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Research Article

Assessment of natural products in the *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonist and antagonist activities

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Abstract. Ecdysteroid agonist and antagonist activities can be detected and quantified with the *Drosophila melanogaster* B_{II} cell bioassay. This bioassay is convenient, sensitive and robust. We report the assessment with this bioassay of the activities of a wide range of compounds representing a number of classes of natural products. Many compounds were inactive over a wide concentration range (10^{-8} to 10^{-4} or 10^{-3} M) or cytotoxic at high concentrations. However, antagonisitic activity was associated with several classes of compounds: cucurbitacins and withanolides (extending previous findings) and phenylalkanoids and certain alkaloids (described for the first time). A withanolide (withaperuvin D) is identified which possesses agonistic activity. Brassinosteroids, which have been ascribed (ant)agonistic properties in the past, were not found to be active in the B_{II} bioassay, either as agonists or antagonists. Possible reasons for the prevalence of antagonists and for the low potency of the majority of them are discussed.

Key words. Agonist; alkaloid; antagonist; bioassay; brassinosteroid; cucurbitacin; ecdysteroid; limonoid; phenylalkanoid; receptor; steroid hormone; triterpenoid; withanolide.

Many plant-derived natural products are known to affect insect growth, development and/or reproduction. Many of these compounds have been postulated to contribute to the deterrence of phytophagous predators. However, in very few cases is the precise mode of action known. Since correct functioning of the ecdysteroid endocrine system in insects is crucial for proper development, a range of molecular structures elaborated by plants are to be expected which interact with, and disrupt, this system in phytophagous insects. In addition to providing useful insights into insect-plant relationships, such compounds have potential as leads for the development of new types of insect pest control agents. We have initiated a strategy to identify such compounds [1]. We have developed a simple microplate-based bioassay [2], which takes advantage of the ecdysteroid-responsive *Drosophila melanogaster* B_{II} cell line, to detect compounds which can either mimic the action of ecdysteroids or antagonise their actions. We have already demonstrated that certain cucurbitacins [3, 4], withanolides [5], limonoids [6] and stilbenoids [7, 8; Meng et al., unpublished data] antagonise the action of 20-hydroxyecdysone. In addition, a number of novel phytoecdysteroids (agonists) have been identified and the bioassay has been used to quantify the potencies of a large number of phytoecdysteroids [9, 10] and to

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develop molecular models for ligand binding to the ecdysteroid receptor complex [11]. There are two major strands to the overall strategy. On the one hand, we have assessed the agonistic and antagonistic activities of extracts of a wide range (ca 5000) of plant species. On the other hand, we have determined whether purified plant secondary compounds of known structure possess activity. We now report the results arising from the second strand of this strategy.

Materials and methods

Compounds. Some compounds were isolated in our own laboratory or purchased, but the majority were most generously provided by other researchers: Prof. H. Achenbach (University of Erlangen, Germany), Prof. G. Burton (Universtiy of Buenos Aires, Argentina), Prof. S. Clouse (University of North Carolina, USA), Dr. E. da Cuhna (University of Strathclyde, UK), Prof. W. Daniewski (Institute of Organic Chemistry, Warsaw, Poland), Dr. M. Feldlaufer (ARS, Beltsville, USA), Dr. A. I. Gray (University of Strathclyde, UK), Dr. S. Habtemariam (University of Greenwich, UK), Dr. J. Harmatha (IOCB, Prague, Czech Republic), Dr. S. Hasegawa (USDA-ARS, Albany, USA), Prof. M. Kawai (Kyoto University, Japan), Prof. J. Koolman [University of Marburg, Germany, providing compounds prepared in the laboratories of Prof. C. Brosa (Institut Quimic de Sarrià, Barcelona, Spain) and Prof. G. Adam (Institut für Pflanzenbiochemie, Halle, Germany)], Prof. T. Konoshima (Kyoto Pharmaceutical University, Japan), Prof. B. Luu (University of Strasbourg, France), Dr. Y. Meng (Zhongshan College, Guangdong, P. R. China), Prof. S. Milosavljevic (University of Belgrade, Yugoslavia), Prof. E. D. Morgan (University of Keele, UK), Dr. Murayama (Sanwa Shoyaku Co. Ltd., Utsunomiya, Japan), Prof. A. Ravelo (Universidad de La Laguna, Tenerife, Spain), Prof. A. B. Ray (Banaras Hindu University, Varanasi, India), Dr. J. G. Roddick (Department of Biological Sciences, University of Exeter, UK), Dr. A. S. S. Rouf (Dhaka University, Bangladesh), Prof. Z. Saatov (Institute of Plant Chemistry, Tashkent, Uzbekistan), Dr. F. Smit (University of Utrecht, The Netherlands), Dr. H. Stebbings (University of Exeter, UK), Dr. H. Stuppner (University of Innsbruck, Austria), Dr. T. Tallamy (University of Delaware, USA), Prof. K. Yamasaki (Hiroshima University, Japan) (see table 1 for sources of individual compounds). Brassinosteroids, cucurbitacins and withanolides were assessed for purity by RP-HPLC and purified by this method if less than 97% pure. Other compounds were used as supplied.

Bioassay. Compounds were assessed for ecdysteroid agonist and antagonist activity at concentrations from 10^{-8} to 10^{-4} or 10^{-3} M in the *D. melanogaster* B_{II} bioassay as described previously [2]. The concentration of 20-hydroxyecdysone used in the assay for antagonist activity was 5×10^{-8} M Each concentration of each compound was assessed in quadruplicate in the agonist and antagonist versions of the bioassay.

In vitro receptor assay. Bacterially-expressed DmEcR, DmUSP, CfEcR and CfUSP were generously provided by Dr. Tarlochan Dhadialla (Rohm & Haas Co.). Stock solutions of test compounds were prepared in methanol. The required amount was dispensed into 1.5-ml Eppendorf tubes in triplicate and the solvent evaporated. The residue was dissolved in 2 μl ethanol. EcR protein (15 μl) and USP (1 μl) were incubated in a total volume of 100 μl (+ 82 μl 10 mM Tris/HCl, pH 7.2, containing 1 mM dithiothreitol and 0.4 mg fatty-acid-free bovine serum albumin) with [24,25,26,27-^{3}H]ponasterone A (0.2 nM, 150 Ci/mmol; ARC Inc.). Non-specific binding was determined by the inclusion of a 500-fold excess of unlabelled ponasterone A. Assays were incubated for 16 h (DmEcR + DmUSP) or 3 h (CfEcR + CfUSP) at 7 °C. Dextran-coated charcoal (25 μl: 500 mg acid-washed charcoal + 50 mg T70 dextran in 20 ml 10 mM Tris/HCl buffer, pH 7.2) was added to each tube and incubated for 5 min in ice-cold water before centrifuging at 13,000 × g for 5 min. Aliquots of the supernatants (100 μl) were radioassayed after the addition of 1 ml scintillation cocktail (Packard Ecoscint). IC_{50} values for compounds were converted to Ki values according to the formula $Ki = IC_{50}/(1 + [ponA]/Kd)$, where [ponA] is the concentration of [^{3}H]ponasterone A used (0.2 nM) and Kd is the equilibrium dissociation constant for the interaction of the receptor complex with [^{3}H]ponasterone A (determined by Scatchard analysis; 0.15 nM for DmEcR/DmUSP and 0.8 nM for CfEcR/CfUSP) [Bourne, unpublished data].

Results and discussion

The structures of the compounds tested are shown in figure 1 and the bioassay results are summarised in table 1. These data, together with those already published, are discussed below.

Alkaloids. Five alkaloids from *Centaurea moschata* (moschamine, *cis*-moschamine, moschamindole, moschamindolol and moschamide [12, 13]) were previously found to possess no ecdysteroid agonist or antagonist activity in the B_{II} bioassay. Among the compounds tested here, three structurally related compounds (isomitraphylline **[6]**, isopteropodine **[7]** and pteropodine **[11]**) showed significant antagonistic activity. Two other related compounds, speciophylline (**13**) and uncarine F (**15**) showed weak activity, but mitraphylline (**8**) was inactive. Veratridine (**16**), one of the insecticidal steroidal alkaloid components of sabadilla

Table 1. Activities of natural products in the B_{II} bioassay for ecdysteroid agonists and antagonists. The number before each compound name refers to the structures in figure 1.

Compound	Maximum conc (M)	Agonist activity	Antagonist activity	Cytotoxicity	Supplier
Alkaloids					
1: aldisin	10^{-4}	inactive	inactive	inactive	Meng
2: atropine	10^{-4}	inactive	inactive	inactive	Sigma (A0132)
3: berberine chloride	10^{-3}	inactive	inactive	$\geq 10^{-5}$ M	Rouf
4: 2-bromoaldisin	10^{-4}	inactive	inactive	inactive	Meng
5: chaconine	10^{-4}	inactive	inactive	at 10^{-4} M	Roddick
6: isomitraphylline	10^{-3}	inactive	active ($EC_{50} = 1.6 \times 10^{-5}$ M)	at 10^{-3} M	Stuppner
7: isopteropodine	10^{-3}	inactive	active ($EC_{50} = 1.5 \times 10^{-5}$ M)	$\geq 10^{-4}$ M	Stuppner
8: mitraphylline	10^{-3}	inactive	inactive	inactive	Stuppner
9: nicotine	10^{-4}	inactive	inactive	inactive	Sigma (N3876)
10: nodosin	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-6}$ M	Meng
11: pteropodine	10^{-3}	inactive	active ($EC_{50} = 1.0 \times 10^{-5}$ M)	$\geq 10^{-4}$ M	Stuppner
12: solanine	10^{-4}	inactive	inactive	at 10^{-4} M	Roddick
13: speciophylline	10^{-3}	inactive	active at 10^{-4} M	at 10^{-3} M	Stuppner
14: strychnine	10^{-4}	inactive	inactive	inactive	Sigma (S0532)
15: uncarine F	10^{-3}	inactive	active at 10^{-4} M	at 10^{-3} M	Stuppner
16: veratridine	10^{-3}	inactive	inactive	$\geq 2.5 \times 10^{-4}$ M	Sigma (V5754)
17: caulerpin	10^{-4}	inactive	inactive	inactive	Meng
18: yohimbine hydrochloride	10^{-3}	inactive	inactive	at 10^{-3} M	Stuppner
Brassinosteroids (N = naturally occurring, S = synthetic analogue)					
19: brassinolide (N)	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Luu
20: castasterone (N)	10^{-3}	inactive	inactive	at 10^{-3} M	Luu
21: 24-epibrassinolide (N)	10^{-4}	inactive	inactive	inactive	Koolman/Clouse/Feldlaufer
22: 24-epicastasterone (N)	10^{-4}	inactive	inactive	inactive	Koolman/Clouse
23: 28-homobrassinolide (N)	10^{-4}	inactive	inactive	inactive	Brosa/Clouse/Luu
24: 22*S*,23*S*-homobrassinolide (S)	10^{-4}	inactive	inactive	at 10^{-4} M	Koolman/Luu
25: 28-homocastasterone (N)	10^{-4}	inactive	inactive	inactive	Koolman
26: 22*S*,23*S*-homocastasterone (S)	10^{-4}	inactive	inactive	inactive	Koolman
27: 22,23,24-trisepibrassinolide (S)	10^{-4}	inactive	inactive	inactive	Koolman/Clouse/Feldlaufer
28: 22,23,24-trisepicastasterone (S)	10^{-4}	inactive	inactive	at 10^{-4} M	Koolman/Clouse
Cardenolides and bufadienolides					
29: digitoxigenin	10^{-4}	inactive	inactive	inactive	Sigma (D9404)
30: digitoxin	10^{-3}	inactive	inactive	$\geq 5 \times 10^{-4}$ M	Sigma (D5878)
31: digoxigenin	10^{-4}	inactive	inactive	inactive	Sigma (D9026)
32: digoxin	10^{-4}	inactive	inactive	inactive	Sigma (D6003)
33: gitoxigenin	10^{-4}	inactive	inactive	inactive	Sigma (G4635)
34: oleandrin	10^{-4}	inactive	inactive	inactive	Sigma (O6500)
35: ouabain	5×10^{-4}	inactive	inactive	inactive	Aldrich (46,441-4)
36: hellebortin A	10^{-3}	inactive	inactive	inactive	Meng
Cucurbitacins					
37: arvenin I	10^{-4}	inactive	inactive	inactive	isolated at Exeter
38:	10^{-3}	inactive	active at 10^{-3} M	inactive	Stuppner
39:	10^{-3}	inactive	active at 10^{-4} M	at 10^{-3} M	Stuppner
40:	10^{-3}	inactive	active at $\geq 10^{-4}$ M	inactive	Stuppner
41:	10^{-3}	inactive	active at 10^{-3} M	inactive	Stuppner
42:	10^{-3}	inactive	inactive	at 10^{-3} M	Stuppner
43: cucurbitacin B	10^{-4}	inactive	active ($EC_{50} = 7.5 \times 10^{-7}$ M)	at $> 10^{-5}$ M	isolated at Exeter
44: cucurbitacin C	10^{-4}	inactive	active ($EC_{50} = 1.1 \times 10^{-6}$ M)	inactive	Tallamy
45: cucurbitacin D	10^{-4}	inactive	active ($EC_{50} = 7.5 \times 10^{-7}$ M)	at $> 10^{-5}$ M	isolated at Exeter
46: cucurbitacin E	10^{-4}	inactive	active ($EC_{50} = 1.1 \times 10^{-6}$ M)	at $> 10^{-5}$ M	Extrasynthese

