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Ecdysteroid antagonists (cucurbitacins) from *Physocarpus opulifolius* (rosaceae)

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Abstract

Methanolic extracts of seeds of 4 species in the genus *Physocarpus* antagonise the action of the insect steroid hormone 20-hydroxyecdysone on a *Drosophila melanogaster* permanent cell line. The active components in the extract of *P. opulifolius* (ninebark) have been identified as cucurbitacin D, cucurbitacin F and 3-*epi*-isocucurbitacin D. The potencies of the individual cucurbitacins have been determined as 5×10^{-7}, 8×10^{-7} and 7×10^{-6} M, respectively (versus 5×10^{-8} M 20-hydroxyecdysone). The distribution of antagonistic activity in plants of *P. opulifolius* has been assessed and HPLC/bioassay has been used to determine the chromatographic profiles of antagonist activity in parts of the growing plant.

Keywords: *Physocarpus opulifolius*; Rosaceae; Ninebark; Cucurbitacin D; 3-*epi*-Isocucurbitacin D cucurbitacin F; Ecdysteroid antagonist; Chemotaxonomy

1. Introduction

Many plant secondary compounds have been shown to affect insect growth, development and/or fecundity and are consequently believed to play a role in the deterrence of insect predators. However, the molecular modes of action of very few of these compounds have been elucidated. One class of secondary compounds which have been postulated to contribute to the deterrence of non-adapted phytophagous insects are the phytoecdysteroids, which are analogues of insect steroid hormones. Over 150 structurally-related phytoecdysteroids have been isolated from a wide variety of plant species (Lafont & Wilson, 1996). All phytoecdysteroids act as hormone agonists of the insect ecdysteroid receptor (Dinan, Hormann, & Fujimoto, 1998). There is growing interest in the receptor as a target for the development of invertebrate-specific pest control agents. To exploit this target it is important to characterise the ligand-binding site and to identify novel agents interacting specifically with the receptor, either as agonists (such as the phytoecdysteroids) or as antagonists. We have screened extracts of >4000 species of plants for the presence of ecdysteroid receptor agonists and antagonists using a simple and effective bioassay (Clément, Bradbrook, Lafont, & Dinan, 1993; Dinan, 1995). One of the genera possessing distinct antagonistic activity was *Physocarpus*. In this paper, we report the isolation of the active constituents in *P. opulifolius* (L.) Maxim., commonly known as ninebark. Using bioassay-guided HPLC purification, we isolated the active compounds and identified them as three cucurbitacins: cucurbitacin D (**1**), cucurbitacin F (**2**) and 3-*epi*-isocucurbitacin D (**3**). Cucurbitacins are reported for the first time from *P. opulifolius* and have only occasionally been isolated from members of the Rosaceae previously.

2. Results and discussion

Although the presence of ecdysteroid agonists (phytoecdysteroids) in plants was first demonstrated over 30 years ago, no definitive ecdysteroid antagonists had been identified until recently, when it was demonstrated that cucurbitacins from *Iberis umbellata* (Cruciferae (Dinan et al., 1997)) and *Cercidiphyllum japonicum* (Cercidiphyllaceae (Sarker, Whiting, Sik, & Dinan, 1997)) and certain withanolides from *Iochroma gesnerioides* (Solanaceae (Dinan, Whiting, Alfonso, & Kapetanidis, 1996)) were active. Further species showing significant

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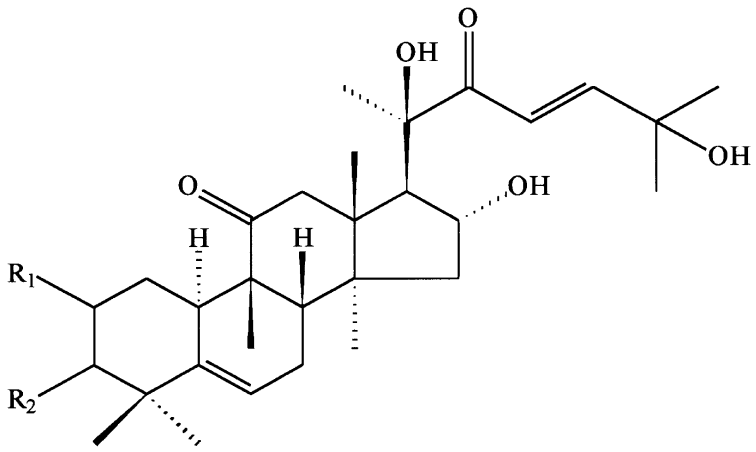

	R_1	R_2
Cucurbitacin D (1)	-OH (β)	= O
Cucurbitacin F (2)	-OH (β)	-OH (α)
3-*Epi*-cucurbitacin D (3)	= O	-OH (β)

Structure 1.

antagonistic activity were found in the genus *Physocarpus*, which was worth investigating in greater detail as it belongs to a separate family and was not known to accumulate either cucurbitacins or withanolides.

