Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor

Laurence DINAN^{*1}, Pensri WHITING^{*}, Jean-Pierre GIRAULT[†], René LAFONT[‡], Tarlochan S. DHADIALLA[§], Dean E. CRESS[§], Bruno MUGAT^{||}, Christophe ANTONIEWSKI^{||} and Jean-Antoine LEPEANT^{||}

^{*}Department of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter, Devon EX4 4QG, U.K., [†]Université René Descartes, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS-URA 400, 45 rue des Saints-Pères, 75270 Paris cedex 06, France, [‡]École Normale Supérieure, Département de Biologie, Laboratoire de Biochimie, CNRS-EP 119, 46 rue d'Ulm, 75230 Paris cedex 05, France, [§]Rohm & Haas Co., Research Laboratories, 727 Norristown Road, P.O. Box 904, Spring House, PA 19477-0904, U.S.A., and ^{||}Institut Jacques Monod, 2 Place Jussieu, F-75251 Paris cedex 05, France

Two triterpenoids, cucurbitacins B and D, have been isolated from seeds of *Iberis umbellata* (Cruciferae) and shown to be responsible for the antagonistic activity of a methanolic extract of this species in preventing the 20-hydroxyecdysone (20E)-induced morphological changes in the *Drosophila melanogaster* B₁₁ permanent cell line. With a 20E concentration of 50 nM, cucurbitacins B and D give 50% responses at 1.5 and 10 μ M respectively. Both cucurbitacins are able to displace specifically bound radiolabelled 25-deoxy-20-hydroxyecdysone (ponasterone A) from a cell-free preparation of the B₁₁ cells containing ecdysteroid receptors. The K_d values for cucurbitacins B and D (5 and 50 μ M respectively) are similar to the concentrations required to antagonize 20E activity with whole cells. Cucurbitacin

B (cucB) prevents stimulation by 20E of an ecdysteroid-responsive reporter gene in a transfection assay. CucB also prevents the formation of the *Drosophila* ecdysteroid receptor/Ultraspindle/20E complex with the *hsp27* ecdysteroid response element as demonstrated by gel-shift assay. This is therefore the first definitive evidence for the existence of antagonists acting at the ecdysteroid receptor. Preliminary structure/activity studies indicate the importance of the Δ^{23-22} -oxo functional grouping in the side chain for antagonistic activity. Hexanorcucurbitacin D, which lacks carbon atoms C-22 to C-27, is found to be a weak agonist rather than an antagonist. Moreover, the side chain analogue 5-methylhex-3-en-2-one possesses weak antagonistic activity.

INTRODUCTION

Insect development is strictly regulated by the interplay between a number of chemically different classes of hormone. The steroid hormones of insects are the ecdysteroids, which are involved at each stage of the insect's life cycle and in the regulation of many developmental, biochemical and physiological processes (reviewed in [1]). Thus, in the search for new agents to control insect pest species, interference with ecdysteroid action is an attractive, but as yet little exploited, target. Not only are the hormonal actions of ecdysteroids specific to invertebrates, but the ecdysteroids are chemically distinct from vertebrate steroid hormones, suggesting that agents specifically disrupting ecdysteroid metabolism/mode of action should not affect vertebrate steroid hormone systems.

Hormone agonists and antagonists are powerful tools for the molecular dissection of hormone action. Steroid hormone antagonists have been identified for the oestrogens, androgens, progestogens and glucocorticoids, and compounds such as tamoxifen have found medical application [2,3]. No unequivocal antagonists have yet been identified in vertebrate systems for vitamin D [4] or in invertebrates for ecdysteroids. The recent rapid advances in the characterization of the ecdysteroid receptors from *Drosophila melanogaster* [5,6] and other insect species [7–10] have made insect systems excellent general models for the study of steroid hormone action [11]. Steroidal ecdysteroid agonists exist in the form of phytoecdysteroids – more than 150 analogues – which have been isolated from various species of plant [12]. The first non-steroidal ecdysteroid agonists have been identified [13] and at least one of these (RH5992) is

being developed as a commercial insecticide. To identify ecdysteroid antagonists among natural products we have initiated a screening programme with a sensitive, robust and convenient microplate-based bioassay on an ecdysteroid-responsive cell line [14,15]. This has identified several plant extracts with potent ecdysteroid antagonist activities. Here we report the bioassay-guided purification and identification of the active principles from one of these extracts and the initial characterization of their molecular mode of action. Cucurbitacins are thus demonstrated to be the first definitive insect steroid hormone antagonists acting at the level of the ecdysteroid receptor.

MATERIALS AND METHODS

Chromatography

General HPLC conditions have been described previously [16]. Analytical and semi-preparative C₁₈, silica and DIOL columns were purchased from Jones Chromatography. Solid-phase extraction (SPE) columns (C₁₈ and silica SEP-PAK) were obtained from the Waters Division of Millipore. Chromatography separation conditions are described below as appropriate.

Bioassay

The microplate-based bioassay for ecdysteroid agonists and antagonists based on the ecdysteroid-specific response of the *Drosophila melanogaster* B₁₁ cell line was performed as described

Abbreviations used: cucB, cucurbitacin B; cucD, cucurbitacin D; EcRE, ecdysteroid response element; 20E, 20-hydroxyecdysone; RP, reverse-phase; SPE, solid-phase extraction.

¹ To whom correspondence should be addressed.

previously [15]. The concentration of 20-hydroxyecdysone (20E) used in the antagonist assay was 50 nM.