Table 1. (Continued)

Compound	Maximum conc (M)	Agonist activity	Antagonist activity	Cytotoxicity	Supplier
47: cucurbitacin F	10^{-4}	inactive	active ($EC_{50} = 8.0 \times 10^{-7}$ M)	at $> 10^{-5}$ M	isolated at Exeter
48: cucurbitacin I	10^{-4}	inactive	active ($EC_{50} = 1.5 \times 10^{-6}$ M)	at $> 10^{-5}$ M	Extrasynthese
49: cucurbitacin R	10^{-3}	inactive	inactive	at $\geq 10^{-4}$ M	Achenbach
50: cucurbitacin U	10^{-4}	inactive	inactive	at $\geq 10^{-4}$ M	Achenbach
51: cucurbitacin U gentobioside	10^{-4}	inactive	inactive	inactive	Achenbach
52: cucurbitacin V gentobioside	10^{-4}	inactive	inactive	inactive	Achenbach
53: 25-acetylcucurbitacin F	10^{-4}	inactive	active ($EC_{50} = 1.2 \times 10^{-5}$ M)	$\geq 10^{-4}$ M	Achenbach
54: 22,23-dihydrocucurbitacin F	10^{-4}	inactive	inactive	$\geq 10^{-4}$ M	Konoshima
55: hexanorcucurbitacin D	10^{-4}	active ($EC_{50} = 2.5 \times 10^{-5}$ M)	inactive	at 10^{-3} M	Achenbach
56:	10^{-3}	inactive	active ($EC_{50} = 1.1 \times 10^{-5}$ M)	at 10^{-3} M	Stuppner
57:	10^{-4}	inactive	inactive	inactive	Stuppner
58:	10^{-3}	inactive	active ($EC_{50} = 7.0 \times 10^{-6}$ M)	at 10^{-3} M	Stuppner
59:	10^{-3}	inactive	nactive	at 10^{-3} M	Stuppner
60: deacetylpicracin	10^{-3}	inactive	active ($EC_{50} = 7.3 \times 10^{-5}$ M)	$\geq 2.5 \times 10^{-4}$ M	Smit
61: 3-*epi*-isocucurbitacin D	10^{-4}	inactive	active ($EC_{50} = 7.0 \times 10^{-6}$ M)	inactive	isolated at Exeter
62: picracin	10^{-3}	inactive	active ($EC_{25} = 5.0 \times 10^{-6}$ M)	$\geq 2.5 \times 10^{-5}$ M	Smit
Insect peptide hormones and neuromodulators					
63: allatostatin IV	10^{-4}	inactive	inactive	inactive	Sigma (A9554)
64: leucokinin I	10^{-4}	inactive	inactive	inactive	Sigma (L2896)
65: leucopyrokinin	10^{-4}	inactive	inactive	inactive	Sigma (L6268)
66: L-noradrenaline	10^{-4}	inactive	inactive	inactive	Sigma (A-7257)
67: DL-octopamine	10^{-4}	inactive	inactive	inactive	Sigma (O0250)
68: proctolin	10^{-4}	inactive	inactive	inactive	Sigma (P4280)
69: tyramine	10^{-4}	inactive	inactive	inactive	Sigma (T7255)
(Iso-)Flavonoids/phyto-oestrogens					
70: apigenin	10^{-3}	inactive	inactive	at 10^{-3} M	Sigma (A3145)
71: biochanin A	10^{-3}	inactive	inactive	at 10^{-3} M	ICN (157745)
72: daidzein	10^{-3}	inactive	weak activity at $\geq 10^{-4}$ M	inactive	Lancaster (14182)
73: fisetin	10^{-4}	inactive	inactive	at 10^{-4} M	Sigma (F4043)
74: genistein	10^{-3}	inactive	inactive	at 10^{-3} M	Sigma (G6649)
75: luteolin	10^{-4}	inactive	inactive	at 10^{-4} M	Sigman (L9283)
76: myricetin	10^{-4}	inactive	inactive	at 10^{-4} M	Sigma (M6760)
77: quercetin	10^{-3}	inactive	inactive	at 10^{-3} M	Sigma (Q0125)
78: zearalenone	10^{-3}	inactive	weak activity at $\geq 10^{-5}$ M	$\geq 2.5 \times 10^{-4}$ M	Sigma (Z2125)
Lignans					
79: bakkenolide A	10^{-3}	inactive	inactive	at 10^{-3} M	Harmatha
80: cubebin	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Harmatha
81: podophyllotoxin	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Harmatha
Limonoids					
82: azadirachtin A	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Morgan
83: entilin A	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Daniewski
84: entilin C	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Daniewski
85: limonin	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Hasegawa
86: nomilin	10^{-3}	inactive	weak activity at 10^{-4} M	at 10^{-3} M	Hasegawa
87: obacunone	10^{-4}	inactive	weak activity at 10^{-5} M	at 10^{-4} M	Hasegawa
88: salannin	10^{-3}	inactive	inactive	at 10^{-3} M	Morgan
89: utilin B	10^{-3}	inactive	inactive	inactive	Daniewski
90: utilin C	10^{-3}	inactive	inactive	inactive	Daniewski
Phenylalkanoids					
91: marginatine	10^{-3}	inactive	active ($EC_{50} = 5.0 \times 10^{-5}$ M)	inactive	da Cunha
92: croweacin	10^{-3}	inactive	active ($EC_{50} = 7.3 \times 10^{-4}$ M)	inactive	da Cunha

Table 1. (Continued)

Compound	Maximum conc (M)	Agonist activity	Antagonist activity	Cytotoxicity	Supplier
93: 2,6-dimethoxy-3,4-methylene-dioxy-1-(2-propenyl)-benzene	10^{-3}	inactive	active ($\mathrm{EC}_{50} = 6.0 \times 10^{-4}$ M)	inactive	da Cunha
94: apiole	10^{-3}	inactive	active ($\mathrm{EC}_{50} = 7.3 \times 10^{-4}$ M)	inactive	da Cunha
95: isoasarone	10^{-3}	inactive	active ($\mathrm{EC}_{50} = 2.5 \times 10^{-4}$ M)	inactive	da Cunha
96: pipermargine	10^{-3}	inactive	active ($\mathrm{EC}_{50} = 3.3 \times 10^{-4}$ M)	inactive	da Cunha
Triterpenoids and saponins					
97: agigenin	10^{-4}	inactive	inactive	inactive	Harmatha
98: aginoside	10^{-4}	inactive	inactive	at 10^{-4} M	Harmatha
99: alisol A	10^{-4}	inactive	inactive	at 10^{-4} M	Murayama
100: 16-ketoalisol A	10^{-4}	inactive	inactive	inactive	Murayama
101: betulin	10^{-3}	inactive	inactive	inactive	ICN (473-98-3)
102:	10^{-4}	inactive	inactive	at 10^{-4} M	Harmatha
103: cycloseversioside E	10^{-4}	inactive	active ($\mathrm{EC}_{50} = \geq 10^{-4}$ M)	inactive	Saatov
104: cycloseversioside F	10^{-4}	inactive	active ($\mathrm{EC}_{50} = \geq 10^{-4}$ M)	inactive	Saatov
105: 6-deoxyaginoside	10^{-4}	inactive	inactive	at 10^{-4} M	Harmatha
106: digitonin	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Harmatha
107: 2,6-dimethylagigenin	10^{-4}	inactive	inactive	at 10^{-4} M	Harmatha
108: dioscin	10^{-4}	inactive	inactive	$> 2.5 \times 10^{-5}$ M	Murayama
109: diosgenin	10^{-4}	inactive	inactive	inactive	Harmatha
110:	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Stuppner
111: betulonic acid	10^{-3}	inactive	inactive	at 10^{-3} M	Stuppner
112:	10^{-3}	inactive	inactive	inactive	Stuppner
113: blepharadol	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-5}$ M	Ravelo
114: chiisanoside	10^{-3}	inactive	weak activity at $\geq 2.5 \times 10^{-4}$ M	inactive	Yamasaki
115: demethylzeylasterone	10^{-4}	inactive	weak activity at $\geq 5 \times 10^{-6}$ M	$\geq 2.5 \times 10^{-5}$ M	Ravelo
116: 7,8-dihydro-7α-hydroxy-6-oxoiguestrol	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-5}$ M	Ravelo
117: elemolic acid	5×10^{-4}	inactive	inactive	$\geq 7.5 \times 10^{-4}$ M	Gray
118: 3α-hydroxytiricalla-7,24-dien-21-oic acid	5×10^{-3}	inactive	inactive	$\geq 7.5 \times 10^{-4}$ M	Gray
119:	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Stuppner
120:	10^{-3}	inactive	inactive	at 10^{-3} M	Stuppner
121:	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Stuppner
122: 6-oxopristimerol	10^{-4}	inactive	inactive	$\geq 5 \times 10^{-5}$ M	Ravelo
123: sapindoside B	10^{-3}	inactive	inactive	$\geq 10^{-5}$ M	Yamasaki
124: β-sitosterol	5×10^{-5}	inactive	inactive	inactive	Meng
125: tricuspidoside B	10^{-3}	inactive	inactive	inactive	Yamasaki
126: tubeimoside I	10^{-4}	inactive	inactive	at 10^{-4} M	Yamasaki
127: tubeimoside II	10^{-4}	inactive	inactive	inactive	Yamasaki
128: tubeimoside III	10^{-4}	inactive	inactive	at 10^{-4} M	Yamasaki
129: ursolic acid	10^{-3}	inactive	inactive	at 10^{-3} M	Stuppner
130: zeylasteral	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-5}$ M	Ravelo
131: zeylasterone	10^{-4}	inactive	inactive	at $\geq 10^{-5}$ M	Ravelo
Withasteroids					
132: angunolide	10^{-4}	inactive	weak activity at $\geq 2.5 \times 10^{-7}$ M	$\geq 2.5 \times 10^{-6}$ M	Saatov
133:	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-5}$ M	Habtemariam
134:	10^{-4}	inactive	inactive	inactive	Habtemariam
135:	10^{-4}	inactive	inactive	$\geq 5 \times 10^{-5}$ M	Habtemariam
136:	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-5}$ M	Habtemariam
137: isophysalin B	10^{-4}	inactive	inactive	at 10^{-4} M	Kawai
138: jaborosalactone S	10^{-4}	inactive	inactive	inactive	Burton
139: physalin A	10^{-4}	inactive	inactive	at 10^{-4} M	Kawai
140: physalin B	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Kawai
141: physalin D	10^{-4}	inactive	inactive	inactive	Kawai
142: physalin F	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Kawai
143: physalin O	10^{-4}	inactive	inactive	inactive	Kawai
144: salpichrolide A	10^{-4}	inactive	inactive	$\geq 10^{-6}$ M	Burton
145: withafastuosin B	10^{-5}	inactive	inactive	at 10^{-5} M	Ray
146: withafastuosin C	10^{-5}	inactive	inactive	at 10^{-5} M	Ray

Table 1. (Continued)

Compound	Maximum conc (M)	Agonist activity	Antagonist activity	Cytotoxicity	Supplier
147: withaferoxolide	10^{-4}	inactive	inactive	inactive	Burton
148: withametelin B	10^{-4}	inactive	inactive	at 10^{-4} M	Ray
149: withametelin F	10^{-4}	inactive	inactive	at 10^{-5} M	Ray
150: withametelin H	10^{-4}	inactive	inactive	inactive	Ray
151: withaperuvin D	10^{-3}	active ($EC_{50} = 2.5 \times 10^{-5}$ M)	inactive	$\geq 10^{-4}$ M	Ray
152: withaperuvin G	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Ray
Others					
153: centaurepensin	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Milosavljevic
154: cnicin	10^{-3}	inactive	inactive	$\geq 10^{-4}$ M	Milosavljevic
155: ellagic acid dihydrate	10^{-4}	inactive	inactive	at 10^{-4} M	Lancaster (4281)
156: isolactarorufin	10^{-3}	inactive	inactive	inactive	Daniewski
157: lactarorufin A	10^{-3}	inactive	inactive	inactive	Daniewski
158: lactarorufin B	10^{-3}	inactive	inactive	inactive	Daniewski
159: maocrystal E	10^{-4}	active ($EC_{50} = 5.0 \times 10^{-6}$ M)	inactive	inactive	Meng
160: marasmic triol	10^{-3}	inactive	inactive	at 10^{-3} M	Daniewski
161: megastigm-5-en-4-one 9-glucoside	10^{-4}	inactive	inactive	inactive	isolated at Exeter
162: paniculoside II	10^{-3}	inactive	inactive	inactive	Yamasaki
163: phlomisoside III	10^{-4}	inactive	inactive	$\geq 2.5 \times 10^{-4}$ M	Yamasaki
164: scopoletin	10^{-3}	inactive	inactive	at 10^{-3} M	Aldrich
165: sinigrin	10^{-3}	inactive	inactive	inactive	Sigma (S1647)
166: taxol	10^{-4}	inactive	inactive	$\geq 10^{-5}$ M	Stebbings
167: octadecyl 1-heptyl-oct-1-enyl ketone	10^{-4}	inactive	inactive	inactive	Meng
168: yokonoside methylester	10^{-4}	inactive	inactive	inactive	Murayama

[prepared from the seeds of *Schoenocaulon officinale* (Liliaceae)], did not possess ecdysteroid (ant)agonistic activity. Two other steroidal alkaloids, chaconine (**5**) and solanine (**12**), were also inactive, as were the nonsteroidal alkaloids atropine (**2**), nicotine (**9**), strychnine (**14**), caulerpin (**17**) and yohimine (**18**).

