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New minor ecdysteroids from *Silene otites* (L.) Wib.

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Abstract

Six minor new ecdysteroid components have been isolated from *Silene otites* (L.) Wib. by a combination of chromatographic methods. Three of them (2-deoxy-20-hydroxyecdysone 3,22-diacetate, 5α-2-deoxy-20-hydroxyecdysone 3-acetate, and 2-deoxy-20-hydroxyecdysone 3-crotonate) are new natural products. © 1997 Elsevier Science B.V.

Keywords: 2-Deoxy-20-hydroxyecdysone 3,22-diacetate; 5α-2-Deoxy-20-hydroxyecdysone 3-acetate; 2-Deoxy-20-hydroxyecdysone 3-crotonate; Ecdysteroids; Phytoecdysteroids

1. Introduction

Ecdysteroids have significant effects on induced hyperglycaemia in experimental mammals [1] and they also potentiate the effect of insulin [2]. There are some pharmacological preparations commercially available, whose active principle is based on their ecdysteroid content. One of them is Ecdisten® [3], originally advertised as a natural compound with tonic effects; however, Slama and Lafont [4] reported novel medical indications. One or two tablets with 5–10 mg of ecdysteroid content is recommended in asthenic and astheno-depressive states, somatic and infectious diseases, neurasthenia, neurosis, hypotension and fatigue [4]. Another preparation is a specific green tea 'Maralan' [5] of the dried green parts of *Leuzea carthamoides* (Herba Leuzea with 0.08–0.22% of 20-hydroxyecdysone content) advertised for increased resistance against stress, stimulation of functions of the central nervous system, to increase appetite and digestion (for review, see Ref. [4]). Ecdysteroids, especially 20-hydroxyecdysone-containing preparations, are used as skin moisturisers, for the softening of the skin [6], and treatment of psoriasis [7].

During the last 10 years, a number of new phytoecdysteroids have been discovered [8,9]. Presently, the search and discovery of new ecdysteroids from plants is continuing and the technical

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procedures for analysis, isolation and structural elucidation are also in progress [10,11].

Hitherto the occurrence of more than 130 phytoecdysteroids has been published; about 40 of them can be detected in various *Silene* species [12,13]; about 31% of phytoecdysteroids are synthesised by *Silene* species. In our pervious work, 16 phytoecdysteroids from *Silene otites* (L.) Wib. were isolated; seven of them are new phytoecdysteroids and five of them are new natural products [13]. Using our experience from the previous isolation work, six additional ecdysteroids have been isolated, in addition to the 16 already known ecdysteroids from *Silene otites* (L.) Wib.

2. Experimental

2.1. Materials and methods

2.1.1. Plants

The aerial parts of *Silene otites* (L.) Wib. were collected in the vicinity of Ásotthalom (a village in the southern part of Hungary, near Szeged). The herb was cleaned, air-dried and milled. Solvents were purchased from Reanal (Budapest, Hungary) in the best analytical grade of purity.

2.1.2. Ecdysteroids

Some of the standard ecdysteroids were isolated in our previous work (e.g., ecdysone, E; 20-hydroxyecdysone, 20E; 2-deoxy-20-hydroxyecdysone, 2d20E) [13–15]. Reference compounds of 2-deoxy-20-hydroxyecdysone 3-acetate (2d20E 3Ac) were prepared by acetylation followed by partial hydrolysis of 2d20E 3,22-diacetate [14]. The same procedure was used with the 5α epimer of 2d20E prepared by equilibration of 2d20E under alkaline conditions as usual.

2.1.3. Thin-layer chromatography (TLC)

TLC plates coated with 0.2 mm silica gel F_{254} (E. Merck, Darmstadt, Germany) were used for normal-phase planar chromatography. RP-TLC plates Whatmann KC18F (Whatmann, Clifton, NJ, USA) were used for reversed-phase TLC.

The solvent systems for normal-phase thin-layer chromatography were: (1) dichloromethane–ethanol (96%) (85:15, v/v); (2) dichloromethane–ethanol (96%) (96:4, v/v); (3) ethyl acetate–methanol–ammonia (85:10:5, v/v/v). The solvent system for reversed-phase thin-layer chromatography was: (4) ethanol (96%)–water (6:4, v/v).