Plant material

Seeds for screening purposes were purchased from commercial seed companies, mainly Chiltern Seeds, Ulverston, Cumbria, U.K. Seeds of *Iberis umbellata* (candytuft) were donated by Suttons Seeds (Torquay, Devon, U.K.). Plants of *I. umbellata* were grown in a domestic garden and harvested when mature (with flowers and fruits).

Reference cucurbitacins

Cucurbitacins E and I were purchased from Extrasynthese SA, Genay, France. Other cucurbitacins were gifts from Professor H. Achenbach (Department of Pharmaceutical Chemistry, University of Erlangen, Erlangen, Germany) and Professor T. Konoshima (Kyoto Pharmaceutical University, Kyoto, Japan).

Alkenyl ketones

Hept-3-en-2-one, hex-4-en-3-one, 5-methylhex-3-en-2-one and 6-methylhepta-3,5-dien-2-one were purchased from Aldrich (Gillingham, Dorset, U.K.) or Lancaster Synthesis (Morecambe, Lancs., U.K.).

Extraction and analysis of plant material

Seed samples were ground in a pestle and mortar; plant material was freeze-dried for 4 days. Samples (approx. 25 mg) were extracted three-times with methanol (1 ml) at 55 °C. The pooled extracts were mixed with 1.3 ml of water and 2 ml of hexane. The hexane phase (containing non-polar lipids and/or pigments) was discarded. Portions of each aqueous methanol phase were analysed for their agonist and antagonist activities by means of bioassay, and for the presence of ecdysteroids by radioimmunoassay [17]. The antisera do not cross-react with cucurbitacins B and D (T. V. Savchenko and L. Dinan, unpublished work). Preliminary chromatographic characterization of the antagonist activities was obtained by monitoring normal-phase (silica, 1 g) and reverse-phase (RP) (C_{18} , 0.5 g) SPE and HPLC separations with the bioassay in antagonist mode.

Isolation of antagonists from candytuft seed

Ground seeds of *Iberis umbellata* (8.4 g) were extracted with methanol (five times with 100 ml at 55 °C) and the residue from the concentrated extracts was partitioned between methanol/water (7:3, v/v, 100 ml) and hexane (twice with 100 ml). The aqueous phase was diluted with water (600 ml) and applied to a 10 g C_{18} SPE cartridge, sequentially eluted with 100 ml each of 25, 50, 80 and 100% (v/v) methanol in water. The antagonists, which were eluted in the 80% (v/v) methanol fraction, were then purified by HPLC on a C_{18} semi-preparative column with a linear gradient from 50 to 100% (v/v) methanol in water (2 ml/min) over 30 min. Final purification of the antagonists was by normal-phase chromatography.

Spectroscopic identification of antagonists

Chemical ionization mass spectra with NH_3 as reagent gas were obtained on a Riber 10-10B apparatus (Nermag) in chemical desorption mode. NMR spectra were obtained on a Bruker AMX500 instrument with standard Bruker microprograms. Chemical shifts are expressed in p.p.m.

Molecular modelling

Molecular modelling was performed with Alchemy III software from Tripos (St. Louis, MO, U.S.A.). Energy minimization was performed *in vacuo* without electrostatic charges. Superimposition of 20E and cucurbitacin D (cucD) was performed with the fitting procedure of the software and by fitting the atoms of the C- and D-rings of the two compounds.

Mode of action of the antagonists

Cell-free receptor binding assay

B_{11} cells were homogenized by sonication and centrifuged (16 000 g for 20 min at 4 °C) as described previously [18]. Aliquots (100 μ l) of the supernatant were incubated in a final volume of 200 μ l with [3H]ponasterone A (180 Ci/mmol; 0.2 nM final concentration) in the presence of known concentrations of A1 or A2 (see below) or a 250-fold excess of unlabelled ponasterone A. K_d values were calculated from IC_{50} values with the LIGAND program [19].

Gel-retardation assays

Gel-shift assays were performed as described by Antoniewski et al. [20] with *D. melanogaster* B_{11} cell nuclear extract, except that incubations and gel separations were performed at 20 °C. The probe was a 27-mer corresponding to the *hsp27* ecdysteroid response element (EcRE) sequence [20,21] labelled at the 5' end with ^{32}P . Gels were vacuum-dried and then substances were detected and quantified by means of a PhosphorImager.

Transfection assays

D. melanogaster S2 cells were transfected in the presence of DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate} with a construct consisting of ten copies of the *hsp27* EcRE coupled to the *Fbp1* minimal promoter (bp -69 to +80) and the *lacZ* reporter gene [22]. After 24 h the cells were exposed to 20E and/or cucurbitacins; after a further 24 h the cells were extracted and the β -galactosidase activity was measured by the method of [22].

Coupled transcription and translation of *Drosophila* ecdysteroid receptor (DmEcR) and Ultraspiracle (DmUSP) proteins

The *Drosophila* EcR and USP cDNAs were subcloned into pGEM-3Z as 3.28 kbp *Fsp1-Hpa1* and 2.2 kbp *EcoRI* fragments respectively. Fragments coding for both the proteins were cloned in the sense orientation with SP6 promoter. Purified circular plasmid DNAs of the two subclones were used as templates in a rabbit reticulocyte-coupled transcription-translation system (Promega). Proteins were synthesized either unlabelled or labelled with [^{35}S]methionine (specific radioactivity 10.2 mCi/mmol). Radiolabelled proteins were used for limited proteolysis experiments. Proteins synthesized by this method are functional by both ligand ([3H]ponasterone A) binding and electrophoretic gel-shift assays (T. S. Dhadialla and D. E. Cress, unpublished work).