Brassinosteroids. Brassinosteroids have been proposed to act as either ecdysteroid agonists [14] or antagonists [15, 16], based on evidence from in vivo and in vitro experiments with insect systems. The apparent similarity of certain structural features between the ecdysteroids and brassinosteroids has been put forward as the basis of these activities. However, where effects are observed, they occur only at high concentrations ($> 10^{-5}$ M) and almost certainly play no role in insect-plant relationships because brassinosteroids occur in plants in only minute quantities [17]. In the B_{II} assay, none of a significant number of brassinosteroids (**19**–**28**) possessed either specific agonist or antagonist activity, although some did show cytotoxicity at high concentrations ($> 10^{-4}$ M). From a biochemical viewpoint, the structural differences between ecdysteroids and brassinosteroids are major (A/B *trans* ring junction in brassinosteroids vs A/B *cis*-junction in ecdysteroids, different locations and orientations of hydroxyl groups, expanded ring-B in some brassinosteroids), so that discernment by the respective receptors between the two classes of molecules should not be surprising. However, this does provide an opportunity to contribute to understanding the structure-activity relationships of the two classes of substances by generating hybrid analogues and determining the minimal chemical changes required to convert a brassinosteroid structure into an ecdysteroid (ant)agonist and vice versa.

Cardenolides and bufadienolides. Certain cardenolides have been shown to inhibit the growth of the silkworm, *Bombyx mori* [18]. None of the compounds tested here (**29**–**35**) possessed agonistic or antagonistic activity. In addition, the structurally related bufadienolide hellebortin A (**36** [Meng et al., unpublished data]) was also inactive.

Cucurbitacins. Antagonistic cucurbitacins or cucurbitanes have been identified from *Iberis umbellata* [3], *Helmsleya carnosiflora* [4] and *Physocarpus opulifolius* [19]. Since EC_{50} values are not reported for the cucurbitacins discussed in Dinan et al. [3], these are included in table 1 together with the data for the compounds reported for the first time here. With previous results [3] came the postulation that the ecdysteroid antagonist activity of cucurbitacins is associated with the presence of an α,β-unsaturated ketone at C-22 and an oxygen-containing functional group at C-3. This is substantiated by the findings here in so far that **57** and **59**, which do not possess the conjugated system in the side chain were inactive and those possessing a 23-en-22-one were all active. However, **58**, which possesses a Δ^{24} double bond, is also biologically active. Since this is also associated with the absence of a C-25 oxygen-containing function, this may contribute to activity [cf. agonistic ecdysteroids where ponasterone A (25-deoxy-20-hydroxyecdysone) is considerably more potent than 20E; ref. 11].

Insect peptide hormones and neuromodulators. The haemocytes of *D. melanogaster* (from which the B_{II} cell line is derived [20]) play very important roles in defence and during development (especially during metamorphosis), and are expected to be responsive to several hormones and modulators. It was thus worthwhile to

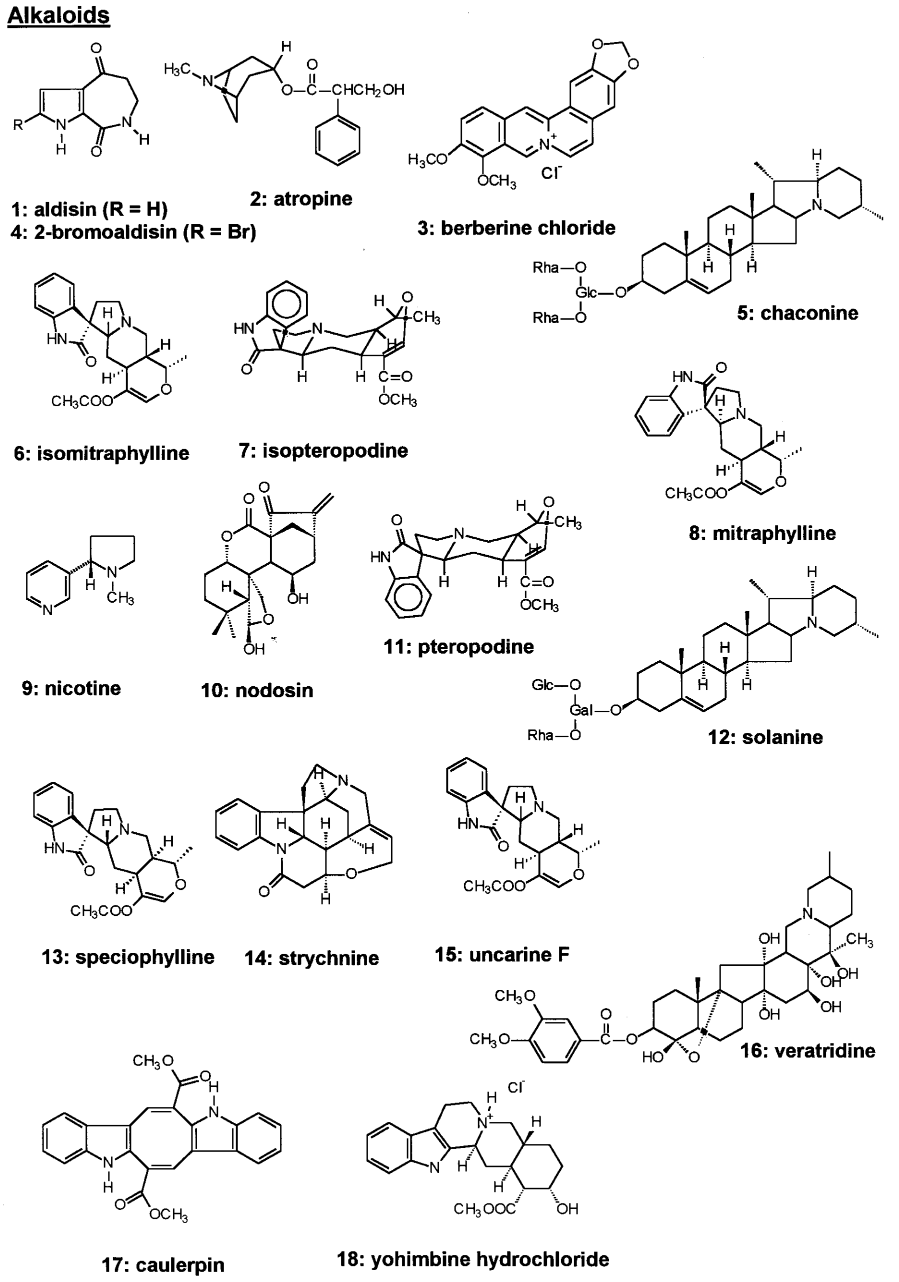
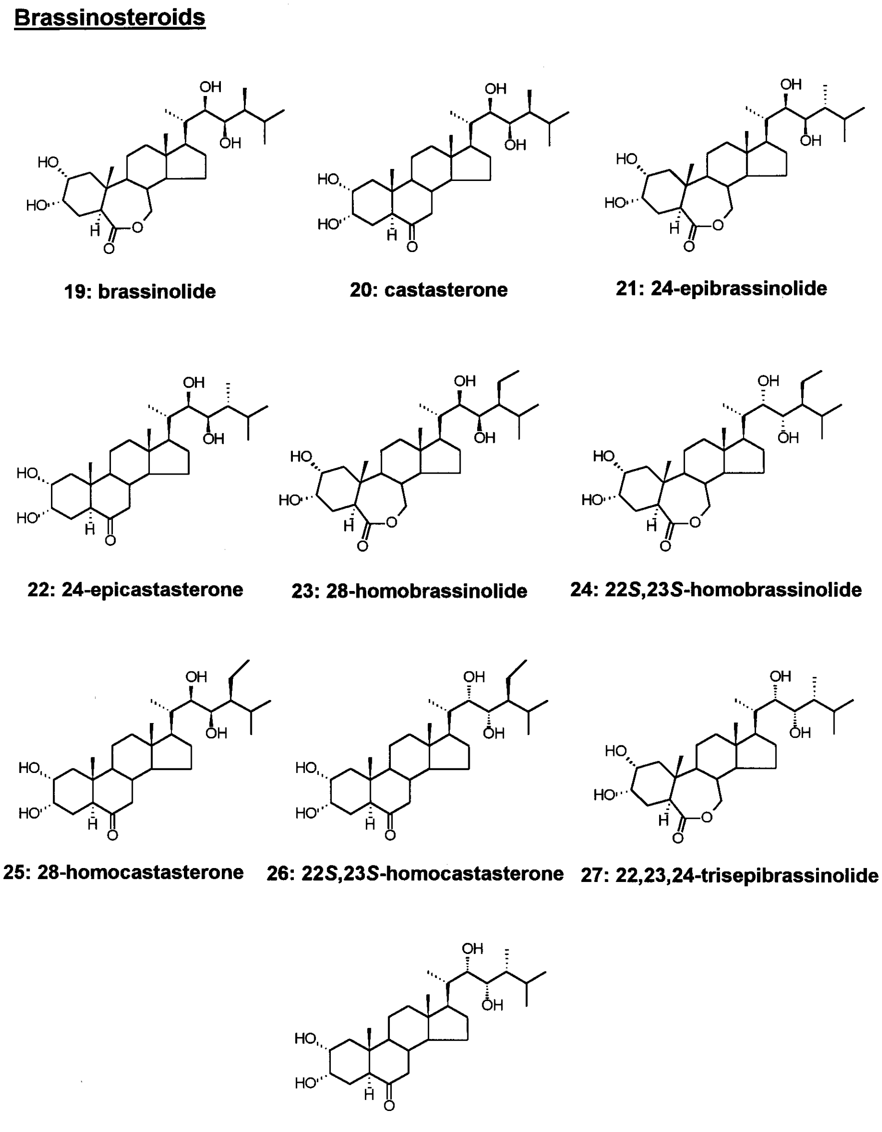


Fig. 1.

determine if any peptides or amino acid derivatives, known to be insect hormones or modulators, could affect ecdysteroid action in the B_{II} cells or produce a response of their own in the cells which might be dramatic enough to develop further bioassays for this activity. None of the tested compounds in these classes (**63**–**69**) produced any observable morphological effect. This does not necessarily mean that the B_{II} cells do not respond to these hormones/modulators, but that the responses operate at the biochemical level and do not manifest themselves as major changes in cell structure. It is also possible that the B_{II} cells rapidly metabolise these types of compounds.

(Iso-)Flavonoids/phyto-oestrogens. A number of these compounds have been shown to interact with vertebrate oestrogen receptors [21]. However, of the compounds assessed here, only daidzein (**72**) and zearalenone (**78**) possessed rather weak activity as ecdysteroid antagonists.

Lignans. The lignan isoamericanin A was previously found to be inactive in the B_{II} bioassay [22]. The three lignans (**79**–**81**) tested here also proved to be inactive as ecdysteroid agonists or antagonists. However, all three were cytotoxic, especially podophyllotoxin (**81**), which is in accord with the pharmacological activity of this potential antineoplastic agent [23].



