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Identification and quantitative analysis of the phytoecdysteroids in *Silene* species (Caryophyllaceae) by high-performance liquid chromatography
Novel ecdysteroids from *S. pseudotites*

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Abstract

Many species in the genus *Silene* (Caryophyllaceae) have previously been shown to contain ecdysteroids and this genus is recognised as a good source of novel ecdysteroid analogues. We have used ecdysteroid-specific radioimmunoassays and the microplate-based *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonist and antagonist activities to identify further phytoecdysteroid-containing species in this genus. The main ecdysteroid components from 10 *Silene* species (*S. antirrhina*, *S. chlorifolia*, *S. cretica*, *S. disticha*, *S. echinata*, *S. italica*, *S. portensis*, *S. pseudotites*, *S. radicosa*, *S. regia*) were isolated and identified, mainly by normal-phase and reversed-phase high-performance liquid chromatography. The amount of each ecdysteroid was determined by comparing chromatogram peak areas with those for reference 20-hydroxyecdysone (20E) on reversed-phase HPLC. 20E is the most abundant ecdysteroid in each of the *Silene* extracts. Polypodine B, 2-deoxy-20-hydroxyecdysone and ecdysone are also common ecdysteroids in these *Silene* species, but the proportions of these ecdysteroids vary between the *Silene* species. HPLC proved to be a quick and effective way to screen *Silene* species, determine ecdysteroid profiles and, hence, identify extracts containing novel analogues. An extract of the aerial parts of *S. pseudotites* was found to contain several new ecdysteroids. These have been isolated and identified spectroscopically (by NMR and mass spectrometry) as 2-deoxyecdysone 22β-D-glucoside, 2-deoxy-20,26-dihydroxyecdysone and 2-deoxypolypodine B 3β-D-glucoside. Additionally, (5α-H)-2-deoxyintegristerone A (5α-^{2}H 91%, 5α-^{1}H 9%) was isolated as an artefact. This study contributes to the understanding of ecdysteroid distribution in *Silene* species and provides further information on the chemotaxonomic significance of ecdysteroids in *Silene* species.

Keywords: *Silene* spp; Plant materials; Phytoecdysteroids; Steroid hormones; Ecdysteroids; Hydroxyecdysone

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1. Introduction

Phytoecdysteroids are analogues of insect steroid hormones occurring in plants. They are believed to contribute to insect deterrence by acting as antifeedants or by interfering in ecdysteroid metabolism or mode of action on ingestion by phytophagous insects [1]. During preliminary screening of plants for phytoecdysteroids using the *Drosophila melanogaster* microplate-based B_{II} cell bioassay and ecdysteroid-specific radioimmunoassays (RIAs), we found that several members of the genus *Silene* (Caryophyllaceae) are rich in ecdysteroids [2], in agreement with previous studies on the chemical constituents of *Silene* species [3–5]. Ecdysteroids usually occur in plants as a complex cocktail of structurally different compounds, but the major components are usually common ecdysteroids like 20-hydroxyecdysone (**1**: 20E) and polypodine B (**2**: polB) [6] (Fig. 1). HPLC is not only a powerful way to separate and isolate these structurally similar compounds, but, by using standardised analytical systems, also a means to quantify common ecdysteroids and as a form of dereplication strategy to identify extracts containing novel analogues. We report here the isolation and identification of the ecdysteroids of *S. regia*, *S. radicosa* and *S. pseudotites* by reversed-phase and normal-phase HPLC analyses, as well as by NMR and mass spectroscopy. We have also identified the main ecdysteroid components in seven further *Silene* species by reversed-phase and normal-phase HPLC. In order to compare the amounts of main ecdysteroid components in these 10 *Silene* species, the main ecdysteroid components were quantified by comparison of the peak area of each ecdysteroid in their chromatograms with those for known amounts of a standard of 20E on reversed-phase HPLC. This study contributes to our understanding of the distribution of ecdysteroids in the genus *Silene*.

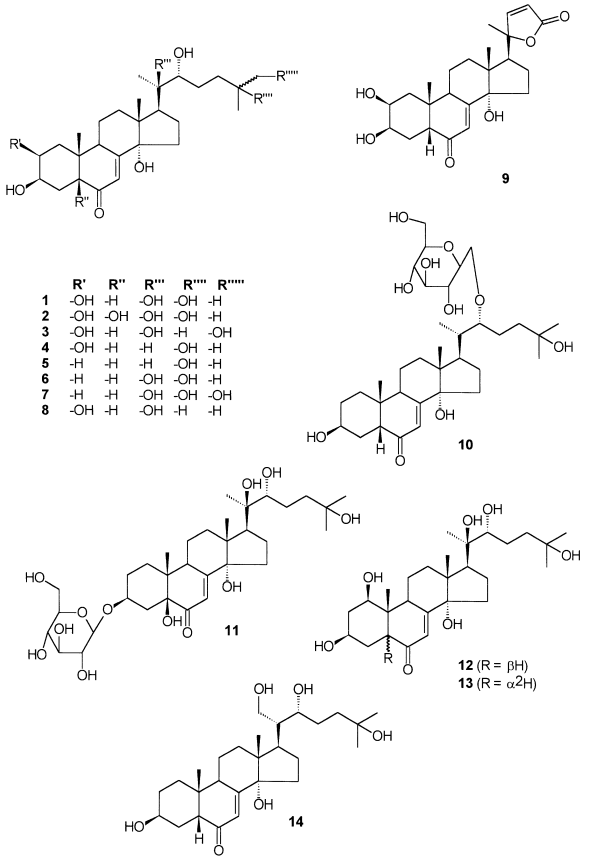

Fig. 1. Structures of the ecdysteroids.

