NEW MINOR ECDYSTEROIDS FROM *Silene viridiflora*

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Two new minor ecdysteroids, the 2,22- and 3,22-diacetates of 20,26-dihydroxyecdysone have been isolated from the aerial part of *Silene viridiflora* L. (Caryophyllaceae).

Keywords: *Silene viridiflora*; Steroids; Ecdysteroids; Natural products; NMR spectroscopy; Isolation; Phytochemistry.

Ecdysteroids are a large family of polyhydroxylated steroids first described as arthropod molting hormones (zooecdysteroids), but later found also in many plants. Phytoecdysteroids are secondary metabolites involved in plant protection against phytophagous insects[1]. Over 300 different ecdysteroids have been identified to date[2]. Ecdysteroid-containing species are found in many plant families[3]. Among angiosperms, the Caryophyllaceae comprise numerous ecdysteroid-positive genera[4], and the *Silene* genus has been extensively investigated[5–8]. The presence of ecdysteroids can be used as a chemotaxonomic marker[4].

Silene viridiflora is a perennial species (size 40–65 cm) growing in Euro-Mediterranean and Central-Asian areas, which is characteristic by small greenish flowers. No phytochemical data are currently available on this species. *S. viridiflora* is included in the section Syphonomorpha and is closely related to *S. italica* (L.) Pers., *S. paradoxa* (L.), *S. nutans* (L.), *S. gigantea* (L.)

and others. The plant shows anti-oncological activity in tests on mice[9] and possesses also hemorheological activity[10].

A preliminary study of this plant has allowed the isolation and identification of its major ecdysteroids, i.e. 20-hydroxyecdysone (0.35% relative to dry weight of the plant), polypodine B (0.25%), 2-deoxy-20-hydroxyecdysone (0.2%), integristerone A (0.2%), sileneoside D (0.1%), sileneoside A (0.08%) and 26-hydroxypolypodine B (0.035%)[11].

EXPERIMENTAL

General Methods

Mass spectra were recorded in chemical ionisation/desorption (CI/D) mode with ammonia gas as the reagent on a Jeol JMS-700 spectrometer. NMR spectra were obtained on a Bruker AMX500 at 300 K. The samples were lyophilised and dissolved in D_2O. $TSPd_4$ (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt) was used as internal reference for ^1H and ^{13}C shifts (δ ±0.2 ppm). Chemical shifts are expressed in ppm. 1D ^1H and ^{13}C spectra and 2D COSY, TOCSY, PFG-HSQC and PFG-HMBC NMR spectra have further allowed the ^1H and ^{13}C assignments.

A HPLC equipment from Thermo Separation was used for a final purification step and for checking compound purity. HPLC (system A) used a Zorbax®-SIL column (250 mm, 4.6 mm i.d., particle size 5 μm, DuPont) eluted with dichloromethane–propan-2-ol–water (125:25:1, v/v/v) with a flow-rate of 2 ml/min. HPLC (system B) used an Ultrasphere®-5ODS2 column (250 mm, 4.6 mm i.d., particle size 5 μm, Beckman) eluted with a linear gradient (40–100% over 30 min) of methanol in water containing 0.1% trifluoroacetic acid, at a flow-rate of 1 ml/min).

Isolation of Ecdysteroids

Air-dried and cut aerial parts (8.5 kg) of *Silene viridiflora* grown in the Siberian Botanical Garden of the Tomsk State University were milled and extracted 5 times with 35 l MeOH each. The extract was concentrated to 2 l and diluted with an equal volume of water. The obtained precipitate was removed by filtration and MeOH was evaporated. The water fraction was extracted successively with 8 l $CHCl_3$, 4 l EtOAc, then 3 l BuOH. After evaporation of the solvents under vacuum 106 g of the EtOAc fraction and 450 g of the BuOH fraction were obtained. From the BuOH fraction, seven known ecdysteroids were isolated as previously described[11].