Brassinosteroids

19: brassinolide

20: castasterone

21: 24-epibrassinolide

22: 24-epicastasterone

23: 28-homobrassinolide

24: 22*S*,23*S*-homobrassinolide

25: 28-homocastasterone

26: 22*S*,23*S*-homocastasterone

27: 22,23,24-trisepibrassinolide

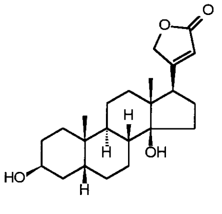
28: 22,23,24-trisepicastasterone

Fig. 1.

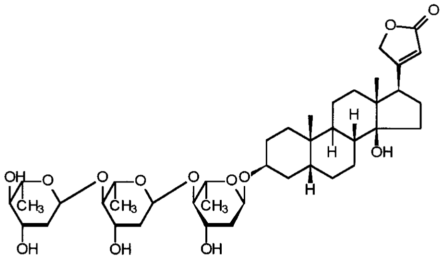
Limonoids. Two limonoids isolated from *Turraea obtusifolia* [6], prieurianin and rohitukin, were found to be responsible for the ecdysteroid antagonistic activity of extracts of the seeds of this species. Azadirachtin (**82**) from *Azadirachta indica* was, however, inactive [2]. Both nomilin (**86**) and obacunone (**87**) possessed weak antagonistic activity, but activity was not strong enough to be able to determine EC_{50} values. A wider range of limonoid structures should be tested, and in ecdysteroid-responsive systems from a range of representative insect species, in order to assess the true significance of this class as ecdysteroid antagonists.

Phenylalkanoids. Phenylalkanoids have a wide range of biological activities, including insecticidal activity [24]. The compounds tested here (**91**–**96**) possessed antagonistic activity, but at rather high concentrations. The most active compound, the phenylalkanoid marginatine (**91**: $EC_{50} = 5.0 \times 10^{-5}$ M), is the least polar compound with the smallest number of oxygen-containing substituents on the aromatic ring. The activities of the

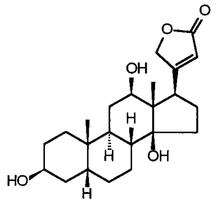
Cardenolides



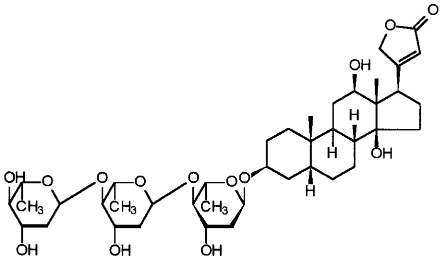
29: digitoxigenin



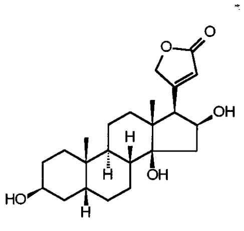
30: digitoxin



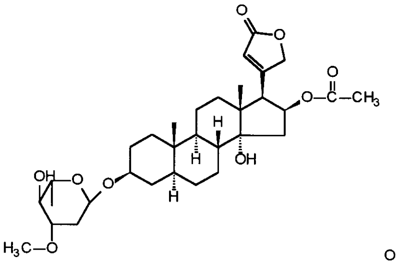
31: digoxigenin



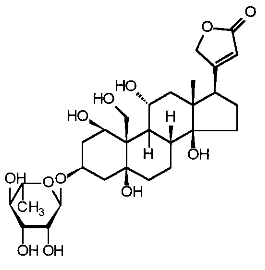
32: digoxin



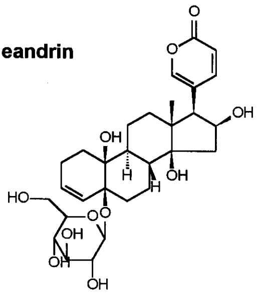
33: gitoxigenin



34:oleandrin



35: ouabain



36: hellebortin A

Fig. 1.

other analogues (phenylpropanoids) are remarkably similar, indicating that the length of the side chain may be a major determinant of activity in the B_{II} assay.

Steroids (non-ecdysteroidal). A wide range of vertebrate-type steroids have proven to be inactive in the B_{II} bioassay, with only androst-4-ene-3,17-dione showing weak antagonistic activity [2; Dinan et al., unpublished data]. Furthermore, two steroidal glycosides from *C. moschata* [25, 26] were inactive. Two bile acids, cholic acid and chenodeoxycholic acid, were also inactive [Dinan et al., unpublished data]. The structure-activity relationship for ecdysteroids in the B_{II} bioassay has been discussed elsewhere [9, 11].

Stilbenoids. Antagonistic stilbenoids have been isolated from *Paeonia suffruticosa* [7], *Iris clarkei* [8] and *Carex pendula* [Meng et al., unpublished data]. The active compounds represent a monomeric stilbene (*cis*-resveratrol) and di-, tri- and tetrameric oligostilbenes. All pos-

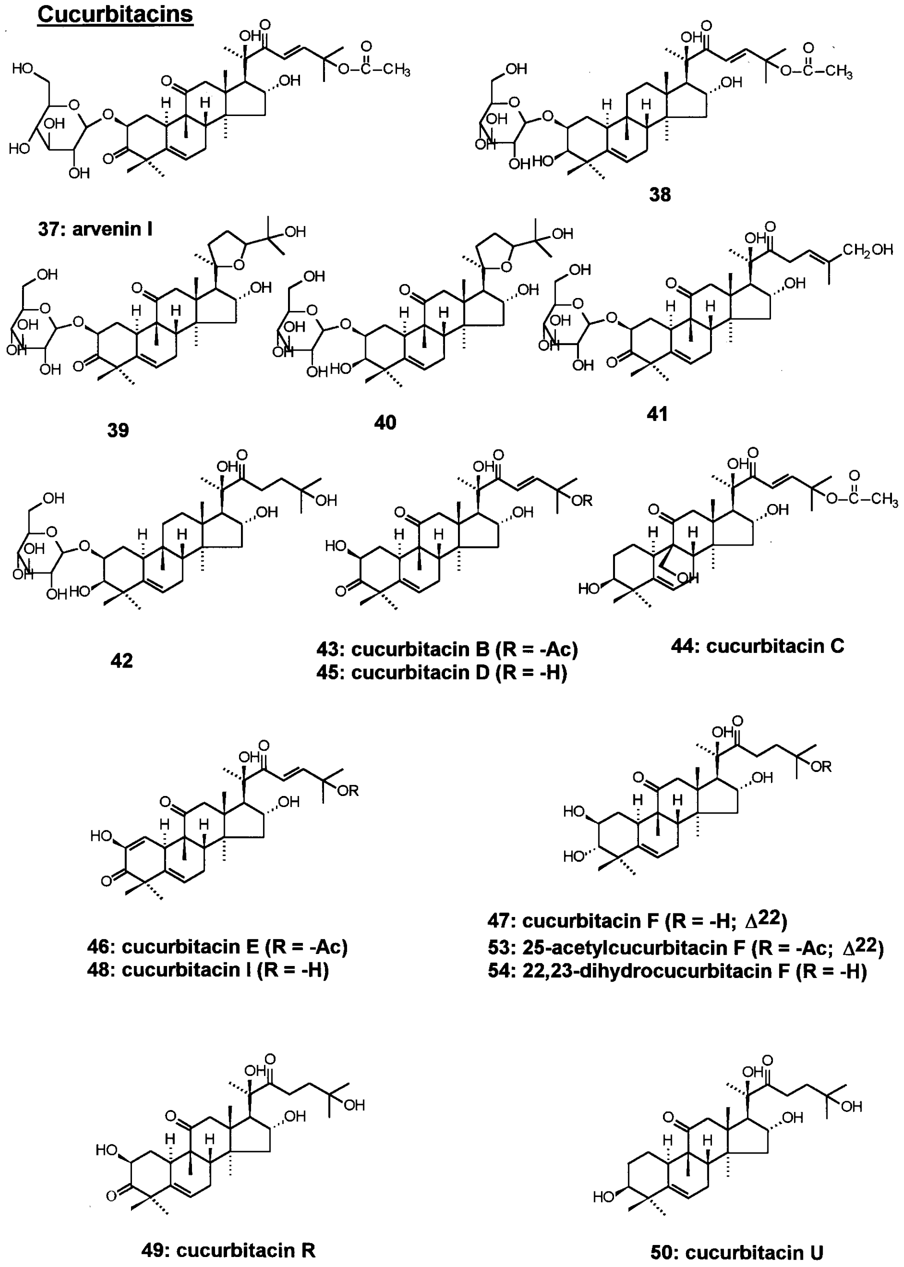
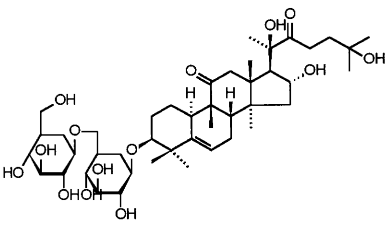


Fig. 1.

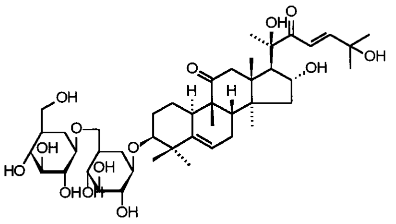
sess significant activity with EC_{50} values of about 10^{-5} M. That molecules of such differing sizes possess similar biological potencies is surprising. However, the stilbenoids from *C. pendula*, at least, have been demonstrated to compete with [^{3}H]ponasterone A (25-deoxy-20-hydroxyecdysone) for the ligand-binding site on the receptor complex [Meng et al., unpublished data].

Triterpenoids and saponins. In view of the activity of ecdysteroids as agonists [11] and cucurbitacins [3] and withanolides [5] as antagonists, the activities of other types of triterpenoid (**97**–**131**) were worth examining. Structurally, these are all very divergent from ecdysteroids, so their lack of agonistic activity was not surprising. Most also possessed no antagonistic activity either, with only chiisanoside (**114**) and demethylzeylasterone (**115**) showing weak activity. None of the compounds possess the stuctural features associated with activity in the cucurbitacins and withanolides.

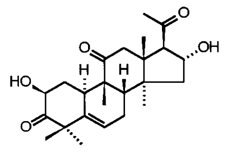
Cucurbitacins cont.



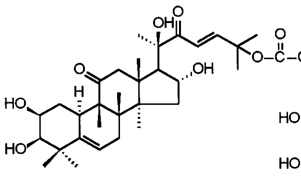
51: cucurbitacin U gentobioside



52: cucurbitacin V gentobioside



55: hexanorcucurbitacin D



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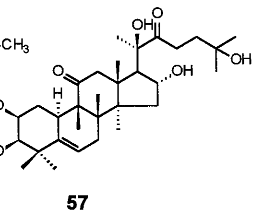

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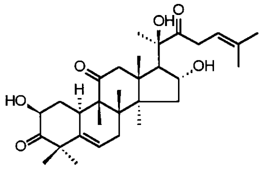

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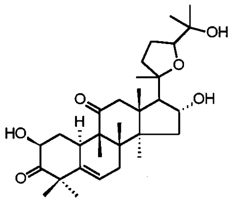

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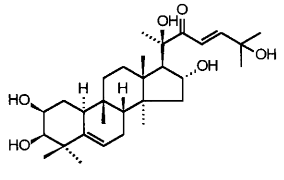
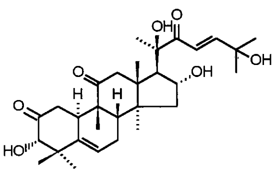
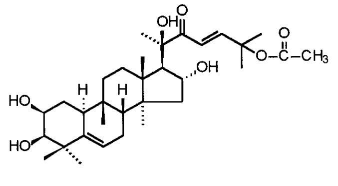


60: deacetylpicracin



61: 3-*epi*-isocucurbitacin D



62: picracin

Fig. 1.

Withasteroids. Several withanolides isolated from *Iochroma gesnerioides* [5] had previously been found to antagonise 20-hydroxyecdysone action on the B_{II} cell line. However, 28-hydroxywithanolide E and 4β-hydroxywithanolide E, from *Physalis peruviana*, were inactive [27]. Empirical examination of the structures of the active withanolides revealed that they possessed an α,β-unsaturated lactone in the side-chain and a 3β-oxygen-containing function. Here, we examined the antagonistic activity of a further 21 withasteroids (**132–152**). Most of these were inactive and none was as active as 2,3-dihydro-3β-hydroxywithacnistine ($EC_{50} = 2.5 \times 10^{-6}$ M), 2,3-dihydro-3β-methoxyiochromolide ($EC_{50} = 5.0 \times 10^{-6}$ M), 2,3-dihydro-3β-methoxywithacnistine ($EC_{50} = 1.0 \times 10^{-5}$ M) or 2,3-dihydro-3β-methoxywithaferin A ($EC_{50} = 3.5 \times 10^{-5}$ M) from *I. gesnerioides*. Angunolide (**132**) possessed weak antagonist activity. The activity of angunolide is surprising because it has neither of the structural features identified previously. However, too much weight should perhaps not be placed on SAR conclusions deriving from very weakly active compounds, owing to the difficulties of differentiating between very weak antagonists and weak cytotoxic agents (both of which generate low densities of small, round cells) in this bioassay.