2. Experimental

2.1. General experimental procedures

UV spectra were determined on a Shimadzu UV-2402 PC spectrophotometer. NMR spectra were obtained on (i) a Bruker Avance DRX400 instrument (in [$^2H_4$]methanol) using standard Bruker microprograms or (ii) a Bruker AMX500 at 300 K; samples were lyophilised and dissolved in ^2H_2O. $TSPd_4$, [2,2,3,3-$^2H_4$]sodium 3-(trimethylsilyl)propionate, was used as internal reference for the proton and carbon shifts. Chemical shifts are expressed in ppm. Mass spectra were recorded in desorption/chemical ionisation (CI/CID) mode with ammonia as the reagent gas on a Jeol JMS-700 spectrometer. Liquid secondary ion mass spectrometry (LSIMS) spectra were obtained at the University of Liverpool by Professor H.H. Rees and Mr. M. Prescott on a VG Quattro triple quadrupole mass spectrometer using a Cs^+ primary beam and glycerol as the matrix. Solid-phase extraction (SPE) C_{18} cartridges [Waters Sep-Pak Vac 35cc (10 g)] were used for pre-HPLC fractionation; HPLC: (a) semipreparative; Gilson

Model 806 HPLC coupled with a Gilson UV–visible detector, (b) analytical; Gilson Model 811 HPLC coupled with a Gilson 160 diode-array detector and using Gilson Unipoint computer program; Sphereclone semipreparative C_{18} column (5 μm particle size; 250×10 mm; Phenomenex, Macclesfield, UK), Spherisorb C_6 semipreparative column (5 μm particle size; 250×10 mm; Jones Chromatography, Hengoed, UK), Apex II Diol semipreparative column (5 μm particle size; 150×4.6 mm; Jones Chromatography) and Spherisorb 5 ODS-2 analytical C_{18} and C_6 column (both 5 μm particle size; 150×4.6 mm; Jones Chromatography). Chromatographic separations were generally monitored at 242 nm.

2.2. Reagents and standard ecdysteroid samples

All organic solvents (BDH, Poole, UK) for HPLC and SPE fractionation were HPLC or analytical grade, as appropriate. Water for HPLC and SPE fractionation was ultra-pure water (Elgasta Maxima Ultra-pure Water System; Elga, UK). Reference ecdysteroids [**1** (20E), **2** (polB), **3** (inokosterone; Ino: a mixture of 25*R*- and 25*S*-isomers), **4** (ecdysone; E), **6** (2-deoxy-20-hydroxyecdysone; 2d20E) and **8** (ponasterone A; ponA)] were samples previously isolated in our laboratory and their identities and purities were confirmed by NMR and chromatographically by NP- and RP-HPLC.

2.3. Radioimmunoassay

RIA was performed according to the procedure described previously [7] using the ecdysteroid-specific antisera, DBL-1 and Black, which were generously donated by Professor Jan Koolman (University of Marburg, Germany). The cross-reactivities of these antisera to a range of phytoecdysteroids are given elsewhere [2].

2.4. Bioassay

Ecdysteroid agonist activities of the extract, SPE fractions and HPLC fractions were assessed with a microplate-based bioassay using the *Drosophila melanogaster* B_{II} cell line [8].

2.5. Plant material

Seeds of *S. regia* Sims were purchased from B&T World Seeds. A voucher specimen has been retained at the Department of Biological Sciences, University of Exeter. Leaves of *S. portensis* L., *S. radicosa* Boiss. & Heldr. in Boiss., aerial parts of *S. italica* (L.) Pers., *S. cretica* L., *S. pseudotites* Besser ex Reichenb., *S. antirrhina* L., *S. disticha* Willd., *S. chlorifolia* Smith. and *S. echinata* Otth. originated at the Siberian Botanical Garden, Tomsk, Russia.

2.6. Extraction of plant material

Seeds of *S. regia* (13.1 g), dried and ground leaves of *S. radicosa* (56.6 g) and *S. portensis* (93.7 g), dried and ground aerial parts of *S. italica* (20 g), *S. cretica* (40 g), *S. pseudotites* (40 g), *S. antirrhina* (13.9 g), *S. disticha* (40 g), *S. chlorofolia* (19 g) and *S. echinata* (40 g) were extracted three or four times (3×24 h) with 4×300 ml, 3×700 ml, 4×900 ml, 3×160 ml, 3×300 ml, 3×330 ml, 3×120 ml, 3×300 ml, 3×150 ml MeOH at 55°C with stirring using a magnetic stirrer (for *S. regia* and *S. radicosa*) and repeated agitation using an ultrasonic bath (for the other eight *Silene* species), respectively. Extracts of each species were pooled.