Antagonist activity was present in extracts of seeds of all four species tested (Table 1). Although the activity in seeds of *P. malvaceus* was higher, seeds of *P. opulifolius* were more readily available in larger amounts, so the active principles were isolated from this source. *P. opulifolius* had not been the subject of phytochemical investigations before. Initial chromatographic analysis by RP-HPLC/bioassay (Fig. 1A) revealed that the antagonist activity coincided with three closely eluting UV-absorbing peaks. These were purified by sequential preparative HPLC on a C_8 column and semipreparative HPLC on a C_{18} column to yield pure **1** and **2**. To obtain **3** in a pure form, it was necessary to also use a third HPLC purification on silica.

Cucurbitacins **1**, **2** and **3** were unambiguously identified on the basis of co-chromatography (HPLC), UV and a series of 1D and 2D NMR experiments including ^{1}H-NMR, ^{13}C-PENDANT (Homer & Perry, 1994), ^{1}H–

Table 1
Assessment of *Physocarpus* spp. for ecdysteroid agonist and antagonist activities

		Radioimmunoassay		Bioassay	
Species	Part	Black	DBL-1	Agonist	Antagonist
Physocarpus intermedicus[a]	seeds	–	–	–	+
Physocarpus malvaceus[b]	seeds	–	0.7	C	++
Physocarpus monogynus[a]	seeds	–	–	–	+
Physocarpus opulifolius[c]	seeds	–	–	–	+
Physocarpus opulifolius[b]	seeds	–	–	–	+
Physocarpus opulifolius[b]	leaves	nd	–	–	++
Physocarpus opulifolius[b]	stems	nd	–	–	+
Physocarpus opulifolius[b]	roots	nd	–	–	+(+)

Source: [a]Gruga Park, Essen, F.R.G., [b]B&T World Seeds, [c]Chiltern Seeds.RIA values are given as μg ecdysone equivalents/g seed; – signifies below the detection limit: nd = not determined.

Bioassay results: 20 μl aliquots were assessed; + = active as neat extract, ++ = active at 10-fold dilution, − signifies not active: C signifies cytoxicity with the neat extract.

