.e. they are 100-fold more active than the cucurbitanes. The activity of cucurbitacins B and D is associated with the presence of an $\alpha,\beta$-unsaturated C-22 ketone, which is absent in the cucurbitanes from *H. carnosiflora*. In these compounds, activity is present in those compounds with an *trans*-$\Delta^{24}$ double bond; carnosiflogenin B with a *cis*-double bond is apparently inactive, although in other respects the structure is identical to that of carnosiflogenin A. In *H. carnosiflora*, the carnosiflosides are accompanied by large amounts of dihydrocucurbitacin F and 25-acetoxy-dihydrocucurbitacin F [6]. These cucurbitacins, which possess an oxo-function at C-22, but which lack the $\alpha,\beta$-unsaturation, possess antagonistic activity ($ED_{50}$ = *ca.* $3 \times 10^{-5}$ M), but are only slightly more potent than the cucurbitane compounds. It is interesting that the (more polar) cucurbitacins in *H. carnosiflora* are found in the free form, whereas the (more apolar) cucurbitanes are conjugated to sugars, suggesting that aqueous solubility may play an important role in determining the antagonistic potency, probably by facilitating mobility to the target site.

Whether the cucurbitacins and cucurbitane glycosides contribute to insect deterrence in *H. carnosiflora* is unknown at present. Cucurbitacins are known to affect the development of certain insect species [9] and are present in certain plant species at concentrations where they might be expected to have an impact on the ecdysteroid receptors. Since the cucurbitane-type compounds are so much less potent than the cucurbitacins from *I. umbellata* they would not be effective unless the ligand specificity of the ecdysteroid receptors of the insect species feeding on *H. carnsosiflora* is altered such that they are much more susceptible than the *D. melanogaster* receptor, or the concentration present in the plant is very high. In this context, it is interesting to note that the total concentration of cucurbitane-type compounds in rhizomes of *H. carnosiflora* is 1.3% of the

dry weight, which corresponds to a concentration in the rhizomes of *ca.* $5 \times 10^{-3}$ M, which is above that required to give antagonistic activity.

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