2.7. Isolation and identification of ecdysteroid components from the seeds of S. regia, leaves of S. radicosa and aerial parts of S. pseudotites

The pooled extracts of the seeds of *S. regia*, leaves of *S. radicosa* or aerial parts of *S. pseudotites* were evaporated to 210 ml, and each of these was made to a 70% aq. methanolic solution by adding 90 ml water. After being defatted with *n*-hexane (4×150 ml), the aqueous phase for each species was concentrated using a rotary evaporator at a maximum temperature of 45°C. The concentrated residues of the three *Silene* species were redissolved in 10% MeOH in water and then were subjected to SPE fractionation using a MeOH–water step-gradient elution with 10, 25, 60, 80 and 100% MeOH in water. Bioassay/RIA of the five fractions of each species revealed the presence of ecdysteroids mainly in the MeOH–water (60:40) (all three *Silene* species) and in the MeOH–water (25:75) fractions (*S. radi-*

cosa and *S. pseudotites*). The MeOH–water (25:75) fractions of *S. radicosa* and *S. pseudotites* were shown to contain only 20E (by co-chromatography with reference 20E). The MeOH–water (60:40) fraction of *S. regia* was subjected to RP-HPLC using a semipreparative C_{18} RP column (isocratic elution with 40% MeOH in water at 2 ml min^{-1} over 50 min, followed by elution with MeOH for a further 10 min) to yield five fractions (P1–P5). P2 and P3 were found to be active in the B_{II} bioassay and were then isolated by RP-HPLC using a C_6 RP column (isocratic elution with 38% MeOH in water, 2 ml min^{-1}); P2 afforded polB (**2**: 2.7 mg), P3 afforded 20E (**1**: 39.6 mg) and 25*S*-inokosterone (**3**: 1.5 mg). The MeOH–water (60:40) fraction of *S. radicosa* was subjected to HPLC fractionation using a semipreparative C_{18} RP column (isocratic elution with 39% MeOH in water at 2 ml min^{-1} over 35 min, followed by elution with MeOH for a further 10 min) to yield five fractions. Fraction 5 was found to be bioactive in the B_{II} bioassay. When assessed by analytical HPLC with a C_6 column coupled to a photodiode-array detector, fraction 5 was found to contain two compounds, which on further fractionation on NP-HPLC using an Apex II Diol semipreparative column afforded polB (**2**: 3.9 mg) and 20E (**1**: 23.5 mg). The MeOH–water (60:40) fraction of *S. pseudotites* was separated on a RP semipreparative C_{18} column, eluted at 2 ml min^{-1} with a linear gradient from 45 to 73% MeOH in water over 42 min, followed by elution with MeOH for a further 10 min. Seven fractions (P1–P7) were obtained. P2, P3, P5 and P7 possessed agonist activity in the B_{II} bioassay. Assessment by photodiode-array detector-assisted HPLC using an analytical C_{18} column (eluted at 1 ml min^{-1} with a linear gradient from 30 to 100% MeOH in water over 30 min, followed by elution with MeOH for a further 10 min), P2, P5 and P7 were the major components of *S. pseudotites* and were isolated almost pure. Further purification of P2, P5 and P7 by RP-HPLC using a semipreparative C_{18} RP column (isocratic elution with 40% MeOH in water at 2 ml min^{-1} over 40 min) afforded 20E (**1**: 28.3 mg), 2d20E (**6**: 42.3 mg) and 2dE (**5**: 65.9 mg), respectively. Further purification of P3 by RP-HPLC using a semipreparative C_{18} RP column (isocratic elution with 40% MeOH in water at 2 ml min^{-1}) and NP-HPLC [Zorbax-Sil semipreparative column, 5 μm particle size, 25 cm×9.4 mm I.D., eluted with dichloromethane–isopropanol–water (125:40:3) at 4 ml min^{-1}, with detection at 245 nm] afforded eight components: 2-deoxy-21-hydroxyecdysone (**14**; 5.05 mg), sidisterone (**9**; 0.49 mg), 2-deoxyintegristerone A (**12**; 0.54 mg) and [5α-^{2}H]2-deoxyintegristerone A (**13**; 0.44 mg), 2-deoxypolypodine B 3β-D-glucoside (**11**: 1.26 mg), E (**4**: 1.29 mg), 2-deoxy-20,26-dihydroxyecdysone (**7**: 1.01 mg) and 2-deoxyecdysone 22β-D-glucoside (**10**: 1.03 mg).

The identities of 20E (**1**), polB (**2**), 25*S*-inokosterone (**3**) and E (**4**) were confirmed by comparing their retention times with those of standard samples on both RP-HPLC using an analytical C_{18} column and NP-HPLC using an analytical Apex II diol column, and comparing their ^{1}H NMR data with those published [9]. The identities of 2dE (**5**), 2d20E (**6**), sidisterone (**9**) and 2-deoxy-21-hydroxyecdysone (**13**) were determined by comparing their ^{1}H NMR data, ^{13}C NMR data and CI-MS data with those published [6,9]. The identities of the new ecdysteroids [2-deoxy-20,26-dihydroxyecdysone (**7**), 2-deoxyecdysone 22-glucoside (**10**), 2-deoxypolypodine B 3β-glucoside (**11**) and [5α-^{2}H]2-deoxyintegristerone A (**13**)] were deduced from their UV, ^{1}H and ^{13}C NMR and mass spectra (see Results and discussion).