Insect peptide hormones and neuromodulators

Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2

63: allatostatin IV

Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2

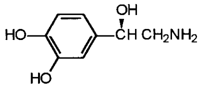
64: leucokinin I

pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2

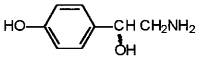
65: leucopyrokinin

Arg-Tyr-Leu-Pro-Thr

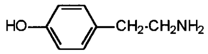
68: proctolin



66: L-noradrenaline

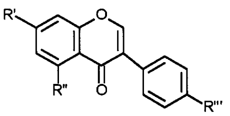


67: DL-octopamine

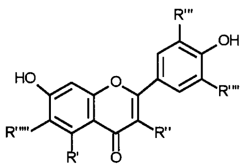


69: tyramine

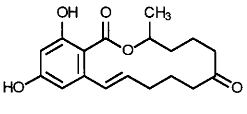
(Iso-)Flavonoids/Phyto-oestrogens



71: biochanin A (R',R'' = -OH; R''' = -OCH$_3$)
72: daidzein (R',R''' = -OH; R'' = -H)
74: genistein (R',R'',R''' = -OH)



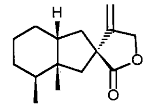
70: apigenin (R' = -OH; R'',R''',R'''',R''''' = -H)
73: fisetin (R',R'''',R''''' = -H; R'',R''' = -OH)
75: luteolin (R',R''' = -OH; R'',R'''',R''''' = -H)
76: myricetin (R',R'',R''',R'''' = -OH; R''''' = -H)
77: quercetin (R',R''' = -H; R'',R'''',R''''' =-OH)



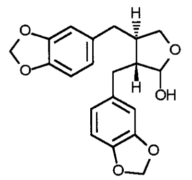
78: zearalenone

Fig. 1.

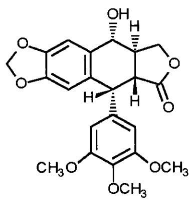
Lignans



79: bakkenolide A

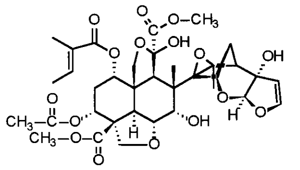


80: cubebin

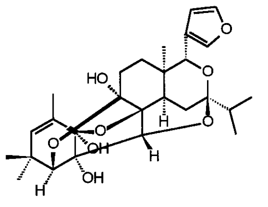


81: podophyllotoxin

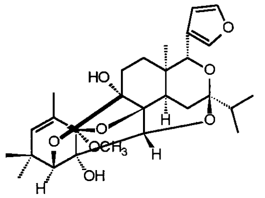
Limonoids



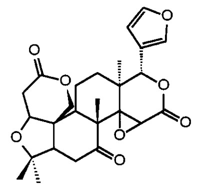
82: azadirachtin A



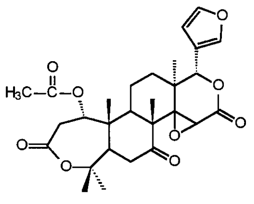
83: entilin A



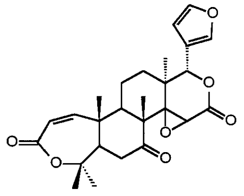
84: entilin C



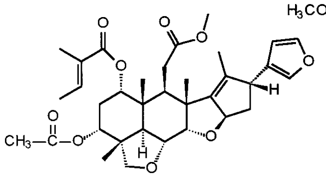
85: limonin



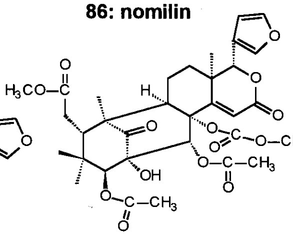
86: nomilin



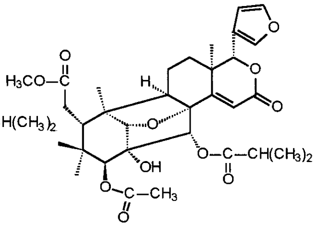
87: obacunone



88: salannin

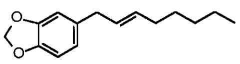


89: utilin B

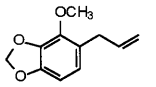


90: utilin C

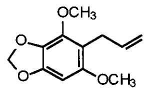
PHENYLALKANOIDS



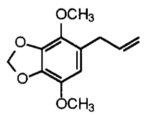
91: marginatine



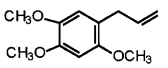
92: croweacin



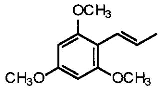
93: 2,6-dimethoxy-3,4-methylenedioxy-1-(2-propenyl)-benzene



94: apiole



95: isoasarone



96: pipermargine

Fig. 1.

The most intriguing finding to come out of the analysis of the withasteroid analogues is the discovery that withaperuvin D (**151**) possessed agonistic activity. This compound has the α,β-unsaturated lactone in the side-chain which is associated with antagonist activity (although the side-chain is linked 17α to the ring system), but also has the very unusual C-3/C-6 epoxide which is probably responsible for imparting the agonistic activity. Further structure-activity studies, together with molecular modelling, are required to resolve this question. Many withanolides are cytotoxic at high concentrations. Of interest is that the cytotoxicities of the four withanolides (**133**–**136**) isolated from *Discopodium penninervium* [28] are remarkably similar to the B_{II} cells as they are to murine macrophage cells [29], both in terms of the concentrations at which cytotoxicity occurs and in the activity sequence: **133** = **136** > **135** > > > **134**.

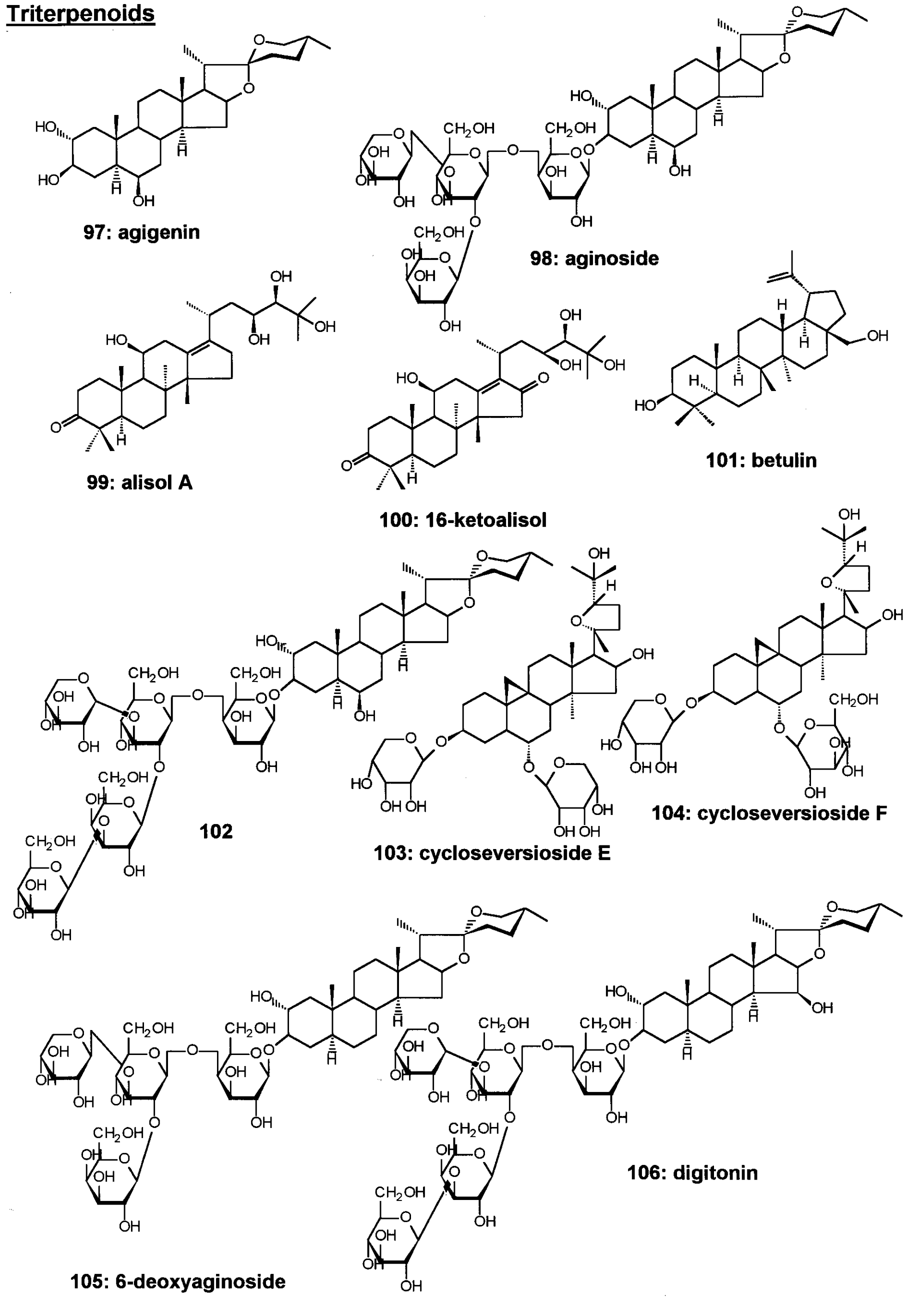
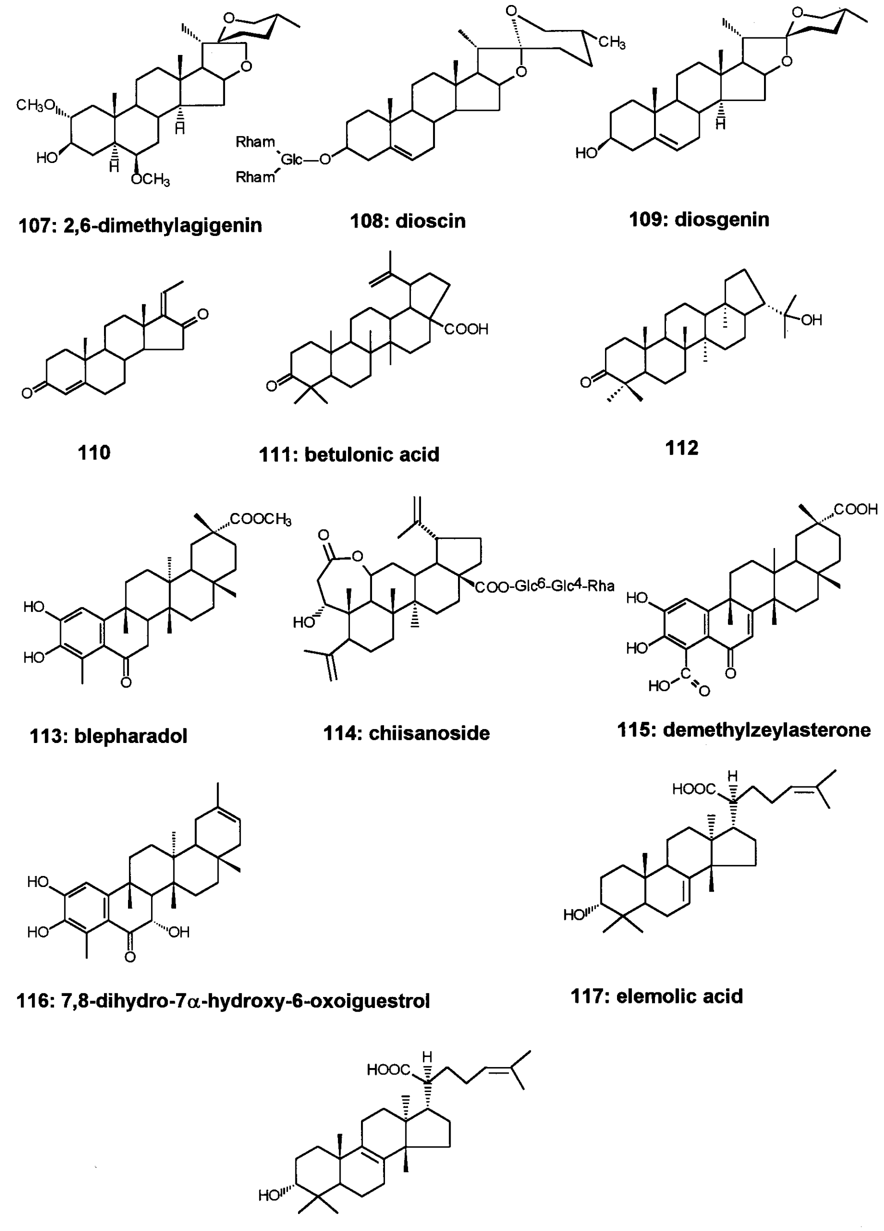


Fig. 1.