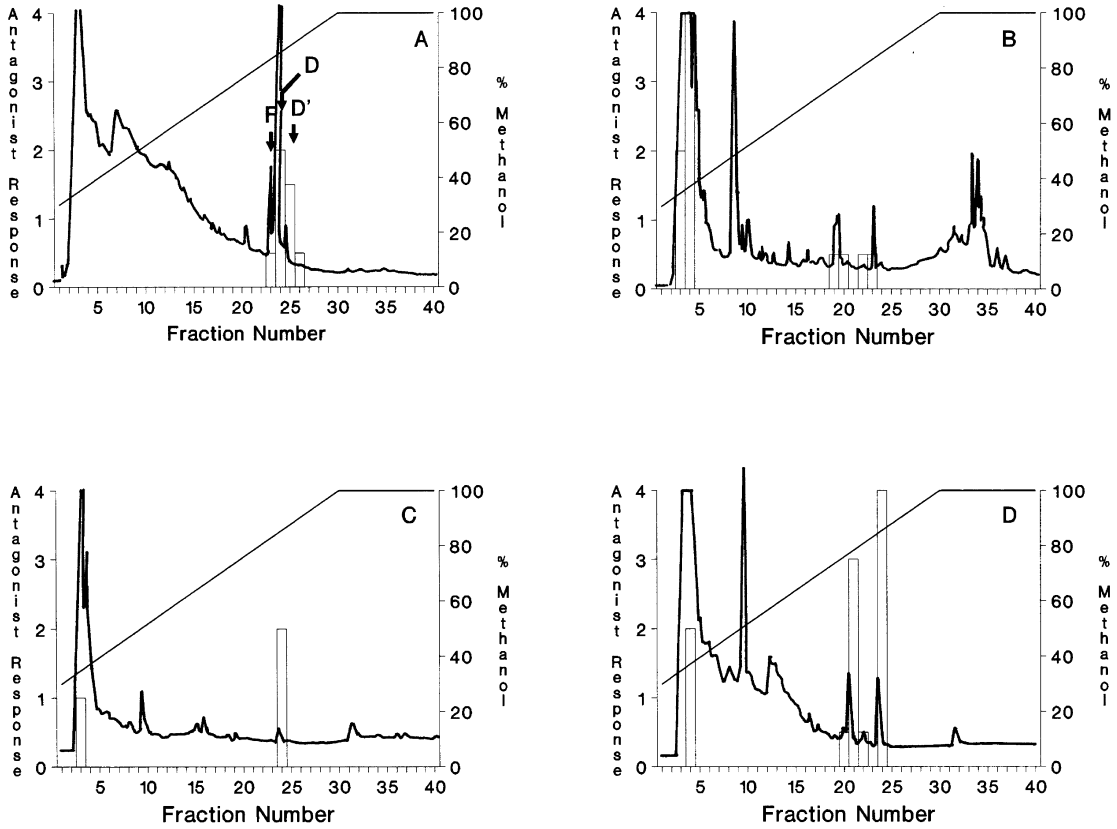

Fig. 1. HPLC/bioassay of a methanolic extract of seeds and plants of *Physocarpus opulifolius*. Portions of the extracts (equivalent to 3 mg d.w.) were separated on a C_{18} reversed-phase analytical column eluted with a gradient of methanol in water at 1 ml/min^{-1} (monitored at 242 nm): (A) seeds, (B) leaves, (C) stems and (D) roots. Fractions of 1 min duration were collected and aliquots (20 μl for seeds, 40 μl for the others) were take for the antagonist version of the B_{II} bioassay. The cellular response is measured turbidometrically at 405 nm. An antagonist response unit corresponds to $(A_{control} - A_{20E})/5$, where $A_{control}$ is the A_{405} value of control cells and A_{20E} is the A_{405} value for cells grown in the presence of 5×10^{-8} M 20-hydroxyecdysone. The arrows in panel A indicate the elution times of the cucurbitacins: cucurbitacin F (F), cucurbitacin D (D) and 3-*epi*-isocucurbitacin D (D′).

^{1}H COSY45, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY. The observed UV and NMR data were also in good agreement with the published data for these compounds (Dreyer & Trousdale, 1978; Kupchan, Meshulam, & Sneden, 1978; Fang, Phoebe, Pezzuto, Fong, & Farnsworth, 1984; Che et al., 1985; Hylands & Magd, 1986; Jacobs, Singh, Reynolds, & McLean, 1990; Konoshima et al., 1993; Halaweish, 1993; Dinan et al., 1997). Complete and unambiguous assignments obtained from detailed 1D and 2D NMR experiments on compounds **2** and **3** are presented for the first time (Table 2).

The antagonistic activities (ED_{50} values) of purified **1**, **2** and **3** to 20-hydroxyecdysone (5×10^{-8} M) on a *Drosophila* cell line are 5×10^{-7}, 8×10^{-7} and 7×10^{-6} M, respectively. All three compounds are cytotoxic at concentrations $> 10^{-5}$ M. Two major regions of interaction of cucurbitacins with the ecdysteroid receptor complex have been proposed previously (Dinan et al., 1997): the 22-oxo-Δ^{23} functional grouping in the side-chain and the A-ring. All three of the cucurbitacins isolated from seeds of *P. opulifolius* share a common side-chain structure possessing the active functional grouping, but differ in the arrangement of the oxygen-containing substituents on the A-ring. Interestingly, the presence of either a 3-oxo group (**1**) or a 3α-OH (**2**) together with a 2β-OH does not markedly change the activity, but transposition of the oxo-group to C-2 in conjunction with a 3α-OH (**3**) reduces activity significantly. The possibility that the three cucurbitacins interact synergistically was tested by comparing the activity of the individual compounds with that of a 73.0:14.8:7.3 mixture (**1**:**2**:**3**), which corresponds to the molar ratio of the three compounds found in the seed extract. Since the dose dependency was very similar to that to the major component (**1**) alone (data not shown), this implies that the activities of the three cucurbitacins are additive rather than synergistic.

