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THE CO-OCCURRENCE OF ECDYSONES WITH BUFADIENOLIDES AND STEROIDAL SAPONINS IN THE GENUS *HELLEBORUS*

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Key Word Index—*Helleborus abchasicus*; *H. atrorubens* hort; *H. bocconei* ssp. *bocconei*; *H. cyclophyllus*; *H. dumentorum* ssp. *dumentorum*; *H. dumentorum* ssp. *atrorubens*; *H. foetidus*; *H. guttatus*; *H. lividus* ssp. *lividus*; *H. lividus* ssp. *corsicus*; *H. multifidus* ssp. *multifidus*; *H. orientalis*; *H. orientalis* hybrids; *H. viridis* ssp. *occidentalis*; Ranunculaceae; ecdysterone; 5β-hydroxyecdysterone; bufadienolides; steroidal saponins.

Abstract—A method is described for the detection of ecdysones in small quantities of plant material. When applied to 14 taxa of *Helleborus* L., ecdysterone and 5β-hydroxyecdysterone were detected and quantified in 11; the other 3, which are morphologically distinct, gave a negative result. The co-occurrence of ecdysones with bufadienolides and steroidal saponins is discussed. The isolation of the ecdysones from the aerial parts of *H. orientalis* hybrids is described.

INTRODUCTION

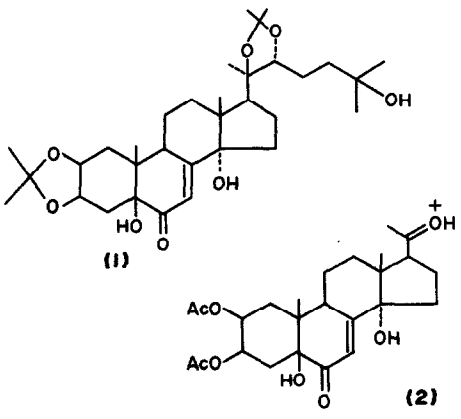
The family Ranunculaceae does not appear to have been investigated for ecdysones apart from their presence having been indicated in *Helleborus niger* by the insect bioassay method [1]. Steroidal saponins and bufadienolides have been reported in the genus Helleborus [2] and the taxonomy is referred to in [3] and [4].

RESULTS AND DISCUSSION

From the aerial parts only, including flowers (deep purple, purple and dark red), of *H. orientalis* hybrids were isolated ecdysterone and 5β-hydroxyecdysterone. Only traces of these two ecdysones were found in a sample of *H. orientalis* Lam. obtained from Cambridge comprising roots and aerial parts including cream coloured flowers. Authentic ecdysterone afforded direct comparison of physical and chemical properties. Evidence for 5β-hydroxyecdysterone came from its MS which indicated an extra hydroxyl group in the nucleus at *m/e* 379 [5]. Its diacetonide (**1**) gave a peak at *m/e* 143 as does ecdysterone diacetonide and this is characteristic of side chain cleavage (at C_{17}-C_{20}) and loss of Me_2CO. Also the triacetate of 5β-hydroxyecdysterone gave a major peak at *m/e* 463 characteristic of two secondary hydroxyl groups in the nucleus (**2**) [6]. Usually the detection of ecdysones in plant extracts is performed by a bioassay [1]. Only 15 g of dried whole plant was sufficient in our procedure for the ecdysones to be detected by MS using material from the polyamide gel column. Identification of individual ecdysones then followed with the aid of TLC, GLC, MS, and other physical data.

Of the 14 taxa of *Helleborus* investigated by this procedure, 11 of them were positive for ecdysones and of these, 7 species had already been reported to contain bufadienolides and 5 of these species contained steroidal saponins [2]. This work [2] was published after our screening procedure for ecdysones had been selected. The TLC system employed by Wissner and Kating [2] and our own method were also applied to the pure ecdysones and to our plant extracts. The ecdysones, the bufadienolides and the saponins were all detected in the plant extracts. All three classes of steroid had different R_f values and each gave different colours with the following reagents: anisaldehyde in sulphuric acid [2], antimony trichloride in chloroform [2] and vanillin in sulphuric acid.