Partial proteolysis of DmEcR/DmUSP heterodimers with proteinase K

Ligand-induced conformational changes in DmEcR were detected as described in [23], with the exception that proteinase K was used as the protease. Both DmEcR and DmUSP were produced as described above. Radiolabelled DmEcR and/or unlabelled DmUSP (4 μ l aliquots) were mixed in a final volume of 100 μ l with T-buffer (10 mM Tris/HCl, pH 7.2). To incubation

mixtures lacking DmUSP or a ligand was added 4 μ l of unprogrammed rabbit reticulocyte lysate or solvent respectively. The protein mixtures were incubated for 60 min at room temperature in the presence of 1 μ M muristerone A (5 β ,11 α ,20-trihydroxy-25-deoxycydysone) or cucurbitacin B (cucB) to allow binding of the ligand. Each mixture was then divided into 20 μ l aliquots, to which were added increasing concentrations of proteinase K (in 2.2 μ l). After incubation at room temperature for a further 20 min, proteolysis was terminated by adding 23 μ l of 2 $\times$ SDS/PAGE sample buffer. Samples were heated at 95 $^{\circ}$ C for 5 min and then subjected to SDS/PAGE on 8–16% gradient gels (Novex, San Diego, CA, U.S.A.). Gels were then treated sequentially with methanol/acetic acid/water (4:1:5, by vol.), ENTENSIFY (NEN Research Products, Boston, MA, U.S.A.) solutions A and B for 45 min, each in accordance with the manufacturer's instructions. The gels were vacuum-dried at 60 $^{\circ}$ C for 2 h and the signals detected by fluorography.

RESULTS

Screening for antagonists

Methanolic extracts from seeds of 1775 species of plant were assessed for ecdysteroid antagonist activity. The undiluted extracts of 42 species showed some activity and eight retained significant activity after at least 10-fold dilution. Of these the extract of *Iberis umbellata* seeds was examined further. Analysis of seeds of other species in the same genus (Table 1) revealed that *I. amara*, *I. coronaria*, *I. hybrida* and samples of *I. umbellata* from various sources were positive, whereas *I. crenata*, *I. gibraltarica*, *I. saxatilis* and *I. sempervirens* were negative. Dilution analysis of the potency of extracts of identical dry weights of portions of mature plants of *I. umbellata* revealed that all portions of the plant contain antagonist activity, with similarly high levels in the leaves, flowers, fruit and seeds, and significantly lower levels in the stems and roots (results not shown). In view of this and practical considerations, the antagonists were isolated from the seeds. A significant antagonistic response is observed in the

bioassay with an amount of seed extract corresponding to 6 μ g (fresh weight) of *I. umbellata* seed.

Bioassay-guided isolation of antagonists

Preliminary characterization

Small aliquots (0.5 ml) of the initial extract (4.3 ml) of 25 mg (fresh weight) of seed contained sufficient activity to allow the determination of the polarity of active principle(s), initially by SPE and then by HPLC. On RP-SPE cartridges, activity was eluted between 50% and 80% methanol/water, whereas on silica SPE cartridges the activity eluted with 5% (v/v) methanol in dichloromethane. Taken together these results indicated a relatively non-polar nature for the antagonist(s). Gradient RP-HPLC separation of the extract revealed two major UV-absorbing peaks (A1 and A2); antagonist activity was found to co-elute with these (Figure 1). The material in each of the activity peaks was separated by normal-phase HPLC; each gave a single peak of UV-absorbing material and co-eluting antagonist activity.

Figure 1 Chromatography of *Iberis umbellata* seed extract

RP-HPLC (ODS-2 analytical column eluted with a methanol/water gradient at 1 ml/min and monitored at 242 nm) separation of a methanolic extract of *Iberis umbellata* seed with UV monitoring (upper panel) and bioassay monitoring (antagonist mode) (lower panel). Fractions of 1 min duration were collected and subjected to bioassay. The retention times of three reference ecdysteroids (E, ecdysone; PoA, 25-deoxy-20-hydroxycydysone) are indicated.

Table 1 *Iberis* species tested for ecdysteroid agonist or antagonist activities

Radioimmunoassay values are given as μ g of ecdysone equivalents/g of seed. Symbols: —, below the detection limit; +, active as neat extract; ++, active as 10-fold dilution; ++++, active as 100-fold dilution. Abbreviations: n.d., not determined; n.l., low radioimmunoassay-positive response but not linear with respect to aliquot size.

Species	Radioimmunoassay			Bioassay	
	Black	White	DBL-1	Agonist	Antagonist
<i>Iberis amara</i> *	—	0.83	0.74	—	+++
<i>Iberis coronaria</i> †	0.46	n.d.	n.l.	—	++
<i>Iberis crenata</i> ‡	—	n.d.	—	—	—
<i>Iberis gibraltarica</i> *	0.39	n.l.	1.36	(+)	—
<i>Iberis hybrida</i> §	—	n.d.	—	—	+(+)
<i>Iberis saxatilis</i> var. <i>candolleana</i> *	—	—	—	—	—
<i>Iberis sempervirens</i> *	—	—	—	—	—
<i>Iberis umbellata</i> §	0.22	n.l.	0.17	—	++
<i>Iberis umbellata</i> *	—	0.26	0.79	—	+++
<i>Iberis umbellata</i> §	0.22	—	0.37	—	++
<i>Iberis umbellata</i> 'Iceberg'*	0.58	1.75	—	—	++

* Source: Chiltern Seeds (Ulverston, Cumbria, U.K.).

† Source: Thompson and Morgan (Ipswich, Suffolk, U.K.).

‡ Source: B&T World Seeds (Fiddington, Somerset, U.K.).

§ Source: Suttons Seeds (Torquay, Devon, U.K.).