After development of the straight-phase plates, ecdysteroids were detected either directly by fluorescent quenching at 254 nm, or by using vanillin–sulphuric acid spray reagent, and the spots were observed either in daylight, or at 356 nm. When the reversed-phase TLC was performed, the separated spots were detected at 254 nm.

2.2. Low-pressure liquid chromatography

2.2.1. Stationary phase

Alumina Brockmann II, neutral (Reanal, Budapest, Hungary) was packed into a glass column (360 × 93 mm). Mobile phases were mixtures of dichloromethane and methanol using a stepwise gradient elution. Kieselgel 60 (63–200 μm) from E. Merck was used to prepare the column (250 × 18 mm), eluted by a stepwise gradient of dichloromethane and 96% ethanol.

2.2.2. Droplet counter-current chromatography (DCCC)

An Eyela DCC-A instrument (Rikakikai, Tokyo, Japan) was used with descending mode of operation with the following system: chloroform–methanol–water (7:13:8, v/v/v); a 0.1 g sample was dissolved in 1.1 ml of the upper phase and a 21 ml h^{-1} flow rate was applied; 7 ml fractions were collected.

2.2.3. High-performance liquid chromatography (HPLC)

Normal-phase HPLC separations were done with Waters HPLC equipment (Millford, MA, USA). Analytical separations used a Zorbax®-SIL (250 × 4.6 mm) column (DuPont) with a flow rate of 1 ml min^{-1}. For preparative separations, Zorbax®-Sil 250 × 9.4 mm column was used with 4 ml min^{-1} flow rate.

The mobile phases were: (1) cyclohexane–2-propanol–water (100:30:1.5, v/v/v); (2)

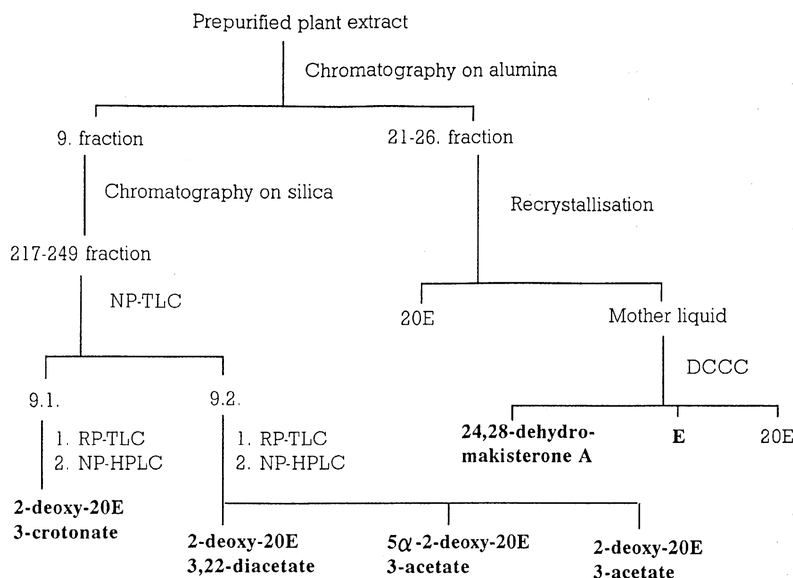

Fig. 1. Fractionation of pre-purified extract of *Silene otites* (L.) Wib. herba.

dichloromethane–2-propanol–water (125:15:1, v/v/v); (3) dichloromethane–2-propanol–water (125:25:2, v/v/v).

2.2.4. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy

Mass spectrometry using the chemical ionisation/desorption (CI/D) mode was carried out on a Riber 10-10D instrument equipped with a direct-inlet probe with an NH_3 reagent gas. NMR spectra (in CD_3OD) were recorded on a 500 MHz Brucker apparatus.