Cucurbitacin triterpenoids, which are typical of the family Cucurbitaceae (Kasai, Matsumoto, Nie, Zhou, & Tanaka, 1988; Jacobs et al., 1990), have also been reported from several plant species belonging to the families Begoniaceae (Doskotch, Malik, & Beal, 1969), Cercidiphyllaceae (Sarker et al., 1997), Cruciferae (Yam-

Table 2
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of **2** and **3** (coupling constant *J*, Hz in parentheses)

Carbon No.		δ_H		δ_C	
		2	**3**	**2**	**3**
1	α	1.92	2.50 dd (19.2, 4.5)	33.3	36.4
	β	1.10	2.21 dd (19.4, 13.6)		
2	α	3.60 ddd (4.3, 9.2, 11.4)	–	71.4	210.9
3	α	–	4.13 s	81.0	
	β	2.98 d (9.2)	–		79.5
4		–	–	41.9	40.9
5		–	–	140.7	140.1
6		5.73 m	5.90 m	119.2	
7	α	1.92	2.05	23.8	23.7
	β	2.40	2.40		
8	β	1.95	2.00	42.6	42.4
9		–	–	48.3	47.9
10	α	2.38	2.98 bdd	34.0	32.4
11		–	–	212.8	212.4
12	α	3.19 d (15.1)	3.13 d (14.8)	48.6	48.7
	β	2.63 d (14.8)	2.66 d (14.6)		
13		–	–	48.2	48.4
14		–	–	50.9	50.7
15	α	1.38	1.42	45.4	45.6
	β	1.85 dd (13.0, 9.0)	1.84 dd (13.0, 9.1)		
16	β	4.38 m	4.41 t (7.5)	71.1	71.3
17	α	2.53 d (6.8)	2.54 d (6.7)	57.6	57.6
18 (Me)	β	0.97 s	0.98 s	19.8	19.9
19 (Me)	β	1.11 s	1.09 s	20.3	19.1
20		–	–	78.2	78.1
21 (Me)	α	1.41 s	1.41 s	23.9	23.9
22		–	–	203.0	203.0
23		6.66 d (15.2)	6.66 d (15.2)	119.1	119.1
24		7.10 d (15.2)	7.10 d (15.2)	155.4	155.5
25		–	–	71.2	71.2
26 (Me)		1.38 s	1.38 s	29.5	29.5
27 (Me)		1.38 s	1.38 s	29.3	29.3
28 (Me)	α	1.29 s	1.37 s	19.1	18.5
29 (Me)	α	0.96 s	1.31 s	21.5	27.7
30 (Me)	β	1.20 s	0.86 s	24.6	24.4

Assignments were confirmed by ^{1}H–^{1}H COSY45, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY experiments.

ada, Hagiwara, Iguchi, Suzuki, & Hsu, 1978; Sarker, Lafont, Sik, & Dinan, 1997), Elaeocarpaceae (Fang et al., 1984), Primulaceae (Yamada et al., 1978), Rubiaceae (Mata, Castaneda, Camacho, & Delgado, 1988), Saxifragaceae (Arisawa, Hatashita, Numata, Tanaka, & Sasaki, 1997), Scrophulariaceae (Stuppner & Wagner, 1989) and Sterculiaceae (Bean et al., 1985). Within the family Rosaceae, cucurbitacins have previously been isolated only from *Cowania mexicana* (Konoshima et al., 1993) and *Purshia tridentata* (Dreyer & Trousdale, 1978), this is the first report on the occurrence of such compounds in the genus *Physocarpus*. Apart from important pharmacological properties (Stuppner & Wagner, 1989), cucurbitacins have been reported to have insect attractant, repellent (Miró, 1995) or antifeedant (Sachdevgupta, Radke, & Renwick, 1993) activities. Most recently, the ecdysteroid antagonistic activity of cucurbitacins isolated from *Iberis* ssp. (Sarker et al., 1997) and of some cucurbitane-type compounds from *Hemsleya carnosiflora* have been reported (Dinan, Whiting, Sarker, Kasai, & Yamasaki, 1997).

Antagonistic activity is present throughout plants of *P. opulifolius* Table 1, with the highest levels present in leaves and with lower levels in roots and stems. The levels of antagonistic activity present in plant parts are equal to or greater than that present in seeds. However, Fig. 1 shows that the antagonist profiles of the plant parts differ from each other and from that of seeds. The root extract (Figure 1D), in addition to an activity peak in fraction 24 corresponding to the cucurbitacins present in seeds, possesses further activity peaks in fraction 21 (corresponding to a distinct UV-absorbing peak) and fraction

4. In stems (Figure 1C), a polar activity peak is observed in fraction 3 in addition to the peak in fraction 24. Most of the activity in the leaf extract (Figure 1B) is present as polar material in fractions 3 and 4. It is possible that this polar material represents the cucurbitacins in a conjugated (glycosylated?) form. This distribution would be consistent with the hypothesis that the cucurbitacins are synthesised in the roots, translocated in the stem and stored in the leaves in a conjugated form. This example also demonstrates the utility of the B_{II} bioassay for monitoring the presence and levels in small plant samples of cucurbitacins, for which there are currently no immunoassays.