Other compounds. Previously, the naphthoquinones, plumbagin and juglone, the chromenes, precocenes I and II, and the sesquiterpene juvenile hormone III were found to be inactive in the B_{II} bioassay [2]. Juvenile hormone III is a natural constituent of certain *Cyperus* spp. [30] and the precocenes, which inhibit juvenile hormone biosynthesis in certain insects, were first isolated from *Ageratum houstonianum* [31]. Among the various compounds (**153–168**) tested here which do not fit into the classes discussed above, only maocrystal E (**159**) was found to be active. This diterpene, which has been isolated from *Isodon* (*Rabdosia*) *eriocalyx* (Labiatae [32]) and *Isodon serra* [Meng et al., unpublished data], possessed significant agonist activity ($EC_{50} = 5.0 \times 10^{-6}$ M).

Competition for the ligand-binding site of the receptor complex. Several of the compounds demonstrating agonist or antagonist activity in the B_{II} bioassay were

Triterpenoids cont.



107: 2,6-dimethylagigenin **108: dioscin** **109: diosgenin**

110 **111: betulonic acid** **112**

113: blepharadol **114: chiisanoside** **115: demethylzeylasterone**

116: 7,8-dihydro-7α-hydroxy-6-oxoiguestrol **117: elemolic acid**

118: 3α-hydroxytricalla-7,24-dien-21-oic acid

Fig. 1.

assessed for their ability to displace [^{3}H]ponasterone A (ponA) from the ligand-binding site of dipteran (*D. melanogaster*) or lepidopteran (*Choristoneura fumiferana*) ecdysteroid receptor (EcR/USP) complexes (fig. 2). Preliminary experiments demonstrated that 2% ethanol in the assay did not affect the level of specifically bound [^{3}H]ponA. The phenylpropanoids were poorly active in the *D. melanogaster* assay, with isoasarone (**95**), pipermargine (**96**) and apiole (**94**) showing only weak displacement at high concentrations. These same compounds were somewhat more active in the *C. fumiferana* assay, but retained the same activity sequence: apiole > pipermargine > isoasarone. The IC_{50} value for apiole (**94**) with the lepidopteran receptor was about 7×10^{-4} M. Marginatine (**91**) was inactive in both assays. Croweacin (**92**) and **93** were not available in

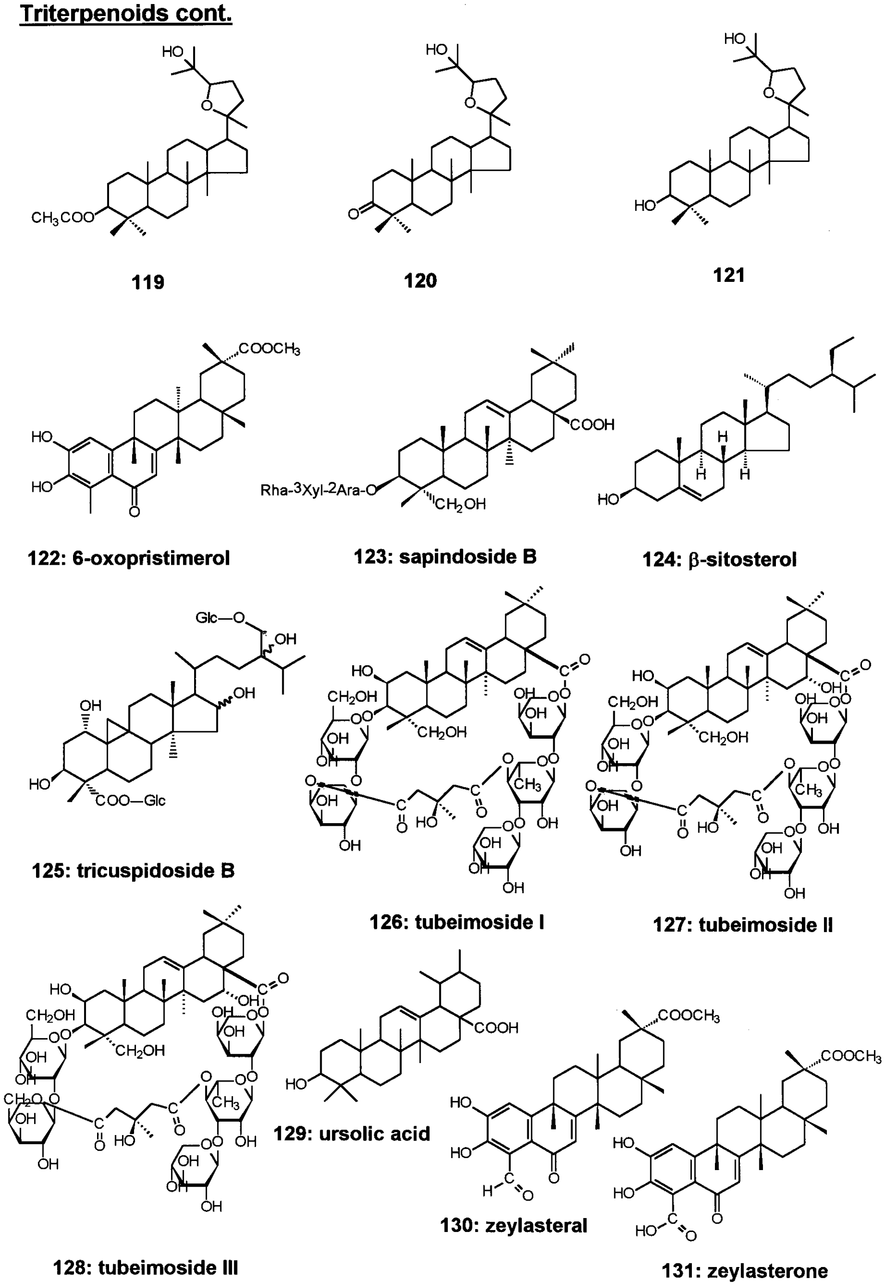


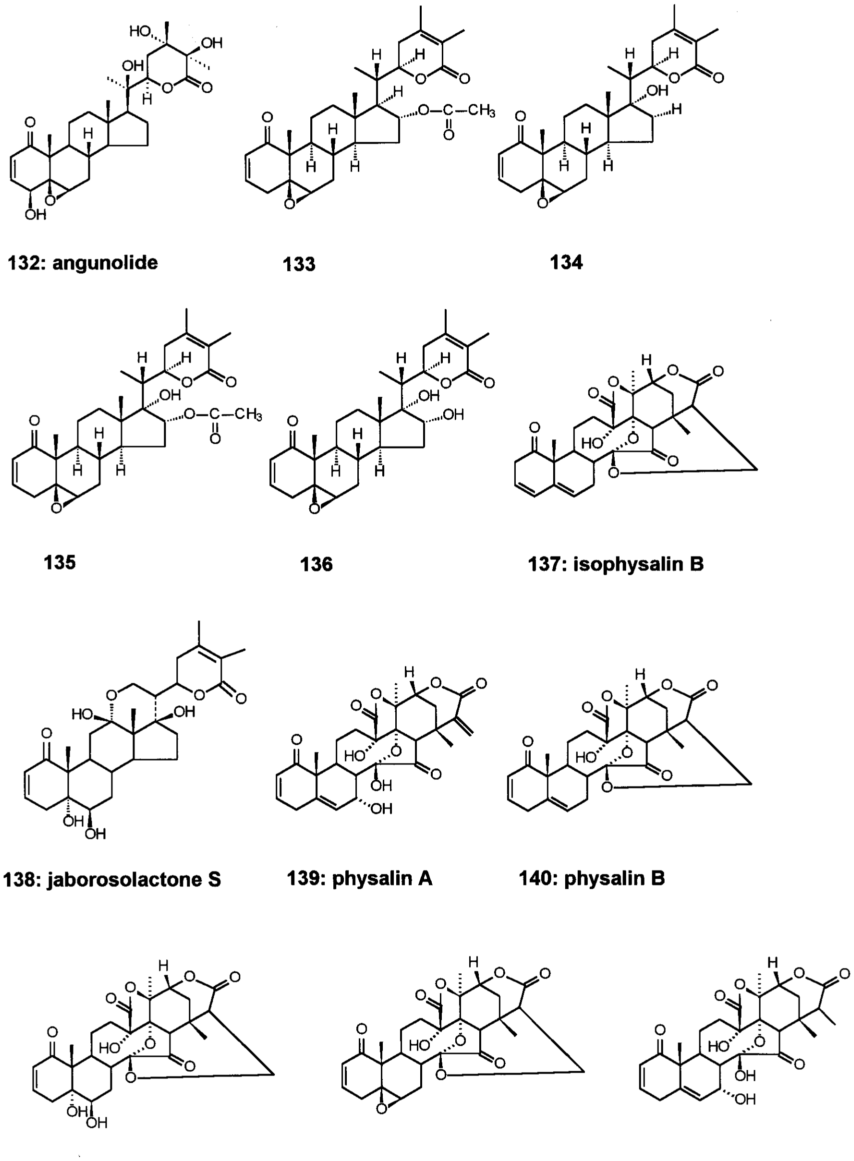
Fig. 1.

sufficient quantities for assay. The activity sequence in the in vitro lepidopteran assay did not correspond to the activity sequence observed in the B_{II} bioassay, where marginatine (**91**) was the most active compound.

In the dipteran assay, isopteropodine (**7**) was active ($IC_{50} = 1.0 \times 10^{-4}$ M, $Ki = 4.3 \times 10^{-5}$ M), which is of the same order as the EC_{50} value in the B_{II} bioassay, while pteropodine (**11**) had no activity even at 10^{-4} M. In the lepidopteran assay, isopteropodine (**7**) was as active ($IC_{50} = 1.0 \times 10^{-4}$ M, $Ki = 8.0 \times 10^{-5}$ M) and pteropodine (**11**) showed weak activity at 10^{-4} M. This contrasts with the B_{II} bioassay, where both compounds had similar activity.

Withaperuvin D (**151**) was active in both assays: $IC_{50} = 6.0 \times 10^{-4}$ M ($Ki = 2.6 \times 10^{-4}$ M) in the dipteran assay and about 1×10^{-3} M in the lepidopteran assay. These activities are at least tenfold less than expected from the B_{II} agonist bioassay. While the lower activity

Withasteroids



132: angunolide **133** **134**

135 **136** **137: isophysalin B**

138: jaborosolactone S **139: physalin A** **140: physalin B**

141: physalin D **142: physalin F** **143: physalin O**

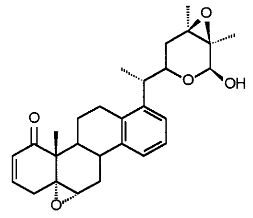
Fig. 1.

in the lepidopteran in vitro assay versus that in the dipteran in vitro assay may be the result of a species difference, the low activity relative to the B_{II} assay indicates that other factors must be important in the potency with intact cells (e.g. metabolism or cellular accumulation). The cucurbitacins deacetylpicracin (**60**) and **58** were inactive in both assays.

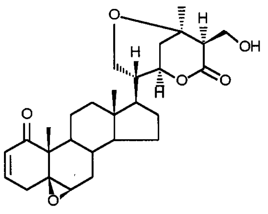
The most active compound was the diterpenoid maocrystal E (**159**), which gave IC_{50} values of 2.0×10^{-5} M ($Ki = 8.6 \times 10^{-6}$ M) in the dipteran assay and 7.2×10^{-5} M ($Ki = 5.8 \times 10^{-5}$ M) in the lepidopteran assay. These values are similar to the EC_{50} value in the B_{II} bioassay and support agonist activity arising through interaction of maocrystal E with the ligand-binding domain of the receptor complex. Hexanorcucurbitacin D (**55**), the other compound which showed agonist activity in the B_{II} bioassay, was also tested with DmEcR/DmUSP and CfEcR/CfUSP and did not compete with [^{3}H]ponasterone A, but the very limited supply of this compound meant that it could only be tested at concentrations up to 10^{-4} M.