2.8. Identification of ecdysteroids from leaves of S. portensis, and aerial parts of S. italica, S. cretica, S. antirrhina, S. disticha, S. chlorofolia and S. echinata

Portions of the methanol extracts (2.5 mg seed equivalents) were dissolved in 200 μl 70% aqueous methanol solution and defatted with hexane (3×150 μl). The aqueous methanol phase was dried and dissolved in 500 μl 30% aqueous methanolic solution, from which 30 μl was separated by a C_{18} analytical column (eluted with a linear gradient from 30 to 100% MeOH in water at 1 ml min^{-1} over 30 min, followed by elution with MeOH for a further 10 min) and monitored by a photodiode-array detector. The ecdysteroid peaks in the chromatograms of each *Silene* species were easily recognised by their UV spectra (λ_{max}: 242–250 nm). Co-injection of refer-

ence ecdysteroids (**1**, **4**, **6** and **8**) confirmed the identities of the peaks. Individual ecdysteroid peaks were collected using a semipreparative C_{18} column (eluted at 2 ml min^{-1} with a linear gradient from 30 to 60% MeOH in water over 40 min). The peak corresponding to 20E in each *Silene* sample was fractionated on both a reversed-phase analytical HPLC system (analytical C_6 column, eluted with an isocratic solution of 28% MeOH in water over 37 min at 1 ml min^{-1}) and a normal-phase HPLC system (Apex II analytical diol column, eluted with a linear gradient from 4 to 20% MeOH in CH_2Cl_2 over 30 min at 1 ml min^{-1}, followed by elution with 20% MeOH in CH_2Cl_2 for a further 10 min). By comparing the retention times of the resulting peaks with those of polB, 20E, 25*S*-inokosterone and 25*R*/*S*-inokosterone and also by co-injection of the peak material with appropriate reference ecdysteroids, the compounds were identified. For the peaks corresponding to 2d20E and E for each *Silene* species, comparison of the retention time and co-injection with those of standards of 2d20E and E on two reversed-phase analytical HPLC systems (a C_{18} column, eluted with a linear gradient from 30 to 100% MeOH in water over 30 min, and a C_6 column eluted isocratically with 45% MeOH in water over 50 min) allowed the identities to be confirmed.

2.9. Quantitative analysis of the ecdysteroid components identified from all 10 Silene species

2.9.1. Calibration of RP-HPLC with standard 20E

HPLC-purified 20E (4.8 mg) was dissolved in 2 ml MeOH. The exact concentration was verified by UV spectrophotometry ($\lambda_{max} = 242$ nm; $\varepsilon = 12\,400$ l mol^{-1} cm^{-1}). Appropriate aliquots of the solution were mixed with water to bring them to 30% methanol in a Hamilton syringe and injected onto RP-HPLC, using the same conditions as described in Section 2.8. Peak areas were integrated and related to the amount of 20E injected. This linear relationship was used to quantify the ecdysteroid components in each *Silene* sample.

2.9.2. Quantification of ecdysteroids in the 10 Silene extracts

Using the same procedures as described in Section 2.8, portions of the extracts were defatted and separated by analytical C_{18} HPLC. The amounts of E, 2dE and 2d20E were calculated based on comparing their peak areas to the calibration curve generated for 20E. For 20E, polB and Ino, because they are not fully resolved on a C_{18} column when eluted with a gradient of MeOH–water, the combined material was further separated by an analytical C_6 column (elution with isocratic mobile phase) or by an Apex II analytical diol column (elution with gradient mobile phase), as described in Section 2.8, and calculating the ratio of the peak areas corresponding to polB, 20E and Ino and the proportions of the total mass (20E + polB + Ino) determined from the C_{18} HPLC chromatogram.

3. Results and discussion

3.1. Ecdysteroid profiles

Preliminary RIA of the methanol extracts of *Silene* samples demonstrated that all the extracts were RIA-positive, indicating that they contain ecdysteroids. Enzymic hydrolysis of portions of each of the extracts with a mixture of hydrolases from *Helix pomatia*, followed by RIA revealed that most *Silene* species are rich in free ecdysteroids and contain only low levels of hydrolysable ecdysteroid conjugates. Most of the *Silene* species possess ecdysteroid agonist activity, but none of them shows antagonist activity, supporting the presence of phytoecdysteroids.