3. Experimental

3.1. General

UV spectra were obtained in MeOH. HPLC was performed on Gilson equipment consisting of two model 303 pumps, a Holochrome detector (set at 230 or 242 nm) and controlled by the Gradient Manager program. NMR spectra were obtained in $CDCl_3$ on a Bruker AVANCE DRX400 instrument using standard Bruker microprograms. Chemical shifts are expressed in ppm.

3.2. Biological material

Seeds of *Physocarpus* spp. were donated by Gruga Park, Essen, F.R.G. or purchased from Chiltern Seeds, Ulverston, Cumbria, UK and B&T World Seeds, Somerset, UK. Plants were grown in the glasshouses of the University of Exeter.

3.3. Initial analysis of plant materials

Seeds were ground in a pestle and mortar. Other plant parts were freeze-dried for 4 days and then powdered. Samples (25 mg) were extracted three times with MeOH (1 ml) at 55°C. The pooled extracts of each sample were mixed with 1.3 ml water and 2 ml hexane. The aq. MeOH phase was analysed for the presence of ecdysteroid agonists and antagonists by RIA, bioassay and HPLC.

3.4. Bioassay

The biological activities (ecdysteroid agonist or antagonist) of extracts, HPLC fractions and purified compounds were determined with a microplate-based bioassay using the *Drosophila melanogaster* B_{II} cell line (Clément et al., 1993). 20-Hydroxyecdysone at a final concentration of 5×10^{-8} M was used in the antagonist bioassay.

3.5. Radioimmunoassay (RIA)

RIAs were performed according to a procedure described previously (Dinan, 1992) using ecdysteroid-specific antisera, DBL-1 and Black. The cross-reactivities of these antisera with a number of phytoecdysteroids are reported elsewhere (Dinan, 1995).

3.6. HPLC/bioassay of plant extracts

Portions of the extracts of the plant parts (equivalent to 3 mg dry wt) were separated on C_{18} reversed-phase column (25 cm × 4.6 mm i.d. Spherisorb ODS-2, 5 μm particle size), eluted at 1 ml/min with a linear MeOH–H_2O gradient from 30 to 100% MeOH over 30 min and then maintained at 100% MeOH for a further 10 min. Fractions of 1 min duration were collected and aliquots (20 or 40 μl) subjected to bioassay.

3.7. Isolation of antagonists

Seeds (22 g) of *P. opulifolius* were milled to a fine powder and then extracted with MeOH (3 × 800 ml at 55°C). The volume of the pooled extracts was reduced to 210 ml, water (90 ml) added and partitioned against *n*-hexane (3 × 200 ml). The defatted aq. MeOH phase was dried, resuspended in H_2O (200 ml) and partitioned against CH_2Cl_2 (4 × 200 ml). The CH_2Cl_2-fraction was found to be active and was subjected to preparative HPLC (C_8 preparative column, eluted with 60% MeOH in water at 5 ml min^{-1}) to yield a mixture of these three cucurbitacins as a single broad peak. This mixture was further analysed by HPLC using a C_{18} semipreparative column, eluted with 60% MeOH in water at a rate of 2 ml min^{-1}, resulting in the isolation of three compounds in pure form (retention times in min: **1** = 27.5, **2** = 24.5 and **3** = 32.0). 3-*epi*-Isocucurbitacin D (**3**) was finally purified by NP-HPLC on a Zorbax Silica analytical column, eluted with 10% EtOH in hexane at 1 ml min^{-1} and monitored at 230 nm.

3.7.1. Cucurbitacin D

Cucurbitacin D (**1**; 33.3 mg). HPLC, UV, ^{1}H NMR and ^{13}C NMR data as reported (Dreyer & Trousdale, 1978; Che et al., 1985; Jacobs et al., 1990; Dinan et al., 1997).

3.7.2. Cucurbitacin F

Cucurbitacin F (**2**; 3.3 mg). UV λ_{max}: 232 nm; ^{1}H NMR and ^{13}C NMR; see Table 2.

3.7.3. 3-epi-Isocucurbitacin D

3-*epi*-Isocucurbitacin D (**3**; 1.2 mg). UV λ_{max}: 230 nm; ^{1}H NMR and ^{13}C NMR; see Table 2.

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