Figure 2 Structures of compounds referred to in the text

Isolation and identification

The antagonist activities were purified from 8.4 g (fresh weight) of seed by SPE and HPLC to yield 5 mg of A1 and 34 mg of A2. The UV/visible spectra of both compounds (in ethanol) were identical, with a major absorbance peak at 230 nm. NMR and mass spectral data were as follows.

A1: δ H (500 MHz, CDCl_3) 1.01 (3H, s, 18-Me β), 1.10 (3H, s, 9-Me β), 1.26 (1H, q, J 13 Hz, 1-H β), 1.31 (3H, s, 4-Me α), 1.37 (3H, s, 4-Me β), 1.38 (3H, s, 14-Me α), 1.40 (6H, s, 26-Me and 27-Me), 1.43 (1H, 15-H α), 1.44 (3H, s, 21-Me), 1.88 (1H, d.d., J 8.7 Hz, 13.4, 15-H β), 1.98 (1H, m, 7-H α), 2.00 (1H, m, 8-H β), 2.35 (1H, d.d.d., J 3.5, 6.1, 12.8, 1-H α), 2.43 (1H, m, 7-H β), 2.57 (1H, d, J 6.8 Hz, 17-H α), 2.72 (1H, d, J 14.7 Hz, 12-H β), 2.77 (1H, broad d, J 12.9 Hz, 10-H), 3.29 (1H, d, J 14.7 Hz, 12-H α), 3.61 (1H, d, J 3.6 Hz, 2-OHe β), 4.38 (1H, s, 16-OH α), 4.40 (1H, t, J 8 Hz, 16-H β), 4.44 (1H, m, J 13.0, 5.9, 3.5 Hz, 2-H α), 5.8 (1H, m, $w_{\frac{1}{2}} = 11$, 6-H), 6.69 (1H, d, J 15.1 Hz, 23-H), 7.13 (1H, d, J 15.1 Hz, 24-H); δ C (125 MHz, CDCl_3) 19.3 (CH₃, C-14Me), 20.1 (CH₃, C-18Me), 20.2 (CH₃, C-9Me), 20.2 (CH₃, C-18Me), 20.2 (CH₃, C-9Me), 21.4 (CH₃, C-4Me α), 24.0 (CH₃, C-21Me), 24.0 (CH₂, C-7), 29.5 (CH₃, C-26Me), 29.5 (CH₃, C-27Me), 29.7 (CH₃, C-4Me β), 34.0 (CH, C-10), 36.2 (CH₂, C-1), 42.5 (CH, C-8), 46.0 (CH₂, C-15), 48.3 (C, C-13), 48.4 (C, C-9), 48.5 (CH₂, C-12), 50.3 (C, C-4), 50.9 (C, C-14), 57.7 (CH, C-17), 71.2 (C, C-25), 71.5 (CH, C-16), 71.8 (CH, C-2), 78.3 (C, C-20), 119.0 (CH, C-23), 120.5 (CH, C-6), 140.7 (C, C-5), 156.0 (CH, C-24), 203.0 (C, C-22), 212.2 (C, C-3), 213.1 (C, C-11); m/z 534 [47%, (M+NH₄)⁺], 517 [20%, (M+H)⁺], 499 [19%, (M+H-H₂O)⁺], 420 (10%), 130 (100%).

A2: δ H (500 MHz, CDCl_3) 1.00 (3H, s, 18-Me β), 1.10 (3H, s, 9-Me β), 1.25 (1H, q, J 13 Hz, 1-H β), 1.30 (3H, s, 4-Me α), 1.36 (3H, s, 4-Me β), 1.38 (3H, s, 14-Me α), 1.46 (1H, 15-H α), 1.46 (3H, s, 21-Me), 1.57 (3H, s, 27-Me), 1.59 (3H, s, 26-Me), 1.89 (1H, d.d., J 8.7 13.4 Hz, 15H β), 2.00 (1H, m, 7-H α), 2.03 (1H, m, 8-

H β), 2.03 (3H, s, MeCO), 2.34 (1H, d.d.d., J 3.5 Hz, 6.3, 12.8, 1-H α), 2.43 (1H, m, $w_{\frac{1}{2}} = 35$, 7-H β), 2.51 (1H, d, J 7.0 Hz, 17-H α), 2.71 (1H, d, J 14.5 Hz, 12-H α), 2.75 (1H, broad d, J 12.9 Hz, 10-H), 3.26 (1H, d, J 14.2 Hz, 12-H α), 3.61 (1H, broad s, 2-OHe β), 4.26 (1H, s, 16-OH α), 4.38 (1H, t, J 8.2 Hz, 16-H β), 4.43 (1H, d.d., J 13.2 Hz, 6.3, 2-H α), 5.81 (1H, m, $w_{\frac{1}{2}} = 11$, 6-H), 6.50 (1H, d, J 15.5 Hz, 23-H), 7.07 (1H, d, J 15.5 Hz, 24-H); δ C (125 MHz, CDCl_3) 19.3 (CH₃, C-14Me), 20.0 (CH₃, C-18Me), 20.2 (CH₃, C-9Me), 21.4 (CH₃, C-4Me α), 22.0 (CH₃, MeCO), 24.0 (CH₃, C-21Me), 24.0 (CH₂, C-7), 26.1 (CH₃, C-27Me), 26.5 (CH₃, C-26Me), 29.5 (CH₃, C-4Me β), 33.9 (CH, C-10), 36.2 (CH₃, C-1), 42.5 (CH, C-8), 45.5 (CH₂, C-15), 48.3 (C, C-13), 48.6 (C, C-9), 48.6 (CH₂, C-12), 50.4 (C, C-4), 50.9 (C, C-14), 58.4 (CH, C-17), 71.5 (CH, C-16), 71.8 (CH, C-2), 78.4 (C, C-20), 79.5 (C, C-25), 120.5 (CH, C-23), 120.6 (CH, C-6), 140.6 (C, C-5), 152.1 (CH, C-24), 170.4 (C, MeCO), 202.6 (C, C-22), 212.2 (C, C-3), 213.2 (C, C-11); m/z 576 [100%, (M+NH₄)⁺], 516 [8%, (M-Ac)⁺], 499 [16%, (M-Ac-H₂O)⁺], 420 (9%).