The above data provide clearer support for the interaction of the agonists (withaperuvin D and maocrystal E) with the ligand-binding site than they do for the antagonists. The EC_{50} value of an agonist in the B_{II} assay will, in most cases, be directly related to its Kd value for the ligand-binding domain. However, the EC_{50} value of an antagonist will also depend on the concentration and

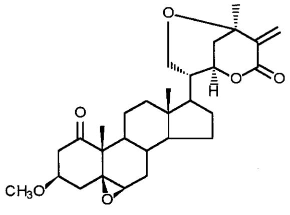
Withasteroids cont.



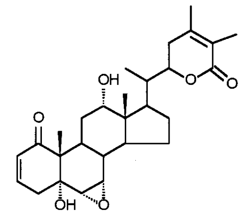
144: salpichrolide A



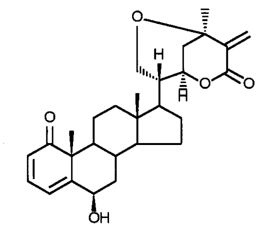
145: withafastuosin B



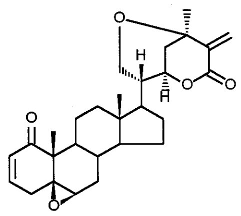
146: withafastuosin C



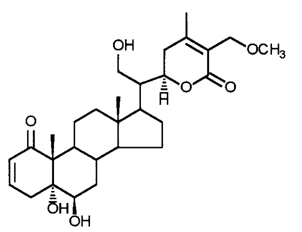
147: withaferoxolide



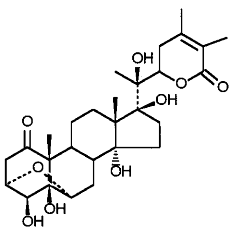
148: withametelin B



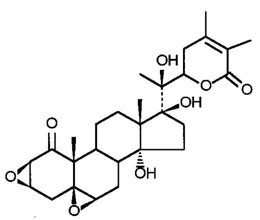
149: withametelin F



150: withametelin H



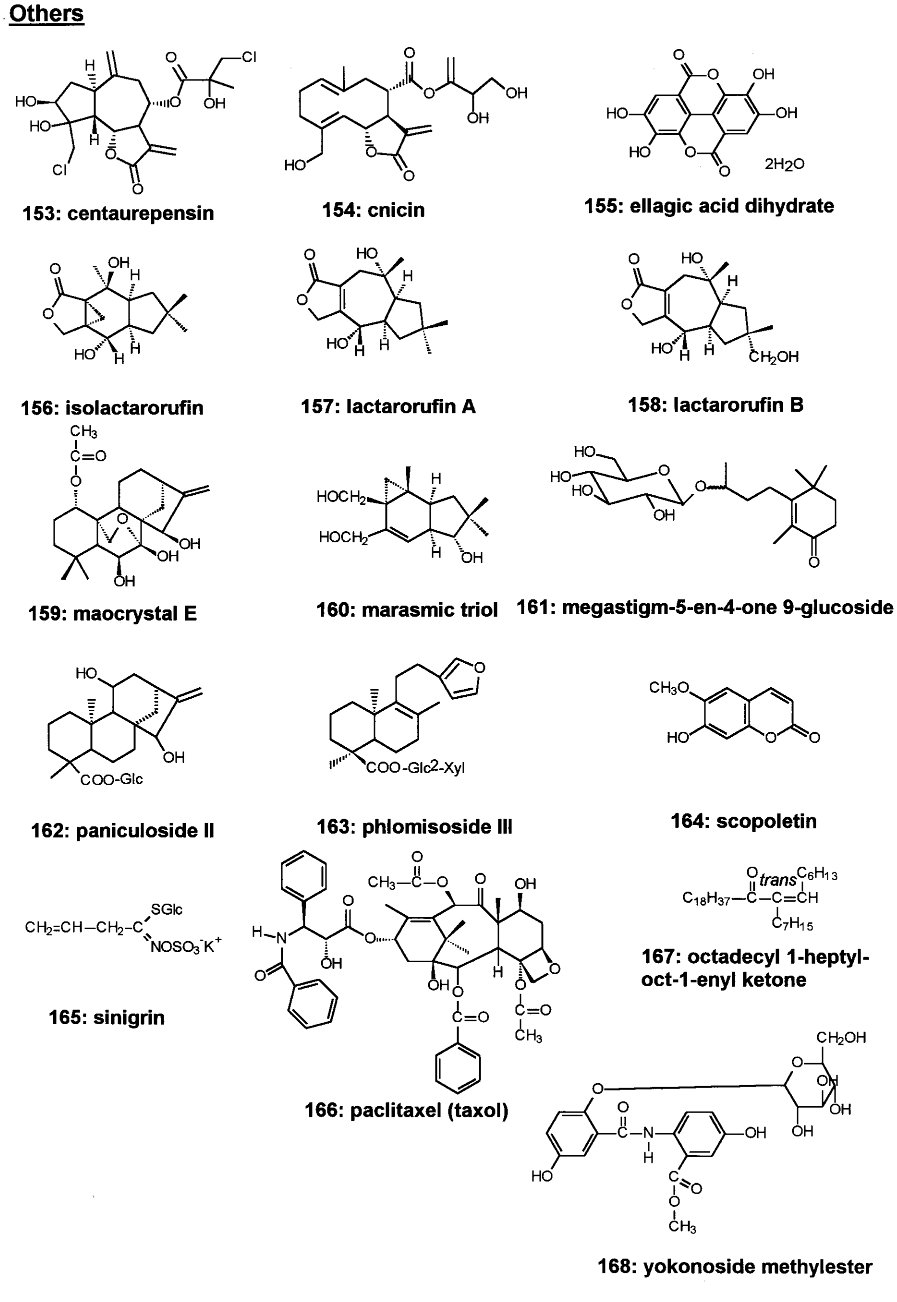
151: withaperuvin D



152: withaperuvin G

Fig. 1.

affinity of the competing ecdysteroid (5×10^{-8} M 20E in the B_{II} assay). Of necessity, one must use a much higher affinity ecdysteroid in the displacement assays (the [^{3}H]ponA used here has a Kd value of 1.5×10^{-10} M with DmEcR/DmUSP and 8.0×10^{-10} M with CfEcR/CfUSP; cf. IC_{50}/Ki values for unlabelled 20E of 1.2×10^{-7} M/5.2×10^{-8} M and 1.6×10^{-7} M/1.3×10^{-7} M with the dipteran and lepidopteran complexes, respectively; [Bourne, unpublished data]). Thus, if an antagonist does interact with the ligand-binding domain, higher concentrations will be required to reveal competition in the EcR/USP assays than might be expected from the EC_{50} in the B_{II} assay. Since solubility becomes a problem with most of these compounds above 10^{-4} or 10^{-3} M in the in vitro assays, no or weak displacement of [^{3}H]ponA should not be taken as



153: centaurepensin

154: cnicin

155: ellagic acid dihydrate

156: isolactarorufin

157: lactarorufin A

158: lactarorufin B

159: maocrystal E

160: marasmic triol

161: megastigm-5-en-4-one 9-glucoside

162: paniculoside II

163: phlomisoside III

164: scopoletin

165: sinigrin

166: paclitaxel (taxol)

167: octadecyl 1-heptyl-oct-1-enyl ketone

168: yokonoside methylester

Figure 1. Structures of the tested compounds.

an inability to compete for the ligand-binding site. Rather, this assay should only be used to categorise compounds which give definite displacement. Other assays (e.g. gel-shift assays) should be used to assess interaction with the receptor complex before it is concluded that an antagonist does not interact with the ligand-binding site.

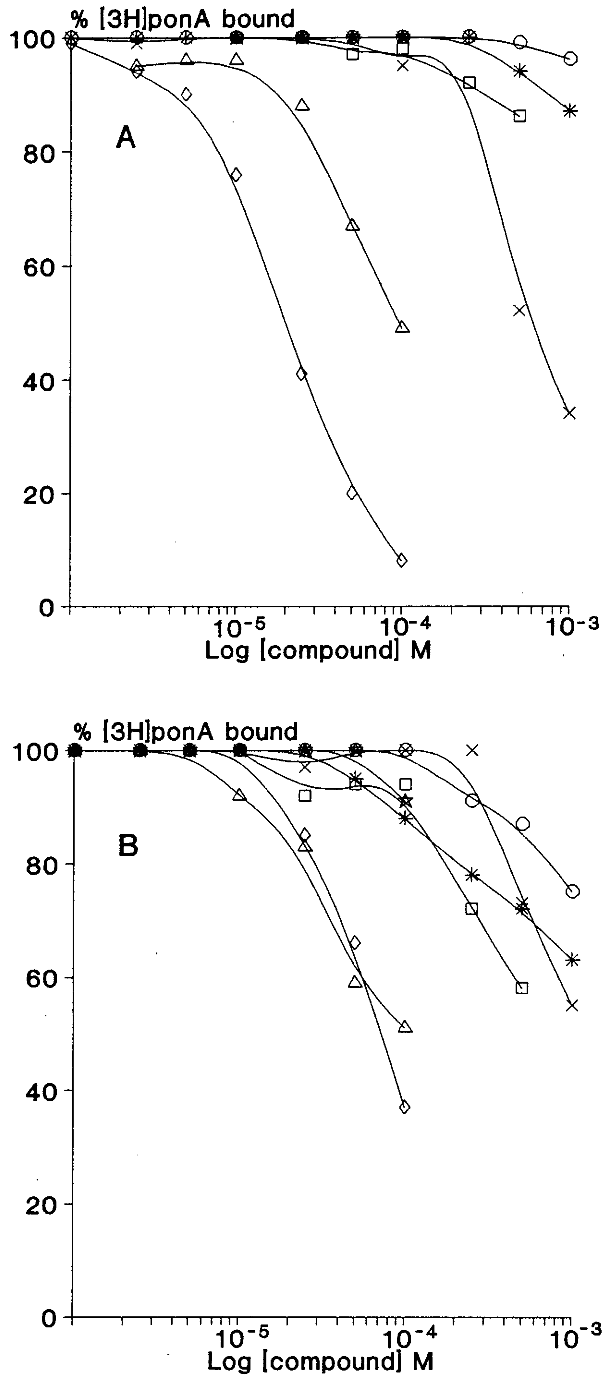


Figure 2. Competition of test compounds with radiolabelled ecdysteroid for the ligand-binding site of the *Drosophila melanogaster* (*A*) and the *Choristoneura fumiferana* (*B*) ecdysteroid receptor complex. Shown are the abilities of maocrystal E (◇), isopteropodine (△), pteropodine (☆), withaperuvin D (×), apiole (□), pipermargine (∗) and isoasarone (○) to compete with [^{3}H]ponasterone A (0.2 nM) for specific binding to EcR/USP. Unbound material was removed by the addition of dextran-coated charcoal.

Lack of agonists. The lack of agonistic compounds among natural products (other than phytoecdysteroids) is somewhat surprising. Although natural agonists do exist in the form of phytoecdysteroids, these are all steroidal analogues of the invertebrate hormones. Ecdysteroid agonist activity has been ascribed to one non-triterpenoid natural product (8-*O*-acetylharpagide [33]), but this was isolated from a plant species (*Ajuga reptans*) which contains high concentrations of ecdysteroids, so whether the activity is inherent in the molecule or derives from contaminating ecdysteroids is not clear. Two types of synthetic non-steroidal ecdysteroidal agonists have been described: bisacylhydrazines [34] and 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutylbenzamide [35]. While non-steroidal agonists may exist in nature, they are clearly rare: our screening of about 5000 species of plants has not identified any non-ecdysteroidal agonists. However, the isolated cases of a cucurbitacin (hexanorcucurbitacin D [3]) and a withanolide (withaperuvin D; this report) show that agonistic analogues can be identified in predominantly antagonistic classes.

Several types of antagonist—several possible sites of action. In contrast to natural agonists, several chemical classes of natural products show antagonistic activity. This can, in part, be ascribed to the nature of the B_{II} bioassay. While agonist activity is almost certainly a consequence of interaction with the ligand-binding site of the ecdysteroid receptor complex and the induction of the normal ecdysteroid-dependent responses, antagonism can be a consequence of interaction at a number of sites in ecdysteroid action, of which the receptor ligand-binding site is only one. Thus, antagonists could (i) interfere with the entry of ecdysteroids into the cells, (ii) interact with the ligand-binding domain of the receptor and prevent ecdysteroid-induced changes in conformation, (iii) interfere with the interaction of EcR and USP, (iv) prevent receptor/DNA interaction or (v) block protein synthesis or morphological responses. Thus, of the different classes of molecules showing antagonistic activity, only a subset will actually interact with the receptor. Initial studies have shown that cucurbitacins [3], stilbenoids [Meng et al., unpublished data] and several industrial chemicals with ecdysteroid antagonist activity [Dinan et al., unpublished data] are able to compete with a radiolabelled ecdysteroid for the ligand-binding site of the receptor complex. More extensive studies on the various classes of antagonists are required to fully elucidate their mode of action. However, molecules possessing very different chemistries are clearly able to interact with the ligand-binding site.

