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ECDYSTEROIDS IN *SPINACIA OLERACEA* AND *CHENOPODIUM BONUS-HENRICUS*

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Key Word Index—*Spinacia oleracea*; *Chenopodium bonus-henricus*; Chenopodiaceae; ecdysteroids; ecdysone, 20-hydroxyecdysone; polypodine B.

Abstract—The roots of *Chenopodium bonus-henricus* and the seeds of *Spinacia oleracea* contain 20-hydroxyecdysone and polypodine B. The seeds of *S. oleracea* also contain a compound with properties similar to those of 24(28)-dehydromakisterone-A and may contain small amounts of ecdysone.

The presence of ecdysone-type hydroxylated steroids have been demonstrated in a great number of plant families. Certain plant species (*Polypodium vulgare, Dacrydium intermedium, Cyanotis vaga, Vitex megapotamica, Ajuga japonica, Serratula inermis, Rhaponticum carthamoides*) can accumulate fairly large amounts (over 1% dry wt) of such compounds [1–5].

In spite of extensive screening programmes involving a few thousand plant species only very vague conclusions can be made on the occurrence of ecdysteroids in the Plant Kingdom. Nevertheless, certain taxonomic entities seem to emerge with a relatively high frequency of accumulation of such compounds, e.g. Polypodiaceae, Podocarpaceae, Taxaceae, Amaranthaceae, and the genera *Cyanotis, Vitex, Ajuga, Serratula* (see refs. [3, 5]). In view of the close relationship of the Chenopodiaceae to Amaranthaceae, one of the prominent ecdysteroid-bearing families, studies were undertaken on *Chenopodium bonus-henricus* L. and *Spinacia oleracea* L., two closely related species, both extensively used as vegetables and renowned for their roborant quality [6–8]. Since ecdysteroids had been reported to exert a specific anabolic effect on various animals [4, 9], it was expected that the alleged roborant activity could eventually be explained by the presence of ecdysteroids.

The present paper deals with the isolation and characterization of the major ecdysteroid-type components of the above species.

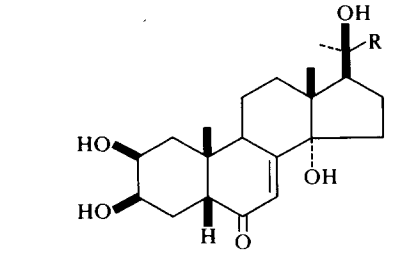
TLC and RIA experiments [10] demonstrated the presence of a series of ecdysteroid-type compounds in the herb and roots of both species as well as in the seeds of *S. oleracea*. Isolation was undertaken from the roots of *C. bonus-henricus* and from the seeds of *S. oleraceae*. The isolated ecdysteroids were identified on the basis of their physical constants and spectral characteristics as well as by direct comparison with authentic samples.

Polypodine B (**2**) and 20-hydroxyecdysone (**1**) were isolated from the roots of *C. bonus-henricus* and the seeds of *S. oleracea*. The latter also yielded an ecdysteroid showing similarities to 24(28)-dehydromakisterone A (**3**) [11]. The possible presence in *S. oleracea* of ecdysone (**4**) was demonstrated by RIA [12] and TLC.

Various genera of the Chenopodiaceae (*Spinacia, Atriplex, Beta, Chenopodium, Kochia, Anabasis*) have earlier been reported to contain saponins, but only a few of these saponins have as yet been characterized unequivocally. They were found with no exception to belong to the triterpene series having in most cases oleanolic acid as aglycone [13–15]. In addition, sterols and some sterol glycosides have frequently been indicated. To our knowledge the compounds **1–3** are the first isolated and characterized ecdysteroids from this family though one *Kochia* sp. was earlier reported to contain ecdysteroids [16]. The presence of the reported compounds in both vegetables provides a possible explanation of their roborant activity, although further work is needed to establish its mechanism and active principle(s).

EXPERIMENTAL

Plant material. C. bonus-henricus roots were collected from wild plants in the Bükk mountains, near Bánkut in Oct. 1980. Voucher specimens were deposited in the Herbarium of Szeged University. Seeds of *S. oleracea* var. Popeye were purchased in Szeged.