The EtOAc extract (106 g) was chromatographed on an Al_2O_3 column (1 m, 10 cm i.d., filled with 3 kg alumina, particle size 0.1 mm) using $CHCl_3$–MeOH (15:1) as eluent. A 25 mg fraction was first eluted, which contained a mixture of 2-deoxyecdysone and a less polar ecdysteroid (TLC, not shown). Then by further elution with the same solvent pure 2-deoxyecdysone (0.85 g, 0.01% of plant dry weight), $C_{27}H_{44}O_5$ (EtOH–H_2O), m.p. 234–235 °C, $[\alpha]_D^{23}$ +93.3 ± 2 (*c* 0.5, MeOH) and 2-deoxy-20-hydroxyecdysone (1.6 g) were isolated; polypodine B (0.85 g) and 20-hydroxyecdysone (1.8 g) were obtained by elution with $CHCl_3$–MeOH (9:1).

The above-mentioned 25 mg of ecdysteroid mixture was chromatographed on a small column, and a fraction (3 mg) was obtained by elution with $CHCl_3$–MeOH (15:1). This fraction was finally purified by HPLC (system A) and gave two baseline-resolved peaks (a and b) of equal intensity (retention times 11.8 and 15.0 min, respectively). The purity of each peak was confirmed by RP-HPLC (system B): peak a was eluted at 9.70 min and peak b at 12.9 min.

RESULTS AND DISCUSSION

The CI/D mass spectra of peaks a and b were almost identical and very complex, with ions at *m/z* 682, 664, 640, 622, 598, 580, 562, 556, 538, 520, 502, 496, 478, 461, 443, 422, 404, 236, 218, 192, 174, 136, 132, 77. These spectra showed many fragments differing by 42 or 60 amu, which was indicative of the presence of acetyl esters, and we knew from previous experiments that with the CI/D mode trans-esterification reactions take place, thus making it difficult to determine the exact molecular weight (MW)[12]. FAB^+ mass spectra gave much simpler patterns with prominent ions at *m/z* 603.3, 581.3 and 563.3, interpreted as $[M + Na]^+$, $[M + H]^+$ and $[M + H - H_2O]^+$, respectively. This was consistent with a MW of 580 amu, i.e. the MW of a diacetate of a compound bearing one extra-OH group as compared with 20-hydroxyecdysone (MW 480), and provided a satisfactory explanation for CI/D spectra. The identification of compounds **1** and **2** (corresponding to peaks a and b), was performed by a combination of NMR procedures which allowed nearly all ^1H and ^{13}C assignments of these compounds (Table I). These compounds (Fig. 1) present the main features of 26-hydroxy compounds[13]: loss of the 26-CH_3 signal, appearance of a CH_2OH signal at δ 3.45 ppm and upfield shift of the 27-CH_3 signal. Compound **1** was identified as 20,26-dihydroxyecdysone 3,22-diacetate due to