RP-HPLC–RIA chromatograms for eight of the 10 *Silene* species are shown in Figs. 2 and 3, with the identified ecdysteroids indicated. The UV profiles show that the profiles of the methanol extracts are relatively simple and that the major components could be separated readily with this HPLC procedure. This laid a foundation for quantifying the peaks according to their peak areas on RP-HPLC. Further separation of the peak material eluting with the t_R of 20E on the C_{18} gradient system on a C_6 analytical column separated polB and Ino from 20E, permitting the ready identification and quantification of each of these three ecdysteroids. Analytical NP-HPLC was also used to confirm the identity and integrity of these three components. Identification of other ecdysteroid components such as 2dE, 2d20E

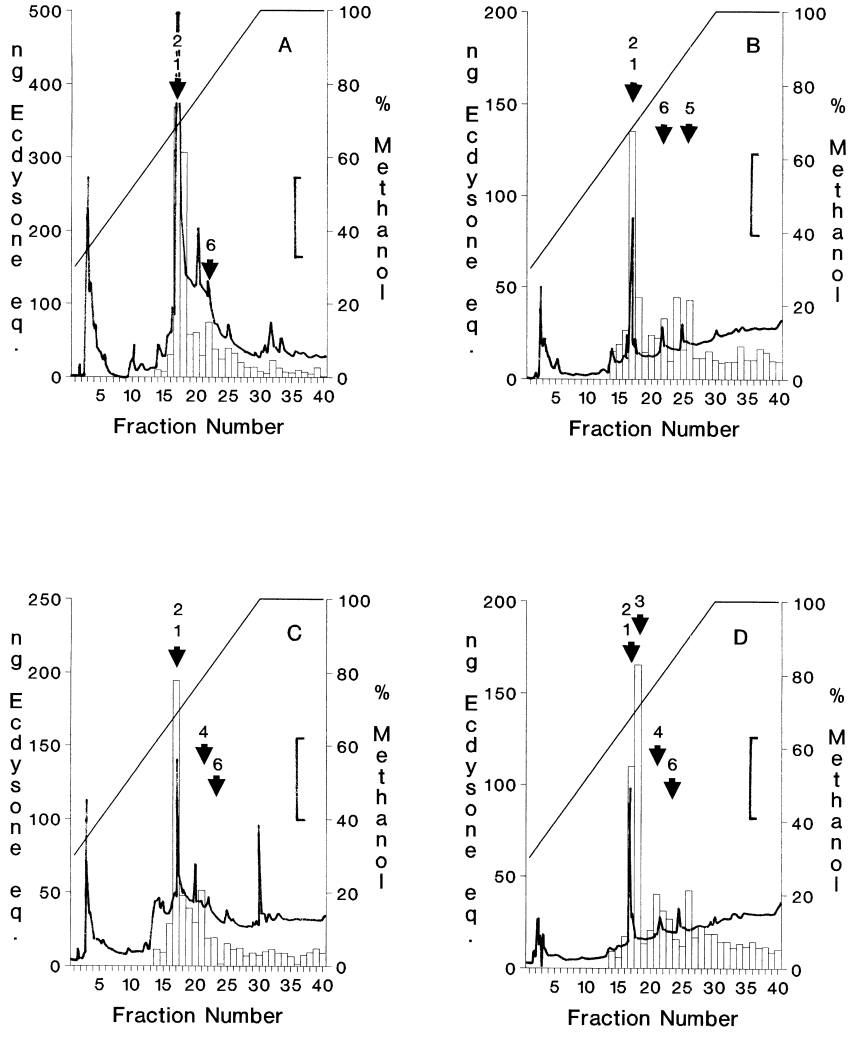

Fig. 2. RP-HPLC–RIA of portions (1 μg ecdysone equivalents with the DBL-1 antiserum) of extracts of aerial parts of (A) *Silene antirrhina*, (B) *S. chlorifolia*, (C) *S. cretica* and (D) *S. disticha*. Samples were separated on a C_{18} analytical column (25 cm×4.6 mm I.D.; 5 μm particle size) eluted at 1 ml min^{-1} with a linear gradient from 30% methanol in water to 100% methanol over 30 min, followed by elution with methanol for 10 min. Column effluent was monitored at 242 nm. Fractions of 1-min duration were collected and appropriate aliquots were subjected to RIA (DBL-1 antiserum). The identities of the major peaks identified are: **1**=20-hydroxyecdysone, **2**=polypodine B, **3**=inokosterone, **4**=ecdysone, **5**=2-deoxyecdysone and **6**=2-deoxy-20-hydroxyecdysone. The vertical bar in each panel represents 0.05 AU.

and E was conducted by comparing retention times and performing co-injections with reference ecdysteroids on both C_{18} and C_6 columns. NP-HPLC could not be used to identify ecdysone, as the amount in the extract was too low in all the *Silene* samples and sensitivity of detection is reduced on NP-HPLC, owing to the high background absorbance of the mobile phase. However, as the identification of these minor components was performed on two columns (C_6 and C_{18}) and each with two mobile phases (isocratic and gradient), the identifications and quantifications should be reliable. The quantitative analyses of ecdysteroids in the 10 *Silene* samples are summarised in Table 1. 20E is the most