2.3. Procedure of isolation

(1) Extraction and pre-purification of the crude extract: the dried aerial parts of *Silene otites* (L.) Wib. were milled (dry material: 3.8 kg), and percolated with 76 l of methanol at room temperature. The methanol extract was evaporated, the dry residue (345.7 g) was dissolved in 1500 ml methanol, and 750 ml acetone was added to the solution. A precipitate was formed, that was separated by decantation, and it was washed three times with 150 ml methanol–acetone (2:1 mixture, v/v). The methanol–acetone extracts were combined, dried, and dissolved in 1300 ml methanol. The same amount (1300 ml) of acetone was added, the precipitate was separated by decantation, washed three times with 150 ml methanol–acetone (1:1 mixture, v/v), then the solutions were combined and dried. The residue (240.4 g) was dissolved in 1200 ml aqueous methanol (50%, v/v) and extracted four times with 500 ml benzene. Ecdysteroids from the organic phase were reextracted twice with 100 ml aqueous methanol (50%, v/v). The aqueous methanol solutions were combined and dried, resulting in 191 g residue.

(2) Fractionation of the pre-purified extract: Fig. 1 shows the isolation scheme of the ecdysteroids from the pre-purified extract. The extracted material (191 g) was dissolved in 350 ml methanol and was adsorbed on 570 g alumina by mixing them, and dried. A total of 1000 g alumina was packed into a column, and the extract-adsorbed alumina was packed to the top of the sorbent. Stepwise gradient elution was performed using mixtures of dichloromethane–methanol (details are given in Table 1), and 500 ml fractions were collected. The progress of elution was monitored by normal-phase TLC using solvent systems Nos. 1 and 3. This paper gives an account of the

Table 1
Monitoring the progress of elution from the alumina column

Fraction Nos.	Eluent
1–5	Dichloromethane
6–13	Dichloromethane–methanol (95:5, v/v)
14–29	Dichloromethane–methanol (9:1, v/v)
30–39	Dichloromethane–methanol (8:2, v/v)
40–42	Methanol

further purification of fractions 9 and 21–26, only.

The purification of fraction 9 from the alumina column was done using silica column chromatography. Fraction 9 (dry residue 0.9 g) was adsorbed on 3 g silica and the column was 30 g. The preparation of the column was done as detailed above, and gradient elution was performed using dichloromethane–96% ethanol, as detailed in Table 2. Fractions were collected, 10 ml each. Monitoring of elution was done using TLC systems Nos. 2 and 3. Fractions 217–249 were combined and further purified on a preparative scale by straight-phase TLC using solvent system No. 2. Bands at $R_F = 0.32$ and 0.51 (marked as 9.1 and 9.2) were scarped and eluted with methanol. The ecdysteroids obtained were further purified by reversed-phase TLC using solvent system No. 4. The bands at $R_F = 0.63$ and 0.41 were scarped, eluted with methanol (marked as 9.1 and 9.2), and further purified by normal-phase HPLC using solvent system No. 1.

The pure components were characterised by their HPLC retention time using two different

Table 2
Monitoring the progress of elution from the silica column

Fraction Nos.	Eluent
1–86	Dichloromethane
87–132	Dichloromethane–ethanol (96%) (99:1, v/v)
133–207	Dichloromethane–ethanol (96%) (98:2, v/v)
208–286	Dichloromethane–ethanol (96%) (96:4, v/v)
287–360	Dichloromethane–ethanol (96%) (94:6, v/v)

Table 3
HPLC retention data

Fraction/compounds	Retention time (min)		
	No. 1	No. 2	No. 3
9.1			
a.	—	14.4	—
b.	—	15.3	—
9.2			
a.	9.3	6.6	—
b.	10.3	14.1	—
c.	11.0	15.0	—
d.	15.9	31.3	—
e.	18.9	77.4	—
f.	22.9	—	—
60–72 Fractions (DCCC)	—	—	17.8
97–100 Fractions (DCCC)	—	—	22.0
References compounds			
2d20E 3,22Ac	9.3	6.4	—
2d20E 3Ac	11.0	15.1	—
2d20E 22Ac	16.0	—	—
2d20E	18.8	—	—
5α 2d20E	22.8	—	—
5β 2d20E	10.2	14.2	—
24,28-dehydromakisterone A	—	—	17.8
E	—	—	22.0
20E	—	—	36.4