Thus A2 was identified unambiguously as cucB and A1 as cucD (Figure 2, structures I and II respectively) by reference to previously published data (e.g. [24,25]).

Effects on cell growth and morphology

Untreated B₁₁ cells grow to form a confluent layer of small, round cells evenly distributed across the bottom of the microplate wells. 20E (50 nM) caused a significant decrease in cell density, together with an increase in cell size and the formation of cellular clumps. Extracts of *Iberis* spp. or active fractions from the purification procedure resulted in small cells, higher cell densities and no clumping. However, the reversal of the 20E effects was not complete: the cells were noticeably slightly larger than untreated cells and the maximum density attained did not correspond to confluency (even though turbidimetric readings were greater than for non-treated controls at more than 2 μ M cucB; Figure 3). The concentrations of cuc B and D required to increase the turbidimetric value (D_{405}) to 50% of the difference between untreated and 20E-treated controls were 1.5 and 10 μ M respectively (Figure 3). Maximal turbidimetric values were obtained with 4 μ M cucB (175%) and 20 μ M cucD (100%). At concentrations above 20 μ M, both cucurbitacins were cytotoxic to B₁₁ cells, bringing about a decrease in cell density and fragmentation of the cells. With higher concentrations of 20E (500 nM or 5 μ M), higher concentrations of cucB were not required for antagonism. The ED₅₀ for cucB was essentially the same for all three concentrations of 20E (Figure 4). However, the maximum absorbance attained was decreased. Concentrations of cucB greater than 10 μ M remained cytotoxic.

Interaction with the ecdysteroid receptor

Ecdysteroid-binding site

Cucurbitacins B and D were able to displace specifically bound radiolabelled [³H]ponasterone A from a cell-free extract of the B₁₁ cells (Figure 5). Again, cucB was more active than cucD. The IC₅₀ value for cucB was 13 μ M, which converts to a K_d of 5 μ M, which is very similar to the concentration of cucB required to bring about a 50% response in intact B₁₁ cells (see above). Under the same conditions the IC₅₀ value for 20E was 42 nM. The K_d for cucB with the *Drosophila* Kc cell cytosolic receptor was 8.8 μ M (results not shown).

Receptor/DmEcRE complex

Western blotting demonstrated that the nuclear extract of B₁₁ cells contained DmEcR and DmUSP (results not shown). In gel-

Figure 3 Concentration-dependences of ecdysteroid antagonists

Concentration dependences of antagonistic activity of cucB ($\square$), cucD ($\triangle$) and 5-methylhex-3-en-2-one (∇) were determined in the B_{11} bioassay. The response is measured as the attenuation of wells at 405 nm relative to control wells with and without 50 nM 20E; 0% is the absorbance of wells containing B_{11} cells growing in the presence of 20E; 100% is the absorbance of wells containing B_{11} cells growing in the absence of 20E.

shift assays (Figure 6), the nuclear extract gave one prominent complex with the radiolabelled *hsp27* probe, which was significantly enhanced by the presence of 20E. The inclusion of cucB inhibited the formation of this complex.

Transfection assays based on *D. melanogaster* S2 cells transfected with a construct consisting of 10 copies of the *hsp27* sequence (containing a known EcRE sequence [21,22]), the *Fbp1* minimal promoter and the *lacZ* reporter gene demonstrated that cucB antagonizes 20E stimulation of gene activity (Table 2).

Partial proteolysis protection experiments

Aliquots of mixtures containing radiolabelled DmEcR (with or without unlabelled DmUSP) in the absence or presence of 1 μ M muristerone A or 1 μ M cucB were subjected to limited proteolysis in the presence of 1, 10 or 100 μ -units/ml proteinase K (Figure 7). Incubation of DmEcR/DmUSP in the absence or presence of ligands and without proteolysis yielded the same electrophoretic pattern as found for DmEcR in lane A of Figure 7 (results not shown). The relative electrophoretic migration of DmEcR is indicated with an arrow (105 kDa). Whereas no protection was observed with 1 μ M cucB, a protected DmEcR fragment (marked with an asterisk) was observed with 1 μ M as well as 0.1 μ M (results not shown) muristerone A.

Molecular comparison of cucD and 20E

Superimposition of the structures of 20E and cucD (Figure 8) reveals the potential for considerable three-dimensional overlap in the region of the C and D rings. The greatest differences are observed in the spatial positioning of the A and B rings, although the oxygen-containing functional groups in the A ring of cucD occupy a similar spatial location to the 2 β - and 3 β -hydroxy groups of 20E. The side chain of cucD is considerably more restricted than that of 20E with regard to possible conformations, owing to the presence of the α,β -unsaturated ketone group. Thus there is enough structural similarity to believe that both these molecules might interact with the same binding site, but they have sufficient differences to suggest why one should be a receptor agonist and the other an antagonist.

Figure 4 Concentration-dependency of antagonism between 20E and cucB

B_{11} cells were incubated for 7 days in the presence of 20E at 50 nM ($\square$), 500 nM ($\triangle$) or 5 μ M (∇) and the indicated concentrations of cucB. Attenuance at 405 nm was used as a measure of the antagonistic response.