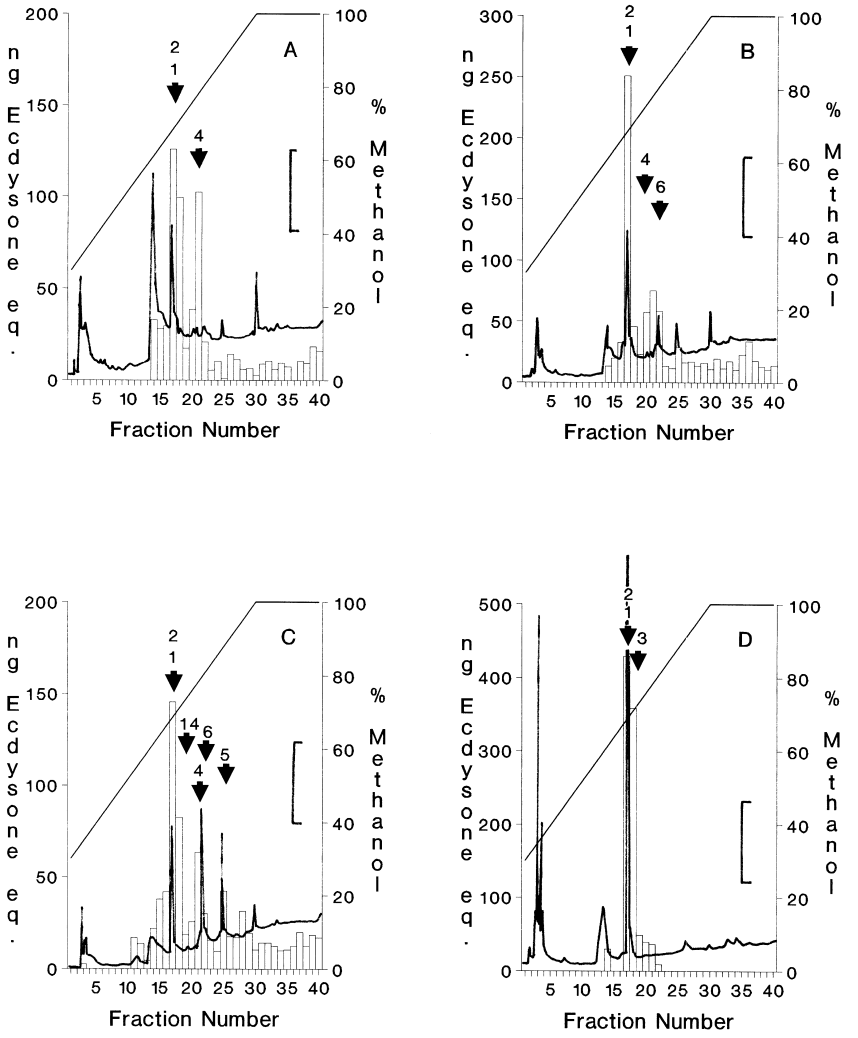

Fig. 3. RP-HPLC–RIA of portions (1 μg ecdysone equivalents with the DBL-1 antiserum) of extracts of (A) *Silene echinata* (aerial parts), (B) *S. italica* (aerial parts), (C) *S. pseudotities* (aerial parts) and (D) *S. regia* (seeds). Conditions as for Fig. 2.

abundant ecdysteroid component in each *Silene* species; its concentration in plant material is in the range 0.12–1.2% of the dry mass. Additionally, polB, 2d20E and E are common ecdysteroids in these *Silene* species, but their concentrations vary extensively. In *Silene pseudotites*, the major ecdysteroids are 20E, 2d20E and 2dE and the contents of 2d20E and 2dE were so high that they approached that of 20E. Additionally, several minor ecdysteroids, including E, were also isolated from *S. pseudotites* (see below). The total concentration of ecdysteroids in the aerial parts of *S. pseudotites* is >2% of the dry mass. A summary of the retention times of the ecdysteroids in various HPLC systems is presented in Table 2.

3.2. Identification of ecdysteroids in S. pseudotites

The compounds from *S. pseudotites* were identified as follows.

Table 1
Quantitative analyses of the major ecdysteroid components from 10 *Silene* species as determined from peak areas on RP-HPLC

Species	Ecdysteroid concentration (%, w/w)						
	1	**2**	**3** (25*R*)	**3** (25*S*)	**4**	**5**	**6**
S. antirrhina	0.119	0.012					0.010
S. chlorifolia	1.00	0.069					0.023
S. cretica	0.118	0.050			t		0.007
S. disticha	0.455	0.076	t	t	0.008		0.009
S. echinata	0.740	0.069			0.061		
S. italica	0.547	0.192			0.022		0.081
S. portensis	0.581	0.027			0.016		0.061
S. pseudotites	1.147	0.021			t	0.366	0.707
S. radicosa	0.276	0.027			t		
S. regia	0.273	0.053		0.026			

t, trace amount.

20-Hydroxyecdysone (**1**): amorphous. UV, ^{1}H NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC.

Polypodine B (**2**): amorphous. UV, ^{1}H NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC.

Ecdysone (**4**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC. CI-MS: 465 $(M+H)^{+}$.

2-Deoxyecdysone (**5**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC. LSIMS (negative-ion mode, with glycerol as matrix): 447 $(M-H)^{-}$, 429 $(M-H-H_2O)^{-}$.