HPLC was done on silica stationary phase using mobile phase systems: No. 1: cyclohexane–2-propanol–water (100:30:1.5, v/v/v), analytical column, 1 ml min^{-1}; No. 2: dichloromethane–2-propanol–water (125:15:1, v/v/v), preparative column, 4 ml min^{-1}; No. 3: dichloromethane–2-propanol–water (125:25:2, v/v/v), preparative column, 4 ml min^{-1}.

mobile phases, as the data are given in Table 3. The chemical structures of the pure ecdysteroids were determined by spectroscopic methods.

Fractions 21–26 from the alumina column were analysed by TLC using solvent systems Nos. 1 and 3. An essential part of 20-hydroxyecdysone was found here, and it was removed by crystallisation in ethyl acetate–methanol (2:1, v/v). One part (4%) of the mother liquid (that contained 0.4 g dry residue) was fractionated by DCCC (droplet counter-current chromatography) in the system mentioned above. The DCCC fractionation was done in four parallel procedures, each of which separated 0.1 g of dry residue. DCCC fractions 60–72 and 97–100 were combined and purified

further by HPLC. The identification of the pure ecdysteroids was done by spectroscopic methods.

3. Results

The dried herb of *Silene otites* (L.) Wib. was extracted by 20-fold excess of methanol, and its ecdysteroids were purified. The crude extract was subjected to two pre-purification steps. The overwhelming majority of polar ballast compounds were removed by two consecutive precipitation steps by acetone, and the apolar components were removed by solvent–solvent extraction using benzene. The pre-purified extract was subjected to coarse column chromatography, where the stationary phase was alumina, and mobile phases various mixtures of dichloromethane and methanol with five consecutive stepwise gradients. The sample-to-stationary phase ratio was kept at about 1:5.

To separate the apolar ecdysteroids (fraction 9), silica column chromatography was employed. Here, a stepwise gradient was used but with dichloromethane–ethanol constituents. The applied five gradient steps were not steeply changed as straight dichloromethane, dichloromethane–ethanol 99:1, 98:2, 96:4 and 94:6 were used (Table 2). The sample-to-stationary phase ratio was about 1:30. Fractions containing ecdysteroids were further purified first on preparative straight-phase TLC (system No. 2) giving two major, ecdysteroid-containing bands (at $R_F = 0.32$ and 0.51, representing 9.1 and 9.2, respectively). After the separated bands had been scarped and eluted from the sorbent, they were analysed by TLC using the same developing systems as in the case of preparative TLC (system No. 2). The bands were not homogenous, due, we suppose, to the decomposition of the ecdysteroids on the adsorbent. Hence, further purification was done using preparative reversed-phase thin-layer chromatography (RP-TLC, system No. 4). Each band consisted of one major and several minor components. The major ecdysteroids of the $R_F =$ 0.63 and 0.41 (9.1 and 9.2, respectively) were further purified by straight-phase HPLC. Figs. 2 and 3 show the HPLC separation of components of 9.1 and 9.2, respectively. Each fraction gives one major and several minor peaks indicating the further decomposition of these compounds. Decomposition of ecdysteroids takes place in aqueous solution during storage also.

The final purification of the ecdysteroids ($R_F =$ 0.63 corresponds to 9.1, while $R_F = 0.41$ corresponds to 9.2) was done using normal phase HPLC (NP-HPLC). To reduce any decomposition, the identification of the isolated compounds was done immediately after their purification using both HPLC (comparison with available synthetic references) and spectroscopic methods. Table 3 gives the retention data in two HPLC systems, and Table 4 the MS data of the isolated ecdysteroids.