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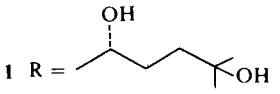

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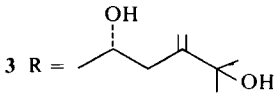

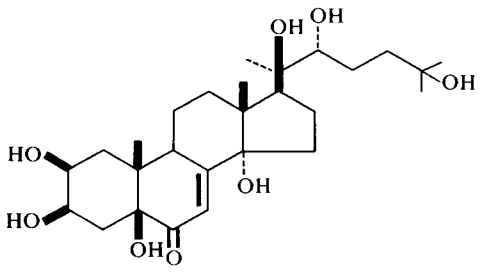

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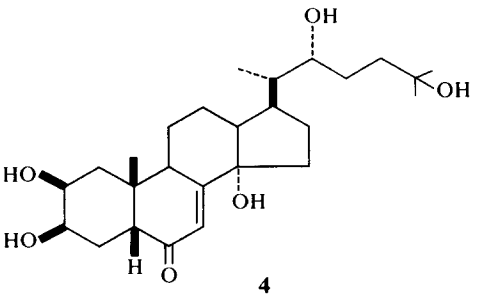

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TLC analysis and RIA measurements. Fresh plant material (10 g) was extracted with 100 ml MeOH (× 2). The extracts were filtered, evapd to dryness (*in vacuo*) and the residue dissolved in 10 ml 70% MeOH. Aliquots (30 μl) were subjected to TLC (Si gel GF_{254}, CH_2Cl_2–EtOH, 4:1), together with authentic ecdysteroids. The plates were examined under short-wavelength UV, and then sprayed with 0.1% vanillin in conc. H_2SO_4.

RIA measurements were made using either anti-polypodine antiserum [10] which has equal sensitivity towards ecdysone and 20-hydroxyecdysone or ecdysone-sensitive antiserum (H-21-B) [12]. Amounts of the plant extracts (10 μl) were dried in Eppendorf tubes (1.4 ml) together with 4000 cpm ^{3}H-labelled ecdysone (New England Nuclear) under N_2. 100 μl 1% anti-polypodine B antiserum in RIA-buffer were added and incubated for 30 min at 38°. The separation of the bound and free radioactivity was done by $(NH_4)_2SO_4$ precipitation [10].

Extraction and isolation of compounds **1**–**3**. Fresh roots (15.8 kg) of *C. bonus-henricus* were milled in a blender then percolated with MeOH (70 l.). The extract was concd under red. pres. to semi-solid consistency. The residue (3.5 kg) was taken up in MeOH (5 l.), when a ppt. formed. The ppt. was filtered and washed with MeOH. The MeOH-soluble portion (305 g) was chromatographed, after evaporation of the solvent, on a column of Brockman grade II, neutral Al_2O_3 (5 kg). Elution with CH_2Cl_2–EtOH (9:1) afforded a crude ecdysteroid mixture containing 20-hydroxyecdysone (**1**) and polypodine B (**2**). From the mixture **1** (200 mg) was sepd by repeated crystallization (EtOAc–MeOH, 2:1) as white needles, mp 237–239° (lit. 237–239°); olive-green colour on TLC with ethanolic vanillin–H_2SO_4 [3]; 2,3,22-triacetate monohydrate mp 151–154° (from MeOH); UV, IR, ^{1}H NMR, ^{13}C NMR [see 17] and MS identical with those of an authentic sample of **1**.

From the combined mother liquors of **1**, polypodine B (**2**) (7 mg) was isolated by repeated prep. TLC (Si gel GF_{254}, CH_2Cl_2–MeOH, 17:3). It crystallized from aq. MeOH in white needles, mp 253–257° (lit. 254–257°). Olive-green colour on TLC with ethanolic vanillin–H_2SO_4; UV, IR, ^{1}H NMR, ^{13}C NMR [17] and MS identical with those of an authentic sample of **2**.

Milled seed (4 kg) of *S. oleracea* was defatted with 18 l. petrol, dried and percolated with 200 l. MeOH. The extract was concd under red. pres. to semi-solid consistency. The residue was taken up in 2.5 l. H_2O–MeOH (7:3). The soln was concd to 1.5 l. then extrd with 500 ml CH_2Cl_2 (× 5) followed by 500 ml CH_2Cl_2–MeOH (2:1) (× 30). The combined CH_2Cl_2–MeOH extracts were evapd *in vacuo*. The residue (20 g) was chromatographed on 335 g polyamide (Woelm). H_2O eluates yielded fractions containing ecdysteroids. The combined fractions were concd *in vacuo* and the residue (6 g) rechromatographed on 500 g Kieselgel (0.05–0.2 mm für Säulenchrom.). Fractions eluted with CH_2Cl_2–EtOH (17:3) were further purified by repeated chromatography on prep. TLC, on a Sephadex LH-20 column (elution with MeOH), and on a Si column (as above). The separation was monitored by TLC. Three ecdysteroids and an aromatic compound were isolated. Two of the ecdysteroids were identified as **1** (858 mg) and **2** (9 mg) respectively; the spectral data of the third, unknown compound, showed striking similarities to 24(28)-dehydromakisterone A (**3**) [11]. The identity awaits further confirmation.

Ecdysone-specific RIA measurements [12] and TLC gave clearly positive results for the presence of ecdysone (**4**) in both the original extract and the mother liquors. However, it was present in such small amounts that it could not be isolated in pure state.