The presence or absence of ecdysones was in agreement with the classification of Hellebores [3,4]. The largest group of species have rhizomes, stems of short duration, are herbaceous and basal leaves are present (acaulescent). Eleven taxa belonging to this group were found to contain ecdysterone and 5β-hydroxyecdysterone and the figures in brackets refer to the amount of these two ecdysones, respectively, expressed as a percentage



on a dry wt basis, when determined by a spectrophotometric method [7]. *H. atrorubens* hort. (0.25; 0.16); *H. bocconei* Ten. ssp. *bocconei* (0.26; 0.15); *H. cyclophyllus* Boiss. (0.27; 0.16); *H. dumentorum* Waldst. & Kit. in Willd. ssp. *dumentorum* (0.29; 0.14); *H. dumentorum* ssp. *atrorubens* (Waldst. & Kit.) Merxm. & Podl. (0.21; 0.11); *H. multifidus* Vis. ssp. *multifidus* (not quantified); *H. multifidus* ssp. *serbicus* (Adamovic) Merzm. & Podl. (0.36; 0.09); *H. orientalis* hybrids (0.45; 0.12); *H. orientalis* Lam. (traces only); *H. abchasicus* A. Braun (0.24; absent); *H. guttatus* A. Braun (0.27; 0.07); *H. viridis* L. ssp. *occidentalis* (Reuter) Schiffner (0.21; 0.06). The following species, in which ecdysones were absent, are morphologically distinct from the foregoing ones: rhizomes are absent; stems are overwintering, ±woody; and leaves are all cauline. *H. lividus* Aiton ssp. *lividus* (one source). *H. lividus* ssp. *corsicus* (Willd.) Tutin (three sources) and *H. foetidus* L. (two sources). Bufadienolides and saponins are absent from *H. lividus* ssp. *corsicus* (*H. lividus* ssp. *lividus* not reported) and *H. foetidus* contained saponins, of an unknown nature, only [2].

EXPERIMENTAL

A Kofler hot bench was used for mp's which are uncorr. Si gel $Pf_{254+366}$ was used for TLC with CH_2Cl_2–EtOH (5:1); ecdysones were visualised by their absorbtion at 254 nm and by spraying with vanillin–H_2SO_4 and heating at 110° for 5 min to give a yellowish-green colour. PLC was with the same adsorbent, 1 mm thick, and continuous development using the above solvent system. Ecdysterone was purchased from Sigma London Chemical Co., Ltd.

Plant material. Fresh whole plants from (i) Cambridge Botanic Garden, in flower: *H. bocconei* Ten. ssp. *bocconei* [3]; *H.* cf. *cyclophyllus* Boiss. [3]; *H. foetidus* L. [3]; *H. lividus* Aiton ssp. *corsicus* (Willd.) Tutin [3]; *H. orientalis* Lam. [3]; *H. abchasicus* A. Braun [4]; *H. guttatus* A. Braun [4] (these latter two species are now regarded as colour forms of the widespread and variable *H. orientalis*, mixed hybrids between such forms being Lentenroses [4]; (ii) Mrs H. Ballard, Old Country, Mathon, Malvern, Worcs.: *H. atrorubens* hort. [4]; *H. foetidus* L. [3]; *H. lividus* Aiton ssp. *corsicus* (Willd.) Tutin [3]; *H. cyclophyllus* Boiss. [3]; *H. dumentorum* Waldst. & Kit. in Willd. ssp. *dumentorum* [3]; *H. dumentorum* ssp. *atrorubens* (Waldst. & Kit.) Merxm. & Podl. [3]; *H. lividus* Aiton ssp. *lividus* [3]; *H. multifidus* Vis. ssp. *multifidus* [3]; *H. multifidus* ssp. *serbicus* (Adamovic) Merxm. & Podl. [3]; *H. orientalis* hybrids (believed to be hybrids of *H. guttatus* and *H. abchasicus* [8]); *H. viridis* L. ssp. *occidentalis* (Reuter) Schiffner [3] and (iii) Chelsea Physic Garden: *H. lividus* Aiton ssp. *corsicus* (Willd.) Tutin [3]. Voucher specimens are held at Cambridge and of Mrs Ballard's material at University of Bath. Immediately on receipt all parts of the plants were chopped into small pieces and dried rapidly in an air flow oven at 65° and the "whole" plant was used for the detection of ecdysones; only *H. orientalis* from Mrs Ballard was in flower, the rest being at a stage before flowering.