On the basis of these data, tentative chemical structures were suggested, which were further assessed by NMR spectroscopy (Table 5). The major component of 9.1 is 2d20E 3-crotonate. Its structure is established on the following basis: ^{1}H-NMR (Table 5) shows the presence of two coupled ethylenic signals and of a methyl group coupled with these signals. Analysis of the coupling constant due to homodecoupling experiments shows a large $3J$ coupling constant between

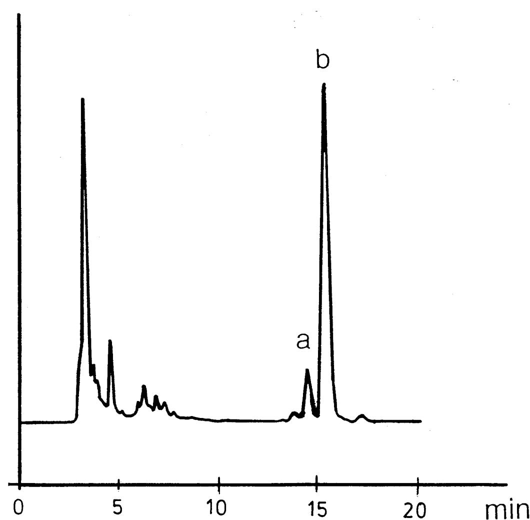

Fig. 2. HPLC separation of fraction 9.1 using Zorbax®-Sil (250 × 4.6 mm). Mobile phase: dichloromethane–2-propanol–water (125:15:1.0, v/v/v) with a flow rate of 1.0 ml min^{-1}.

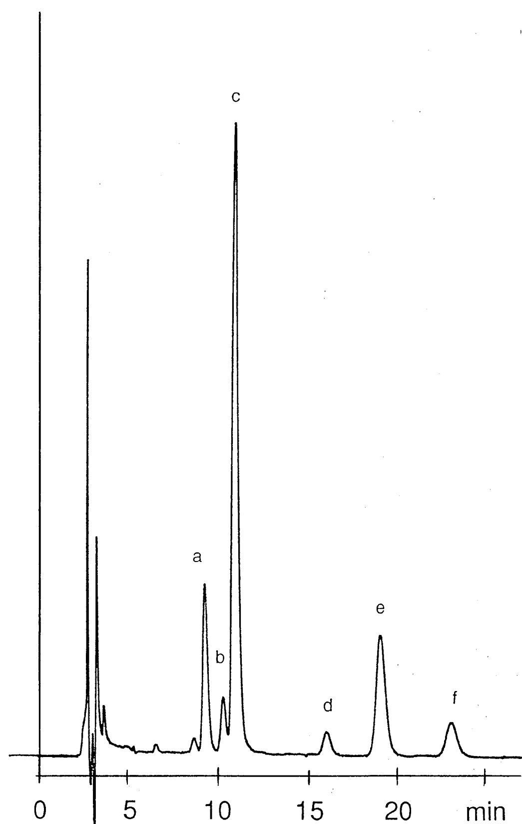

Fig. 3. HPLC analysis of fraction 9.2 using Zorbax®-Sil (250 × 4.6 mm). Mobile phase: cyclohexane–2-propanol–water (100:30:1.5, v/v/v) with a flow rate of 1.0 ml min^{-1}.

the two ethylenic signals typical of trans-stereochemistry with respect to the double bond for these two ethylenic protons. A coupling constant ($3J$ CH_3–CHb = 7.1 Hz) and a small allylic constant ($4J$ CH_3–CHa = 1.6 Hz) are observed for the methyl group and the ethylenic protons. All these elements are in agreement with a crotonate structure for the ester group. Finally, these crotonate groups are assigned to the 3 position, indicated by the large downfield shifts observed for the 3-He signal.

Three pure ecdysteroids were isolated from the 9.2 band (Fig. 3, peaks a, b, c), namely, 2d20E 3,22-diacetate, 5α-2d20E 3-acetate and 2d20E 3-acetate.

Their characterisation relies on the comparison with synthetic reference compounds and on MS data. The peak that was identified as 2d20E 3,22-diacetate was ascertained on the following basis: ^{1}H-NMR (Table 5) shows the presence of two acetyl groups (singlet methyl signals at $\delta = 2.04$ and 2.10 ppm). The positions of these two acetyl groups are assigned to the 3 and 22 positions respectively, which is indicated by the large downfield shifts observed for the 3-He and 22-H.