Is the activity biologically significant? Whether the antagonistic activities associated with the various types of molecule are significant in insect/plant relationships is difficult to assess at this stage. The interaction is possibly serendipitous and plays no role in nature. However, these studies do suggest a possible role for these active molecules and suggest further experiments to test their real contribution to insect/plant interactions. Furthermore, the various classes of weak antagonists may interact synergistically with other chemicals to provide adequate protection to a plant.

Why not very potent? That the EC_{50} values for the various types of antagonists are $> 10^{-6}$ M is striking. When plants produce secondary compounds which are not direct analogues of biologically active compounds endogenous to insects for defence purposes, they could, at one extreme, produce a small amount of a compound which is highly active or, at the other extreme, a large amount of a compound with a low activity in order to achieve the same deterrent effect. For the plant, there would be a difference in the energy and resources required for these two strategies. However, if the secondary compounds consist only of carbon, hydrogen and oxygen, then energy from photosynthesis and an adequate supply of the elements are probably not limiting factors. For the insect, there are two pressures: one is to develop a detoxification/avoidance mechanism to deal with the secondary compound and, second, to detoxify the amount present to below toxic levels. Once the insect has developed a detoxification mechanism, the metabolic cost of dealing with a large amount of compound will be more significant than dealing with a small amount. Thus, this would provide a selection pressure for the plant to produce a large amount of less active compound. Since lower affinity is associated with lower specificity, lower-affinity compounds may interact with the ecdysteroid receptors from a wider range of insect/invertebrate species. For compounds which involve nutritionally restricted elements (containing e.g. N or P), a different situation may prevail and the selection pressure would generate lower concentrations of more active compounds. Therefore, most carbon-, hydrogen- and oyxgen-containing compounds from plants can be expected to possess low activity and may only be useful as leads, and not as insecticides in their own rights. Plants from more extreme environments may provide higher-affinity compounds. In cases where the plant secondary compounds are direct analogues of insect chemicals (e.g. phytoecdysteroids), the plant should produce analogues which are of similar affinity to the endogenous insect compounds so that they can compete effectively at the site of action. In addition, the insect will a priori possess metabolic pathways to deal with the endogenous compounds, which would also be effective on the exogenous analogues. This does not preclude the possibility that insects could develop further metabolic options for dealing with exogenous analogues of their own biologically active molecules (as is seen with the inactivation and excretion of phytoecdysteroids as C-22 long-chain fatty acyl esters in tolerant insect species; see Blackford and Dinan [36] and references cited therein), but the existence of these extra pathways would provide a selection pressure for the plant to produce larger amounts of the same or more effective (normally higher-affinity or ones whose structure is not compatible with the insect's detoxification strategy) phytoecdysteroids, or to change to a completely different protection strategy. This concurs with the observations that many ecdysteroid antagonists are only just high affinity (ca 10^{-6} M), while many phytoecdysteroids have considerably higher affinity [11].

Conclusions. Members of several chemically diverse classes of natural products can antagonise the action of 20-hydroxyecdysone on the B_{II} cell line of *D. melanogaster*: cucurbitacins, withanolides, stilbenoids, limonoids, alkaloids and phenylalkanoids. Examples of non-ecdysteroidal natural products possessing agonist activity are rare in nature; in this study, only the diterpene maocrystal E and the withasteroid withaperuvin D were found to act as ecdysteroid agonists in the B_{II} bioassay. Several of the active compounds are able to displace a radiolabelled ecdysteroid from the ligand-binding site of dipteran and lepidopteran ecdysteroid receptor complexes. This extends previous findings that antagonistically active cucurbitacins compete with ecdysteroids for the ligand-binding domain of the ecdysteroid receptor complex.

The majority of active cucurbitacins and withanolides antagonise the action of 20-hydroxyecdysone, but individual analogues of cucurbitacins (hexanorcucurbitacin D) and withanolides (withaperuvin D) demonstrate that certain stuctural modifications result in agonistic activity. Although empirical observation has suggested which structural features of cucurbitacins and withanolides are responsible for antagonistic activity, systematic QSAR studies are required for all classes of antagonists to provide fuller understanding. This is especially true for the alkaloids and phenylalkanoids, where potency and/or activity sequence data between the B_{II} assay and the in vitro EcR/USP assays are not fully in accord.

Natural ecdysteroid antagonists possess predominantly moderate to low affinity for the receptor. Their significance in insect-plant relationships should be investigated further. There may be an evolutionary advantage to plants producing larger amounts of lower-affinity antagonists, rather than smaller amounts of high-affinity ones.

Acknowledgments. This research was supported by the Biotechnology and Biological Sciences Research Council, EU-INTAS (Contract 96-1291) and Rohm & Haas Co. (Spring House, Pa.). The generous donations of natural products are very gratefully acknowledged.

1 Dinan L. (1995) A strategy for the identification of ecdysteroid receptor agonists and antagonists from plants. Eur. J. Entomol. **92:** 271–283

2 Clément C. Y., Bradbrook D. A., Lafont R. and Dinan L. (1993) Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. Insect Biochem. Mol. Biol. **23:** 187–193

3 Dinan L., Whiting P., Girault J.-P., Lafont R., Dhadialla T. S., Cress D. E. et al. (1997) Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. Biochem. J. **328:** 643–650

4 Dinan L., Whiting P., Sarker S. D., Kasai R. and Yamasaki K. (1997) Cucurbitane-type compounds from *Hemsleya carnosiflora* antagonize ecdysteroid action in the *Drosophila melanogaster* B_{II} cell line. Cell. Mol. Life Sci. **53:** 271–274

5 Dinan L., Whiting P., Alfonso D. and Kapetanidis I. (1996) Certain withanolides from *Iochroma gesnerioides* antagonize ecdysteroid action in the *Drosophila melanogaster* cell line. Entomol. Exp. Appl. **80:** 415–420

6 Sarker S. D., Savchenko T., Whiting P., Šik V. and Dinan L. (1997) Two limonoids from *Turraea obtusifolia* (Meliaceae), prieurianin and rohitukin, antagonise 20-hydroxyecdysone action in a *Drosophila* cell line. Arch. Insect Biochem. Physiol. **35:** 211–217

7 Sarker S. D., Whiting P., Dinan L., Šik V. and Rees H. H. (1999) Identification and ecdysteroid antagonist activity of three resveratrol trimers (suffruticosols A, B and C) from *Paeonia suffruticosa*. Tetrahedron **55:** 513–524

8 Keckeis K., Sarker S. D. and Dinan L. (2000) Resveratrol-type oligostilbenes from *Iris clarkei* antagonize 20-hydroxyecdysone action in the *Drosophila melanogaster* B_{II} cell line. Cell. Mol. Life Sci. **57:** 333–336

9 Harmatha J. and Dinan L. (1997) The biological activity of natural and synthetic ecdysteroids in the B_{II} bioassay. Arch. Insect Biochem. Physiol. **35:** 219–225

10 Dinan L., Savchenko T., Whiting P. and Sarker S. D. (1999) Plant natural products as insect steroid receptor agonists and antagonists. Pestic. Sci. **55:** 331–335

11 Dinan L., Hormann R. E. and Fujimoto T. (1999) An extensive ecdysteroid CoMFA. J. Computer-Aided Mol. Design **13:** 185–207

12 Sarker S. D., Savchenko T., Whiting P., Šik V. and Dinan L. N. (1997) Moschamine, *cis*-moschamine, moschamindole and moschamindolol: four novel indole alkaloids from *Centaurea moschata*. Nat. Product Lett. **9:** 189–199

13 Sarker S. D., Dinan L., Šik V., Underwood E. and Waterman P. G. (1998) Moschamide: an unusual alkaloid from the seed of *Centaurea moschata*. Tetrahedron Lett. **39:** 1421–1424

14 Spindler K.-D., Spindler-Barth M. and Turberg A. (1992) Action of brassinosteroids on the epithelial cell line from *Chironomus tentans*. Z. Naturforsch. **47c:** 280–284

15 Hetru C., Roussel J.-P., Mori K. and Nakatani Y. (1986) Activité antiecdystéroïde de brassinostéroïdes. C. R. Acad. Sci. Paris Sér. II **302:** 417–420

16 Lehmann M., Vorbrodt H.-M., Adam G. and Koolman J. (1988) Antiecdysteroid activity of brassinosteroids. Experientia **44:** 355–356

17 Adam G., Schmidt J. and Schneider B. (1999) Brassinosteroids. Prog. Chem. Org. Nat. Products **78:** 1–46

18 Fukuyama Y., Ochi M., Kasai H. and Kodama M. (1993) Insect growth inhibitory cardenolide glycosides from *Anodendron affine*. Phytochemistry **32:** 297–301

19 Sarker S. D., Whiting P., Šik V. and Dinan L. (1999) Ecdysteroid antagonists (cucurbitacins) from *Physocarpus opulifolius* (Rosaceae). Phytochemistry **50:** 1123–1128

20 Gateff E., Gissmann L., Shrestha R., Plus N., Pfister H., Schröder J. et al. (1980) Characterization of two tumorous blood cell lines of *Drosophila melanogaster* and the viruses they contain. In: Invertebrate Systems in vitro, pp. 517–533, Kurstak E., Maramorosch K. and Dübendorfer A. (eds,) Elsevier/North Holland, Amsterdam

21 Kardinaal A. F. M., Waalkens-Berendsen D. H. and Arts C. J. M. (1997) Pseudo-oestrogens in the diet: health benefits and safety concerns. Trends Food Sci. Technol. **8:** 327–333

22 Sarker S. D., Šik V. and Dinan L. (1998) Isoamericanin A: a neolignan from *Trianthema turgidifolia*. Biochem. Syst. Ecol. **26:** 681–683

23 Canel C., Moraes R. M., Dayan F. E. and Ferreira D. (2000) Podophyllotoxin. Phytochemistry **54:** 115–120

24 Perrett S. and Whitfield P. J. (1995) Anthelmintic and pesticidal activity of *Acorus gramineus* (Araceae) is associated with phenylpropanoid asarones. Phytother. Res. **9:** 405–409

25 Sarker S. D., Dinan L., Šik V. and Rees H. H. (1998) Moschatine: an unusual steroidal glycoside from *Centaurea moschata*. Phytochemistry **48:** 1039–1043

26 Sarker S. D., Lafont R., Girault J.-P., Šik V. and Dinan L. (1998) (20*R*)-15α-Hydroxy-8β,9α,14α,17α-pregn-4-en-3-one 20-*O*-β-D-glucopyranoside: a minor constituent of the seeds of *Centaurea moschata*. Pharm. Biol. **36:** 202–206

27 Dinan L., Sarker S. D. and Šik V. (1997) 28-Hydroxywithanolide E from *Physalis peruviana*. Phytochemistry **44:** 509–512

28 Habtemariam S., Gray A. I. and Waterman P. G. (1993) 16-Oxygenated withanolides from the leaves of *Discopodium penninervium*. Phytochemistry **34:** 807–811

29 Habtemariam S. (1997) Cytotoxicity and immunosuppressive activity of withanolides from *Discopodium penninervium*. Planta Med. **63:** 15–17

30 Toong Y. C., Schooley D. A. and Baker F. C. (1988) Isolation of insect juvenile hormone III from a plant. Nature **333:** 170–171

31 Bowers W. S. (1983) The precocenes. In: Invertebrate Endocrinology, vol. 1, Endocrinology of Insects, pp. 517–521, Downer R. G. H. and Laufer H. (eds), Liss, New York

32 Li C., Zhou J. and Sun H. (1985) Structures of new diterpenoids, maocrystal A–E from *Rabdosia eriocalyx*. Acta Bot. Yunn. **7:** 115–116

33 Elbrecht A., Chen Y., Jurgens T., Hensens O. D., Zink D. L., Beck H. T. et al. (1996) 8-*O*-Acetylharpagide is a nonsteroidal ecdysteroid agonist. Insect Biochem. Mol. Biol. **26:** 519–523

34 Hsu A. C.-T., Fujimoto T. T. and Dhadialla T. S. (1997) Structure-activity study and conformational analysis of RH-5992, the first commercialized nonsteroidal ecdysone agonist. ACS Symp. Ser. **658:** 206–219

35 Mikitani K. (1996) A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide shows apparent insect moulting hormone activities at molecular and cellular levels. Biochem. Biophys. Res. Commun. **227:** 427–432

36 Blackford M. and Dinan L. (1997) The tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) detoxifies ingested 20-hydroxyecdysone, but is susceptible to the ecdysteroid agonists RH-5849 and RH-5992. Insect Biochem. Mol. Biol. **27:** 167–177