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VIOLAXANTHIN ESTERS FROM *VIOLA TRICOLOR* FLOWERS

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Key Word Index—*Viola tricolor*; Violaceae; violaxanthin ester; β-hydroxy acids.

Abstract The pattern of fatty acid esters of violaxanthin and the minor xanthophylls in the petals of *Viola tricolor* (yellow varieties) is unusually complex. This is due to the fact that β-hydroxy acids (12:0, 14:0, 16:0) take part in the esterification in addition to the usual acids (12:0, 14:0, 16:0, 18:0).

The xanthophylls in flower petals are usually esterified with saturated C_{12}–C_{18} acids ([1–3]; for review see [4]). The yellow varieties of *Viola tricolor* contain as their main pigment violaxanthin (*ca* 75% of total carotenoid) which occurs in an unusually complex ester pattern as has been shown by TLC [5]. In the present investigation the acid moieties of these violaxanthin esters have been analysed.

The source of carotenoid esters were whole petals or isolated lipid globules (plastoglobuli) in which the esters are mainly localized [6]. Violaxanthin diesters were the main components while monoesters and free violaxanthin only occurred in trace amounts. The diesters were separated by Si gel TLC into three fractions in comparable concentrations. The corresponding fatty acids upon saponification or transmethylation of these fractions were the usual saturated acids in the less polar fraction, saturated and, additionally, unknown more polar acids in the middle fraction in a molar ratio of *ca* 1:1, and exclusively polar acids in the polar fraction. The saturated acids were 12:0, 14:0, 16:0, and 18:0 acids as identified by GC. The polar acids were β-hydroxy 12:0, 14:0, and 16:0 acids as revealed by MS and ^{1}H NMR. This means that in the three ester fractions the two hydroxyl groups of violaxanthin were either both esterified with the usual acids, or one hydroxyl with a usual acid and one hydroxyl with a β-hydroxy acid, or both hydroxyls with β-hydroxy acids, respectively. The β-hydroxy group itself was not esterified as was shown by acetylation. The minor xanthophylls of *Viola tricolor*, lutein, lutein epoxide, and neoxanthin, apparently showed corresponding ester patterns.

This is to our knowledge the first report of xanthophyll β-hydroxy acid esters from flower petals.

EXPERIMENTAL

Flower petals or lipid globules [6] from *Viola tricolor* L. subsp. *maxima* were extracted with acetone. Violaxanthin esters were separated by Si gel TLC using petrol–Et_2O–Me_2CO (10:2:1), free fatty acids with $CHCl_3$–EtOAc–HCO_2H (40:6:3), and fatty acid methyl esters with petrol–Et_2O (2:1). Saponification was performed using ethanolic KOH. Acetylation was done in pyridine using Ac_2O. Fatty acid methyl esters were prepared by transmethylation using NaOMe or by methylation using CH_2N_2. Methyl esters were separated by GC (column, 2.4 m × 3.2 mm, packed with 10% EGSSX, isothermal at 210°, carrier gas N_2 at 30 ml/min, FID).

EIMS (probe) 70 eV, *m/z* (rel. int.): 103, formed by α-cleavage to the hydroxy group (100), 74, formed by the McLafferty rearrangement triggered by the ester carbonyl group [7] (34). CIMS (NH_3, probe) 200 eV, *m/z* (rel. int.): 304 $[M + NH_4]^+$ from C_{16} methyl ester (7), 287 $[M + H]^+$ from C_{16} (2), 276 $[M + NH_4]^+$ from C_{14} (100), 269 $[M + H - H_2O]^+$ from C_{16} (4), 259 $[M + H]^+$ from C_{14} (40), 248 $[M + NH_4]^+$ from C_{12} (46), 241 $[M + H - H_2O]^+$ from C_{14} (66), 231 $[M + H]^+$ from C_{12} (19), 213 $[M + H - H_2O]^+$ from C_{12} (29), 209 $[M + H - H_2O - MeOH]^+$ from C_{14} (8), 181 $[M + H - H_2O - MeOH]^+$ from C_{12} (5).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.008 (1 H, *br s*, H_β), 3.716 (3 H, s, COOMe), 2.823 (1 H, *d*, J = 4 Hz, OH), 2.514 and 2.410 (AB-part of ABX-spectrum, 1 H each, CH_2 (α), J_{AB} = 16.2 Hz, J_{AX} = 3 Hz, J_{BX} = 9 Hz (X = H_β)), chemical shift and large value of J_{AB} is indicative for the α-position to the carbonyl), *ca* 1.53 (1 H), ca 1.43 (2 H) and *ca* 1.258 (*ca* 17 H, CH_2–CH_2), 0.880 (3 H, tr, $J \approx 6.8$ Hz, Me). Upon addition of D_2O to the solution the OH-signal disappeared and the multiplet signal of H_β became slightly sharper.