The structural elucidation of the isolated compounds suggests a pathway for the decomposition of ecdysteroids: the characteristic changes are deacetylation of the ecdysteroid 22-acetates, as well as $5\beta \rightarrow 5\alpha$ isomerisation, that seem both to take place continuously during the purification process.

The first, so-called coarse column chromatographic separation resulted in certain 20-hydroxyecdysone-rich fractions (Nos. 21–26). From these fractions, the majority of 20-hydroxyecdysone was obtained by crystallisation. The mother liquid was fractionated by DCCC, and two of the combined fractions (fractions 60–72 and 97–100) were further purified by HPLC. Two additional ecdysteroids were isolated: 24,28-dehydromakisterone A (fractions 60–72) and ecdysone (fractions 97–100). Their structural identification was done on the basis of their chromatographic characteristics and MS/NMR data compared with those of authentic reference molecules.

4. Discussion

In general, the plant ecdysteroids consist of a few major components with numerous components in trace amount [10]. The increase of the number of identified phytoecdysteroids through the last decade has been due to the development of more efficient analytical methods that allow not only the major but also the minor components of the different plant species to be isolated.

Our previous work has dealt with the isolation of several major ecdysteroids (such as 2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone 22-acetate, 20-hydroxyecdysone) from *Silene otites* (L.) Wib. These ecdysteroids are present in the plants in the

Table 4
Data of chemical ionisation mass spectra (CI/D, NH_3) (recorded between m/z 200 and 600)

Compound	Molecular weight	Main ions $(m/z)^+$	
9.1 b	532	550 $(M+H+NH_3)^+$	100%
		533 $(M+H)^+$	36%
		515 $(M+H-H_2O)^+$	8
		447 $(M+H-\text{crotonic acid})^+$	3%
		432 $(550-C_{22}–C_{27})^+$	6%
		417 $(M+H-C_{22}–C_{27})^+$	3%
9.2 a	548	566 $(M+H+NH_3)^+$	100%
		549 $(M+H)^+$	27%
		531 $(M+H-H_2O)^+$	4%
		506 $(M+H+NH_3–\text{acetic acid})^+$	8%
		489 $(M+H-\text{acetic acid})^+$	2%
		471 $(489-H_2O)^+$	1%
		453 $(489-2H_2O)^+$	1%
		406	10%
		389	3%
9.2 b	506	524 $(M+H+NH_3)^+$	27%
		507 $(M+H)^+$	60%
		489 $(M+H-H_2O)^+$	55%
		471 $(M+H-2H_2O)^+$	100%
		453 $(M+H-3H_2O)^+$	30%
		447 $(M+H-\text{acetic acid})^+$	3%
		429 $(489-\text{acetic acid})^+$	2%
		406 $(524-C_{22}–C_{27})^+$	22%
		389 $(M+H-C_{22}–C_{27})^+$	18%
9.2 c	506	524 $(M+H+NH_3)^+$	100%
		507 $(M+H)^+$	86%
		489 $(M+H-H_2O)^+$	6%
		471 $(M+H-2H_2O)^+$	8%
		453 $(M+H-3H_2O)^+$	3%
		406 $(524-C_{22}–C_{27})^+$	15%
		389 $(M+H-C_{22}–C_{27})^+$	5%
DCCC fraction 60–72	492	510 $(M+H+NH_3)^+$	36%
		493 $(M+H)^+$	71%
		475 $(M+H-H_2O)^+$	18%
		457 $(M+H-2H_2O)^+$	100%
		439 $(M+H-3H_2O)^+$	18%
		380 $(510-C_{22}–C_{27})^+$	16%
		363 $(M+H-C_{22}–C_{27})^+$	14%
		345 $(363-H_2O)^+$	7%
DCCC fraction 97–100	464	482 $(M+H+NH_3)^+$	44%
		465 $(M+H)^+$	48%
		447 $(M+H-H_2O)^+$	100%
		429 $(M+H-2H_2O)^+$	38%
		411 $(M+H-3H_2O)^+$	1%

range 0.01–1%, when the ecdysteroid content is calculated on the dry weight basis. Applying a more effective separation method, additional ecdysteroids have been isolated which are present in *Silene otites* (L.) Wib. only in trace amounts, i.e., less then 0.001% [13].