Structure/activity relationship

The biological activities of 10 cucurbitacins are compared in Table 3. Comparison of compounds **I** to **IV** indicates that the presence or absence of a Δ^1 -double bond does not significantly affect antagonistic activity, whereas the presence of a 25-acetoxy group enhances activity. However, the relative activities of **VII** and **VIII** lead to the opposite conclusion about acetylation at C-25. Because the functional groups in the A ring of **VII** and **VIII** are different from those of **I** to **IV**, overall interaction at both ends of the molecule might be important for activity. Further studies are required to resolve this. High antagonist activity is associated only with the presence of a Δ^{23} -22-oxo functionality: no activity is observed with **V** and **VI** and very low activity with **IX**. This conclusion is supported by the biological activity of **X**, which possesses a truncated side chain and consequently lacks the α,β -unsaturated ketone. This compound possesses weak

Figure 5 Competition between ecdysteroids and cucurbitacins for the ligand-binding site of the ecdysteroid receptor

Displacement of specifically bound [3 H]ponasterone A from a cell-free ecdysteroid receptor preparation by cucB ($\square$), cucD ($\triangle$) and 20E (∇). A cell-free extract was incubated with 0.2 nM [3 H]ponasterone A in the presence of the indicated concentrations of competitor and the specifically bound radioactivity was determined by dextran-coated charcoal assay. Data points are the means of triplicate assays.

Table 2 Effect of cucB on reporter gene expression

D. melanogaster S2 cells were transfected with 10 × *hsp27* EcRE coupled to the *Fbp1* minimal promoter and the *lacZ* reporter gene. Cells were exposed to 20E and/or cucB 24 h later and the relative β-galactosidase activities of cell extracts were measured after a further 24 h.

Treatment	Relative β-galactosidase activity
Control transfected cells	16.4 ± 1.1 (n = 4)
Cells + 1 μM 20E	1117.0 ± 219.1 (n = 4)
Cells + 1 μM 20E + 1 μM cucB	902.8 ± 42.6 (n = 4)
Cells + 1 μM 20E + 10 μM cucB	826.3 ± 124.3 (n = 4)
Cells + 1 μM 20E + 50 μM cucB	49.6 ± 4.7 (n = 4)
Cells + 1 μM cucB	36.0
Cells + 10 μM cucB	68.2
Cells + 50 μM cucB	16.3

Figure 6 Effect of 20E and cucB on DmEcR/DmUSP/EcRE interaction

Gel-shift assays for the effect of 20E (1 μM) and cucB (100 μM) on the interaction of DmEcR/DmUSP in a nuclear extract of B₁ cells (μg protein) with [³²P]*hsp27* EcRE. The additions of nuclear extract, 20E and cucB are summarized at the bottom. The dried gel was processed in a PhosphorImager and the relative intensities of the relevant bands were quantified (percentage binding).

Figure 7 Partial proteolytic protection of DmEcR/DmUSP

In vitro-expressed [³⁵S]methionine-labelled DmEcR with or without unlabelled DmUSP (see top panels for a summary of the additions) was incubated with muristerone A or cucB (1 μM) and then subjected to proteolysis with proteinase K at 1 μ-unit/ml (lanes b), 10 μ-units/ml (lanes c) or 100 μ-units/ml (lanes d). Lane A was a control containing an equivalent amount of unhydrolysed [³⁵S]DmEcR.

Figure 8 Comparison of the three-dimensional shapes of 20E and cucD

CPK model (upper) and Dreiding model (lower) of the superimposition of 20E (in red) and cucD (in green), both shown without hydrogen atoms for clarity.

agonist activity. Thus it seems that the ring system contributes to the interaction with the ligand-binding site, whereas the Δ²³-22-oxo functionality confers antagonistic activity. Several simple unsaturated ketones that structurally mimic the side chain portion of cucurbitacins were tested for activity. The one with the greatest structural similarity, 5-methylhex-3-en-2-one, possessed weak antagonistic activity between 50 and 500 μM, becoming cytotoxic at 1 mM (Figure 3). The other alkenyl ketones possessed no agonistic or antagonistic activity, although hex-4-en-3-one and hept-3-en-2-one were cytotoxic at 100 μM and higher.

DISCUSSION

The cucurbitacins are a group of triterpenoid compounds isolated predominantly from the Cucurbitaceae, but also from a few genera within other plant families including the Cruciferae [26]. They are renowned for their bitter taste but they also possess a

Table 3 Potencies of cucurbitacins in the B₁₁ bioassay

Bioassay results: + + +, full response; + +, moderate response; +, slight response; —, no response; C, cytotoxic.

Compound	Cucurbitacin	Activity	Concentration (M) ...	Response			
				10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
I	Cucurbitacin B	Antagonist	—	—	+	+ + +	C
II	Cucurbitacin D	Antagonist	—	—	—	+ +	C
III	Cucurbitacin E	Antagonist	—	—	+	+ + +	C
IV	Cucurbitacin I	Antagonist	—	—	—	+ +	C
V	Cucurbitacin R	None	—	—	—	—	C
VI	Cucurbitacin U	None	—	—	—	—	C
VII	Cucurbitacin F	Antagonist	—	—	+	+ + +	C
VIII	25- <i>O</i> -Acetylucurbitacin F	Antagonist	—	—	—	+	C
IX	22,23-Dihydrocucurbitacin F	Antagonist	—	—	—	—	+
X	Hexanorcucurbitacin D	Agonist	—	—	—	+	+ + +

number of potent pharmacological effects, deriving largely from their cytotoxic and antitumoral properties [27]. Other biological roles have also been attributed to the cucurbitacins, of which the most relevant here are their strong antifeedant activity towards a number of insect species [28,29], while acting as a feeding attractant to diabrotic beetles [30]. Moreover some plant species containing cucurbitacins present insecticidal activity towards various insect species, including *Diabrotica*. Thus cucurbitacins seem generally detrimental to insect development and even in the exceptional case of *Diabrotica* spp. they are toxic at higher concentrations, even if lower concentrations can be tolerated.