Table 2
HPLC retention times for ecdysteroids

Ecdysteroid	Retention time (min)				
	System A	System B	System C	System D	System E
1	10.3	18.0		9.4	18.5
2	10.3	15.4		7.0	13.5
3 (25*R*)	10.3	20.7		10.8	18.0
3 (25*S*)	10.3	22.0		11.3	18.0
4	13.3		13.4	7.9	13.5
5	17.5				8.3
6	14.6		14.5		10.5
7					23.7
8	16.8		16.8	4.2	5.5
9					8.0
10					23.5
11					38.0
12					14.0
13					22.5
14					12.5

System A: Spherisorb analytical C_{18} column (5 μm particle size, 25 cm×4.6 mm I.D.) eluted at 1 ml min^{-1} with a linear gradient from 30 to 100% MeOH over 30 min. System B: Spherisorb analytical C_6 column (5 μm particle size, 15 cm×4.6 mm I.D.) eluted at 1 ml min^{-1} with MeOH–water (28:72). System C: Spherisorb analytical C_6 column (5 μm particle size, 15 cm×4.6 mm I.D.) eluted at 1 ml min^{-1} with MeOH–water (45:55). System D: APEX II analytical Diol column (5 μm particle size, 15 cm×4.6 mm I.D.) eluted at 1 ml min^{-1} with a linear gradient from 4 to 20% MeOH in CH_2Cl_2 over 30 min. System E: Zorbax-Sil semipreparative column (5 μm particle size, 25 cm×9.4 mm I.D.) eluted at 4 ml min^{-1} with dichloromethane–isopropanol–water (125:40:3).

2-Deoxy-20-hydroxyecdysone (**6**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC. LSIMS (negative-ion mode, with glycerol as matrix): 463 $(M-H)^{-}$, 445 $(M-H-H_2O)^{-}$.

2-Deoxy-20,26-dihydroxyecdysone (**7**): CI-MS: 498 $(M+H+NH_3)^{+}$, 481 $(M+H)^{+}$, 480 $(M)^{+}$, 463 $(M+H\text{-}H_2O)^{+}$. ^{1}H NMR data (in $^{2}H_2O$) present the typical features of 2-deoxy and 20,26-dihydroxy compounds as reported [5]. 18-Me 0.863 (s); 19-Me 0.976 (s, broad, $w_{1/2}$ 4 Hz); 21-Me 1.235 (s); 27-Me 1.161(s); 26-CH_2-OH 3.45 (s); 7-H 5.96 (d, 2.2); 3-H 4.10 (s, broad $w_{1/2}$ 20 Hz); 22-H 3.44 (d, 10.5); 9-H 3.15 (s, broad $w_{1/2}$ 25 Hz); 5-H 2.39 (d,d, broad, 12.5, 3.4); 17-H 2.33 (t, 9.7).

Sidisterone (**9**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [4,9], co-chromatographs with reference on NP- and RP-HPLC. CI-MS: 434 $(M+H+NH_3)^{+}$, 417 $(M+H)^{+}$, 416 $(M)^{+}$, 399 $(M+H-H_2O)^{+}$.

2-Deoxyecdysone 22β-D-glucoside (**10**): CI-MS: 628 $(M+H+NH_3)^{+}$, 611 $(M+H)^{+}$, 610 $(M)^{+}$, 593 $(M+H-H_2O)^{+}$. ^{1}H NMR data (in $^{2}H_2O$) present the typical features of 2-deoxyecdysone compounds as reported [5]. The identity of the sugar as a β-D-glucopyranose was worked out from the characteristic signal for the anomeric proton at δ 4.53 (d, $J=8$ Hz) and ^{1}H–^{1}H coupling patterns observed by ^{1}H NMR. While in the ^{13}C NMR spectrum the chemical shift for C-1 (δ 101.8) supported the presence of the β-D-glucopyranose unit, the attachment was confirmed from the ^{1}H–^{13}C long-range coupling between the anomeric proton (1′-H) and C-22 in the heteronuclear multiple-bond correlation spectrum. This attachment is also confirmed by observation of an nOe 1′-H⇔22-H. Thus, the structure was assigned unambiguously. 18-Me 0.744 (s); 19-Me 0.990 (s, broad, $w_{1/2}$ 4 Hz); 21-Me 0.956 (d, 6.8); 26-Me 1.238 (s); 27-Me 1.243 (s); 7-H 5.97 (d, 2.1); 3-H 4.10 (s, broad $w_{1/2}$ 23 Hz); 22-H 3.73 (d, broad 10.5); 9-H 3.14 (m, broad $w_{1/2}$ 25 Hz); 5-H 2.41 (d,d, broad, 12.3, 3.2); 17-H 2.18 (m); 1′-H 4.53 (d, 8); 2′-H 3.29 (d,d, 9.1, 8.1); 3′-H 3.48 (t, 9.3); 4′-H 3.38 (t, 9.3); 5′-H 3.45 (m); 6′-H 3.70 (d,d, 12.4, 6.2); 6′-H 3.89 (d,d, 12.3, 2.3).