Table 5
^{1}H-NMR data for 2d20E and derivatives

	2d20E 3-crotonate $CDCl_3$	2d20E 3,22-diAc $CDCl_3$	2d20E CD_3OD	2d20E 22Ac CD_3OD
3-He	5.13 (sb,$w_{1/2}=12$)	5.07 (sb,$w_{1/2}=12$)	3.98 (sb,$w_{1/2}=12$)	3.98 (sb,$w_{1/2}=12$)
5-H	2.39 (d,d)	2.37	2.38 (d,d,12,5)	2.43 (d,d,12,5)
7-H	5.85 (d,2.5)	5.84 (d,2.5)	5.80 (d,2.5)	5.79 (d,2.5)
9-Ha	3.11 (m,$w_{1/2}=22$)	3.09 (m,$w_{1/2}=22$)	3.20 (m,$w_{1/2}=22$)	3.20 (m,$w_{1/2}=22$)
17-H	2.37	2.38	2.39 (m)	2.39 (m)
22-Hb	3.44 (d,10)	4.85 (d,d,11,2)	3.33 (d,d,11,2)	4.87 (d,d,11,2)
18-Me	0.86 (s)	0.84 (s)	0.89 (s)	0.87 (s)
19-Me	0.98 (s)	0.97 (s)	0.96 (s)	0.95 (s)
21-Me	1.21 (s)	1.26 (s)	1.19 (s)	1.28 (s)
26-Me	1.24 (s)	1.20 (s)	1.19 (s)	1.15 (s)
27-Me	1.25 (s)	1.22 (s)	1.20 (s)	1.16 (s)
3-CH_3CO	—	2.04 (s)	—	—
22-CH_3CO	—	2.10 (s)	—	2.08 (s)
	<u>CH</u>$_3$CH = 1.87 (d,d,7.1,1.6)	—	—	—
	C<u>Hb</u> = 6.95 (d,q,16,7.1)	—	—	—
	= C<u>Ha</u>5.83 (d,q,16,1.6)	—	—	—

For ^{1}H-NMR data for 2d20E 3Ac and 5α-2d20E 3Ac, see Ref. [6]. Multiplicity of signals: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad signal; $w_{1/2}$, width at half-height in Hertz; δ in ppm.

The isolation of these minor ecdysteroids requires an efficient multi-step purification method. Recent work deals with the isolation of six ecdysteroids that have hitherto not been isolated from this plant. Their structural elucidation has also been done and the structures of the three new ecdysteroids are shown in Fig. 4.

The pre-purification of apolar ecdysteroids of *Silene otites* (L.) Wib. required the use of a RP-TLC step after having already performed extraction, fractionated precipitation and adsorption chromatography.

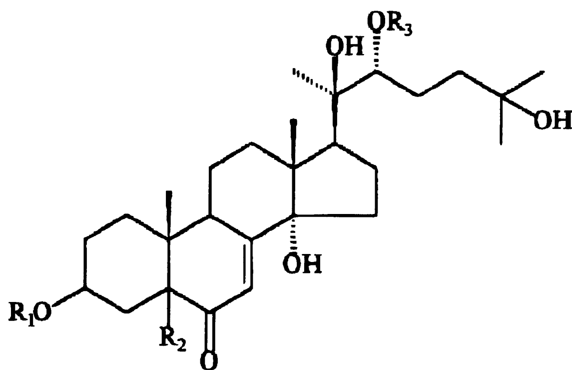

Fig. 4. Chemical structures of new ecdysteroids.