The compounds we have isolated from seed of *I. umbellata* have unequivocally been shown to correspond to cucurbitacins B and D by a wide range of spectroscopic techniques. Gmelin [31] was the first to investigate the cucurbitacins in seed of *I. umbellata*, finding predominantly cucurbitacins B and D, together with much smaller amounts of cucurbitacins E and I, 1,2-dihydrocucurbitacin J and 1,2-dihydrocucurbitacin K. The distribution of antiecdysteroid activity within the genus *Iberis* (Table 1) coincides with the known occurrence of cucurbitacins [27,31]. However, because the identities of the major cucurbitacins vary between different members of this genus, it was clear that cucurbitacins other than B and D must also possess significant antiecdysteroid activity. This prompted us to initiate studies on the structure/activity relationship to assess the contribution of structural features to the antagonistic activity and to identify more potent cucurbitacins.

Ecdysteroids induce a very characteristic and specific response in the *D. melanogaster* B₁₁ cell line [32]; a significant proportion of the cells initially elongate, and then the cells form large phagocytotic clumps. Consequently, cell density decreases and cell size increases relative to untreated controls. In the absence of 20E, the cucurbitacins have no observable effects on the B₁₁ cells, except at high concentrations where they are cytotoxic. The cucurbitacins largely prevent the occurrence of the 20E-induced changes, such that the cell density is increased and cell size is decreased relative to ecdysteroid-treated controls. However, it is noticeable that the cells are not as small as untreated controls and the cell density is not as high (even though the *D*₄₀₅ values are greater). Thus a combination of agonist and antagonist is not exactly equivalent to no treatment. This might reflect the observation that steroid hormone receptors, in the absence of ligand, act as repressors of gene activity [33,34].

The interaction of the cucurbitacins with the ecdysteroid receptor, rather than at some other point in ecdysteroid action,

is clearly demonstrated by a number of lines of evidence. First, both cucurbitacins are able to displace specifically bound ponasterone A from a cell-free receptor preparation of the B₁₁ cells. The calculated *K*_d values for the binding of the two cucurbitacins to the receptor are very similar to the concentrations required to antagonize 20E action on whole cells. Secondly, in a transfection assay involving an ecdysteroid-responsive gene construct possessing several copies of *hsp27* EcRE, the stimulatory effect of 20E on gene expression is prevented by the simultaneous presence of cucB. By itself, cucB does not significantly affect levels of expression of the reporter gene. Lastly, gel-shift assays reveal that the cucurbitacins prevent the formation of DmEcR/DmUSP complexes with the same EcRE.

It is probable that other ecdysteroid antagonists remain to be identified that interact either with the receptor or at other sites involved in ecdysteroid-regulated gene expression. We have already identified several antagonistic withanolides [35] that, owing to their structural similarities to cucurbitacins and ecdysteroids, probably also bind to the ligand-binding site of the ecdysteroid receptor. The recent detection of an orphan nuclear receptor (XR78E/F) that inhibits the ecdysteroid response [36] suggests that agonists of this receptor (if they exist) are ecdysteroid antagonists.

DmEcR expressed *in vitro* is partly protected against enzymic hydrolysis in the presence of 1 μ M muristerone A. Similar protection is not afforded by 1 μ M cucB. This indicates that the antagonistic cucurbitacins do not bring about the same conformational changes in the receptor protein as agonistic ecdysteroids.

The presence of a Δ^{23-22} -oxo function in the side chain seems to be very important for antagonistic activity of cucurbitacins. Cucurbitacins lacking a carbon-carbon double bond in the side chain possess very poor antagonistic activity. The absence of C-22 to C-27 (compound X) actually results in a molecule with weak agonist activity, and an analogue of the side chain (5-methylhex-3-en-2-one), which has an α,β -unsaturated ketone, is a weak antagonist. Such unsaturated ketones are well known as Michael acceptors, giving rise to the possibility that cucurbitacins might interact covalently with the ecdysteroid receptor if there is a suitably positioned nucleophilic group within the ligand-binding site to attack the C-22 carbonyl group. Circumstantial evidence for covalent attachment is provided by the lack of competition between 20E and cucB (Figure 4). If non-covalent interaction of cucB with the receptor were occurring, one would expect that higher concentrations of cucB would be required to antagonize the higher concentrations of 20E. An α,β -unsaturated

ketone is also present in the side chain of many withanolides, some of which have been shown to antagonize 20E action in *B₁₁* cells [35]. However, only withanolides possessing an unusual C-3 oxygen-containing function possess antagonistic activity, indicating that interactions at both ends of the molecule are important.

Finally, the low levels of ecdysteroids detected by RIA in most of the *Iberis* extracts are intriguing. If confirmed by other techniques, they might contribute to the efficacy of the antagonists. Because antagonists can only be expected to be active when the hormone and the receptor are present and the titres of ecdysteroids and ecdysteroid receptors are developmentally regulated, an antagonist alone would be developmentally disruptive only at certain stages of development. However, the ingestion of ecdysteroids has been shown to induce the synthesis of the ecdysteroid receptor [37]; thus the co-occurrence of low levels of phytoecdysteroids might be a mechanism to extend the efficacy of the cucurbitacins in the defence of *Iberis* spp.