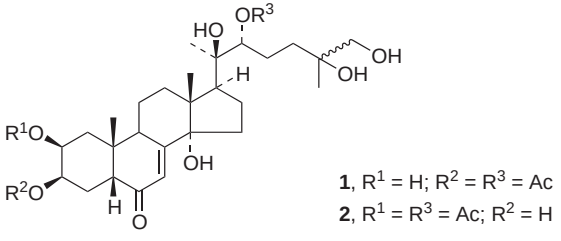

FIG. 1
Structures of compounds **1** and **2**

TABLE I
^{1}H NMR and ^{13}C data for compounds **1** and **2** in D_2O

Carbon	Compound **1**	Compound **2**	Proton	Compound **1**	Compound **2**
C1	39.0	39.2	1-Ha	1.48	1.53
			1-He	1.99	1.96
C2	68.5	74.5	2-Ha	4.13 (m, $w_{1/2}$ = 22)	5.08 (m, $w_{1/2}$ = 22)
C3	73.9	67.8	3-He	5.17 (m, $w_{1/2}$ = 8)	4.22 (m, $w_{1/2}$ = 8)
C4	n.d.[a]	n.d.[a]	4-Ha	1.78	1.80
			4-He	1.85	1.83
C5	53.4	53	5-H	2.35 (dd, 4.2, 13.5)	2.41 (dd, 5, 13)
C6	n.d.[a]	n.d.[a]			
C7	123.9	123.8	7-H	5.98 (d, 2.5)	5.99 (d, 2.5)
C8	n.d.[a]	n.d.[a]			
C9	36.5	36.6	9-Ha	3.11 (m, $w_{1/2}$ = 24)	3.18 (m, $w_{1/2}$ = 24)
C10	40.6	41.0			
C11	n.d.[a]	n.d.[a]	11-Ha	1.74	1.73
			11-He	1.86	1.86
C12	33.5	33.8	12-Ha	1.76	1.76
			12-He	1.96	1.96
C13	50.0	50.2			
C14	87.8	88.1			
C15	32.8	33.1	15-Hα	1.67	1.67
			15-Hβ	2.06	2.06
C16	22.2	22.9	16-Hα	1.88[b]	1.88[b]
			16-Hβ	1.80[b]	1.80[b]
C17	52.1	52.1	17-H	2.31 (t, 9.5)	2.31 (t, 9.5)
C18	19.6	19.7	18-Me	0.86 (s)	0.86 (s)
C19	25.7	25.6	19-Me	1.02 (s)	1.02 (s)
C20	79.9	79.9			
C21	22.8	23.0	21-Me	1.34 (s)	1.34 (s)
C22	84.2	82.9	22-H	4.85 (dd, 10.5, 2)	4.85 (dd, 10.5, 2)
C23	n.d.[a]	n.d.[a]	23-Ha	1.56	1.56
			23-Hb	1.76	1.76
C24	37.4	37.4	24-Ha	1.75	1.75
			24-Hb	1.46	1.46
C25	75.9	76.0			
C26	71.4	71.3	26-CH_2OH	3.42 (s)	3.42 (s)
C27	24.7	24.6	27-Me	1.15 (s)	1.15 (s)
2-CH_3CO	–	23.6/176.5	2-CH_3CO	–	2.127 (s)
3-CH_3CO	23.2/176.6	–	3-CH_3CO	2.176 (s)	–
22-CH_3CO	23.2/177.5	23.4/177.6	22-CH_3CO	2.165 (s)	2.167 (s)

[a] n.d., not detected. [b] Interchangeable signals.

two new singlets in the acetate region and a downfield shift for both 3-H and 22-H signals, and C-3 and C-22. Compound **2** was identified as 20,26-dihydroxyecdysone 2,22-diacetate due to two new singlets in the acetate region and a high-frequency shift of 2-H and 22-H signals as well as C-2 and C-22.

With time, a slow equilibration of compounds **1** and **2** was observed and, after 50 days in D_2O (at 4 °C), each NMR tube contained an equimolar mixture of **1** and **2**, which is consistent with classical observations for acetyl group migration between positions 2 and 3. It is therefore difficult to decide whether **1** or **2** is the original ecdysteroid present in the plant.

Silene viridiflora is indeed an ecdysteroid-rich species: altogether, these compounds account for at least 100 g, i.e. 1.2% of the plant dry weight. The EtOAc extract contained ca. 5% of all ecdysteroids (in fact almost all 2-deoxyecdysone, but only 8.6% of 2-deoxy-20-hydroxyecdysone, 5.4% of 20-hydroxyecdysone and 3.8% of polypodine B).

This species is, however, unusual as far as its ecdysteroid pattern is concerned. Like *S. otites*[7], it contains a rather complex cocktail of ecdysteroids, however there is no single major component, but several compounds of similar relative abundance. Among these ecdysteroids, we can underline relatively high amounts of glycosides which account for 15% of total ecdysteroids. This was previously observed for *S. brahuica*[5], the source of a wide array of glycosides, including glucose and galactose conjugates. *S. viridiflora* contains galactosides, which are probably formed by a broadly-specific UDP-galactosyl transferase[14]. Moreover, *S. viridiflora* ecdysteroids cover a wide spectrum of polarities, as they range from very polar glycosides to non-polar ecdysteroids such as compounds **1** and **2**. The significance of such a complex mixture in insect–plant relationships is presently unclear at present. This will deserve extensive studies of structure–activity relationships of ecdysteroids for their effect on insect taste receptors[15].

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