The earlier steps of isolation consisted of adsorption chromatography. During this step, two kinds of reaction on ecdysteroids acetates could take place by the effect of adsorbent (first of all, aluminium oxide). One such reaction is the 3↔2 acyl migration (when a 2,3-diol is present [14]) and deacetylation of 22-acetates (when a 20,22-diol is present). These reactions have been observed in the cases of other steroids [16–21]. We have observed the deacetylation of 22-acetates during our earlier work [22]. In our case, partial conversion of 2d20E 322Ac into 2d20E 3Ac is suggested, which means that the original concentration of the former product is probably underestimated.

The simultaneous occurrence of these two ecdysteroid acetates in the same column fraction provides good evidence that deacetylation takes place. It is therefore difficult to decide whether 2d20E 3Ac is originally present in the plant or if it is an artefact. Despite the above mentioned deacetylation reaction, we consider that 2d20E 3Ac is a genuine compound as it occurs in 32% in the pre-purified crude extract (not shown).

The same question may be asked with regard to the occurrence of 5α compound. It is well known that 5β↔5α isomerisation can take place under

slightly alkaline condition, and that the 5α configuration is the most stable in the case of 2-deoxyecdysteroids [23]. Therefore, the isolation of such ecdysteroids might be due to their formation during the drying process of the plant, the purification procedure or the subsequent storage.

Two of the three newly isolated ecdysteroid acetates, 2-deoxy-20-hydroxyecdysone 3,22-diacetate and 5α-2-deoxy-20-hydroxyecdysone 3-acetate, are new natural products. We isolated earlier three ecdysteroids acetates (all of them 22-acetates [13]) from this plant, and the ecdysteroid acetates of *Silene otites* (L.) Wib. have hereby been expanded by three new members.

From the isolated apolar *Silene otites* ecdysteroids, 2-deoxy-20-hydroxyecdysone 3-crotonate is also a new natural product. This is a new type of ester derivative of ecdysteroids because, hitherto, in plants only sulphates, acetates, cinnamates and benzoates of ecdysteroids have been found. The biological function of these various ecdysteroid ester derivatives is probably protection against insects [24].

Minor compounds from other 9.1 fractions that were present in amounts too low for their complete structural elucidation were only analysed by HPLC and MS, and these data suggest that another three esters are present, e.g., 2d20E 3-tiglate (MW 546).

No doubt a more extensive analysis would lead to the identification of a very complex family of esters with short-chain fatty acids.

We have isolated two further minor ecdysteroids from the plant. For isolation of these ecdysteroids, we applied our methods elaborated earlier [13,25]. The minor ecdysteroids were enriched from the mother liquid of crystallisation using DCCC. The DCCC fractionation has been used following the first simple separation methods (such as solvent–solvent partition, fractionated precipitation) and adsorption chromatography. After DCCC, the purification required the use of a more efficient separation method (such as HPLC) [13]. As DCCC offers a selectivity that differs from that of adsorption chromatography, their combination is very efficient. In the present study, these allowed the isolation of the ecdysone and 24,28-dehydromakisterone A (Fig. 4).

Although the occurrence of ecdysone was published in some *Silene* species [12], previously we failed to isolate it from *Silene otites* (L.) Wib. Using the above detailed combined chromatographic procedure, we hereby report the detection of the presence of low concentrations of ecdysone in *Silene otites* (L.) Wib. Most probably, ecdysone is rapidly metabolised by the enzymes of *Silene otites* (L.) Wib; its life-span in the plant must therefore be moderately short and does not accumulate. The 24,28-dehydromakisterone A is a rather common plant ecdysteroid [26].

The size of the phytoecdysteroid family is rapidly growing, and the present data suggest that a single plant species may contain very complex mixtures of minor ecdysteroids. It would be of interest to determine whether all compounds are similarly distributed within the plant or if some specialisation exists between, e.g., leaves, stems and flowers. Up to now, such studies have mainly considered quantitative aspects of major compounds only [27].

The described pharmacological effects of ecdysteroids [4] suggest their great potential importance in human medicine, a fact which is still neglected. In this paper, the isolation and structural elucidation of novel ecdysteroids are reported. These novel compounds are relatively hydrophobic. Either their isolation in larger quantity or their semisynthetic preparation will produce substances in quantity enough for the pharmacological investigations to facilitate their application as cosmetics and medicinal products.

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