Isolation of ecdysterone and 5β-hydroxyecdysterone. H. orientalis hybrids, dried aerial parts including flowers (deep purple, purple and dark red forms) was powdered in a Christy–Norris fixed beater mill: 1 kg was extracted with MeOH in a Soxhlet apparatus and the concentrate was made aqueous. Lipophylic material was extracted with *n*-hexane and then $CHCl_3$ before the ecdysones were extracted from the aq. phase with *n*-BuOH. The concentrate was adsorbed on Celite and subjected to column chromatography (5 × 60 cm) using Si gel (Woelm, Activity I) with $(Me)_2CO$ as eluent. Two ecdysones (A and B) were detected by TLC and PLC afforded these by extraction with EtOH and EtOAc, respectively. Compound A was applied in MeOH to a column of Polyamide (Woelm) and eluted with H_2O and detected by TLC, R_f 0.35. Compound A, ecdysterone, $C_{27}H_{44}C_7$, crystallised as needles from $(Me)_2CO$:MeOH (5:1); 0.065% of dry wt., mp 230–235° (lit. [9] 234°); UV λ_{max}^{MeOH} 245 nm; MS *m/e* 480 (M^+) and diacetonide *m/e* 560 (M^+) 403, 385, 341 [6]; IR ν_{max}^{KBr} cm^{-1}: 3370, 1640; NMR (DMSO-d_6): δ 0.75 (*s*, C-18), 0.85 (*s*, C-19), 1.08 (*s*, C-21), 1.10 (*s*, C-26), 5.65 (C-7) [10]. These data were identical with those for authentic ecdysterone. Compound B, 5β-hydroxyecdysterone, $C_{27}H_{44}O_8$ crystallised from $(Me)_2CO$ without purification via polyamide gel; 0.001% of dry wt., mp 254–256) (lit. [9] 254–257°); UV λ_{max}^{MeOH} 245 nm; MS *m/e* 478 ($M^+ - H_2O$); diacetonide [6] *m/e* 576 (M^+) (**1**), 419, 401, 357, 143; triacetate [6] *m/e* 463 (**2**), 445, 401; IR ν_{max}^{KBr} cm^{-1}: 3350, 1670; NMR (DMSO-d_6): 5.62 (C-7).

Detection of ecdysones. 15 g of dried powdered material (whole plant before flowering) was extracted with MeOH and subjected to chromatography on a polyamide gel column (2 × 30 cm). Elution with H_2O furnished two ecdysones (A and B) which were visualised on Si gel $Pf_{254+366}$ plates, as above. The two were separated by PLC and the extracts were subjected to MS by direct probe insertion. The fragmentation patterns revealed two types of steroid nucleus with an identical side chain structure. A: *m/e* 363, 345, 327, 99, 81, 43; B: *m/e* 379, 361, 343, 99, 81, 43 [5].

GLC. Part of the polyamide eluate was evaporated in a Teflon capped tube at 50° in a vacuum oven. The residue was dissolved in C_5H_5N and 40 μl trimethylsilylimidazole was added and the mixture heated at 100° for 1 hr [11] in a capped teflon tube. The stainless steel column was 50 cm × 3 mm id, packed with 3% SE 30. The injection port and detection temp were 280°, column temp 240° and the flow rate (N_2) 50–60 ml/min. The completely derivatised ecdysones appeared as a major peak, a smaller peak was probably due to partially derivatised molecules. The same result was obtained when starting with authentic ecdysterone. The quantitative determination was by spectrophotometry [7].

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