We thank Suttons Seeds (Torquay, Devon, U.K.) for providing seeds of *Iberis umbellata*, Ada Tsitsekli for assistance in performing the cell-free binding assays, and Professor H. Achenbach (University of Erlangen, Erlangen, Germany) and Professor T. Konoshima (Kyoto Pharmaceutical University, Kyoto, Japan) for providing reference cucurbitacins. This research was supported by grants from the European Community (SCI*123-C) and the Biotechnology and Biological Sciences Research Council. Part of the work was carried out while L.D. was a recipient of an EMBO Short-term Fellowship.

REFERENCES

- Koolman J. (ed.) (1989) *Ecdysone: From Chemistry to Mode of Action*, Thieme Verlag, Stuttgart
- Henderson, B. E., Ross, R. K. and Pile, M. C. (1993) *Science* **259**, 633–638
- Parczyk, K. and Schneider, M. R. (1996) *J. Cancer Res. Clin. Oncol.* **122**, 383–396
- McDonnell, D. P., Vegeto, E. and Gleeson, M. A. G. (1993) *Bio/Technology* **11**, 1256–1261
- Luo, Y., Amin, J. and Voellmy, R. (1981) *Mol. Cell. Biol.* **11**, 3660–3675
- Koelle, M. R., Talbot, W. S., Segreaves, W. A., Bender, M. T., Cherbas, P. and Hogness, D. S. (1991) *Cell* **67**, 59–77
- Imhof, M. O., Rusconi, S. and Lezzi, M. (1993) *Insect Biochem. Mol. Biol.* **23**, 115–124
- Cho, W.-L., Kapitskaya, M. and Raikhel, A. S. (1995) *Insect Biochem. Mol. Biol.* **25**, 19–27
- Fujiwara, H., Jindra, M., Newitt, R., Palli, S. R., Hiruma, K. and Riddiford, L. M. (1995) *Insect Biochem. Mol. Biol.* **25**, 845–856
- Swevers, L., Drevet, J. R., Lunke, M. D. and Iatrou, K. (1995) *Insect Biochem. Mol. Biol.* **25**, 857–866
- Guild, G. and Richards, G. (1992) *Nature (London)* **357**, 539
- Lafont, R. D. and Wilson, I. D. (1996) *The Ecdysone Handbook*, 2nd edn. The Chromatographic Society, Nottingham
- Wing, K. D. (1988) *Science* **241**, 467–469
- Clément, C. Y. and Dinan, L. (1991) in *Insect Chemical Ecology*, (Hrby, I., ed.), pp. 221–226, Academia, Prague
- Clément, C. Y., Bradbrook, D. A., Lafont, R. and Dinan, L. (1993) *Insect Biochem. Mol. Biol.* **23**, 187–193
- Dinan, L. (1988) *J. Chromatogr.* **436**, 279–288
- Dinan, L. (1995) *Eur. J. Entomol.* **92**, 271–283
- Dinan, L. (1985) *Arch. Insect Biochem. Physiol.* **2**, 295–317
- Munson, P. J. (1983) *Methods Enzymol.* **92**, 543–576
- Antoniewski, C., Laval, M., Dahan, A. and Lepesant, J.-A. (1994) *Mol. Cell. Biol.* **14**, 4465–4474
- Riddihough, G. and Pelham, H. R. B. (1987) *EMBO J.* **6**, 3729–3734
- Antoniewski, C., Mugat, B., Delbac, F. and Lepesant, J.-A. (1996) *Mol. Cell. Biol.* **16**, 2977–2986
- Yao, T.-P., Forman, B. M., Ilang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P. and Evans, R. M. (1993) *Nature (London)* **336**, 476–479
- Che, C. T., Fang, X., Phoebe, Jr., C. H., Kinghorn, A. D. and Farnsworth, N. R. (1985) *J. Nat. Prod.* **48**, 429–434
- Jacobs, H., Singh, T., Reynolds, W. F. and McLean, S. (1990) *J. Nat. Prod.* **53**, 1600–1605
- Lavie, D. and Glotter, E. (1971) *Fortschr. Chem. Organ. Naturst.* **29**, 307–362
- Miró, M. (1995) *Phytotherapy Res.* **9**, 159–168
- Nielsen, J. K., Larsen, L. M. and Sorensen, H. (1977) *Phytochemistry* **16**, 1519–1532
- Huang, X., Renwick, J. A. A. and Sachdev-Gupta, K. (1993) *J. Chem. Ecol.* **19**, 1645–1663
- Metcalf, R. L. and Lampman, R. L. (1989) *Experientia* **45**, 240–247
- Gmelin, R. (1963) *Arznei. Forsch.* **13**, 771–776
- Gateff, E., Gissman, L., Shrestha, R., Plus, N., Pfister, H., Schröder, J. and zur Hausen, H. (1980) in *Invertebrate Systems In Vitro* (Kurstak, E., Maramorosch, K. and Dübendorfer, A., eds.), pp. 517–533, Elsevier/North Holland, Amsterdam
- Dobens, L., Rudolph, K. and Berger, E. M. (1991) *Mol. Cell. Biol.* **11**, 1846–1853
- McDonnell, D. P., Vegeto, E. and O'Malley, B. W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10563–10567
- Dinan, L., Whiting, P., Alfonso, D. and Kapetanidis, I. (1996) *Entomol. Exp. Appl.* **80**, 415–420
- Zelhof, A. C., Yao, T.-P., Evans, R. M. and McKeown, M. (1995) *Biochemistry* **92**, 10477–10481
- Deak, P., Zavorsky, P. and Maróy, P. (1988) *Insect Biochem.* **18**, 847–852