2-Deoxypolypodine B 3β-D-glucoside (**11**): CID-MS: 480 $(M-\text{sugar})^{+}$, 463 $(M+H-\text{sugar}-H_2O)^{+}$, 180 $(\text{sugar})^{+}$. ^{1}H NMR data (in $^{2}H_2O$) present the typical features of 2-deoxy and 5,20-dihydroxy ecdysteroids as reported [5]. The identity of the sugar as a β-D-glucopyranose was worked out as for compound **10** (see above). The β-D-glucopyranose unit attachment was confirmed from the observation of an nOe 1′-H⇔3-H. Thus, the structure was assigned unambiguously. 18-Me 0.875 (s); 19-Me 0.905 (s); 21-Me 1.233 (s); 26-Me 1.221(s); 27-Me 1.233 (s); 7-H 5.99 (d, 2.1); 3-H 4.31 (s, broad $w_{1/2}$ 12 Hz); 22-H 3.44 (d 10.5); 9-H 3.243 (m); 17-H 2.32 (t, 9.3); 1′-H 4.56 (d, 8); 2′-H 3.30 (d,d, 9, 8); 3′-H 3.51 (t, 9.3); 4′-H 3.39 (t, 9.3); 5′-H 3.45 (m); 6′-H 3.71 (d,d, 12.4, 5.9); 6′-H 3.90 (d,d, 12.3, 2).

2-Deoxyintegristerone A (**12**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [9], co-chromatographs with reference on NP- and RP-HPLC. CI-MS: 498 $(M+H+NH_3)^{+}$, 481 $(M+H)^{+}$, 463 $(M+H-H_2O)^{+}$, 445 $(M+H-2H_2O)^{+}$, 427 $(M+H-3H_2O)^{+}$, 409 $(M+H-4H_2O)^{+}$.

[5α-^{2}H]2-Deoxyintegristerone A (**13**): ^{1}H NMR data (in $^{2}H_2O$) present the typical features of a 2-deoxy-1β,20-dihydroxy-5α-ecdysteroid as reported [5]. 18-Me 0.863 (s); 19-Me 0.844 (s); 21-Me 1.224 (s); 26-Me 1.224 (s); 27-Me 1.233(s); .7-H 5.99 (d, 2.3); 1-H 3.744 (d,d, 11.9, 4.3); 3-H 3.744 (t,t, 11.6, 4.7); 22-H 3.427 (d 10.6); 9-H 2.95 (s, broad $w_{1/2}$ 23 Hz); 5-H 2.543 (d,d, 12.5, 3.1); 17-H 2.31 (t, 9.9). The 5α-configuration was confirmed by nOe between 9-H and 5-H. The presence of predominantly (91%) deuterium at C-5 was demonstrated by the reduced abundance of the 5-H signal in the ^{1}H NMR spectrum. It seems probable that [5α-^{2}H]2-deoxyintegristerone A (**13**) is an artefact generated during NMR in deuterated solvent, since it is known that (5β-H) 2-deoxyecdysteroids readily undergo epimerisation at C-5 to form the 5α-epimer [10].

2-Deoxy-21-hydroxyecdysone (**14**): amorphous. UV, ^{1}H and ^{13}C NMR data as reported [6,9], co-chromatographs with reference on NP- and RP-HPLC.

3.3. Chemotaxonomic significance

The *Silene* genus comprises more than 700 different species and its taxonomy appears very complex

[11]. It is, however, the most extensively studied genus with regard to the presence of phytoecdysteroids. The abundance and diversity of ecdysteroids existing in *Silene* species suggest that ecdysteroids in this genus might have a chemotaxonomic value [12–15]. It is clear that the species in certain sections of the genus (Siphonomorpha, Dipterospermae, Silene, Otites) have a very high probability of containing ecdysteroids, while others (Auriculatae, Conomorpha, Eudianthe, Heliospermae, Inflatae) appear to contain only ecdysteroid-negative species [12]. The 10 ecdysteroid-positive species examined here come from diverse sections of the genus and we considered whether the ecdysteroid profile could be related to taxonomic position. 20E and polB are major ecdysteroids in all 10 species examined, so these cannot be used as taxonomic markers; the occurrence of other ecdysteroids, especially the minor ecdysteroids, could possibly provide a fingerprint characteristic of the section, subsection or species. The genus has been divided into 44 [16] or 29 [17] sections. We shall relate the chromatographic profiles to the more recent of these studies. *S. italica* and *S. regia* occur in the Siphonomorpha. In terms of ecdysteroids in addition to 20E and polB, the profiles of these two samples are different. However, the samples represent different parts of the two species. *S. chlorifolia* and *S. radicosa* are both allocated to the section Sclerocalycinae. In addition to 20E and polB, which are common to both species, *S. chlorifolia* contains 2d20E and 2dE as significant ecdysteroids, while only a small amount of E was detectable in *S. radicosa* in addition to 20E and polB.

S. pseudotites (section: Otites) is a hybrid between *S. otites* and *S. colpophylla* [17]. It possesses high ecdysteroid levels like *S. otites* [4–6,18], but the identification of at least three new ecdysteroids from this species in the present study demonstrates that the profiles are not the same. The ecdysteroid content of *S. colpophylla* has not yet been investigated.

S. portensis (Rigidulae) and *S. disticha* (Silene) possess similar profiles even though they occur in different sections. *S. cretica* (Behenantha) possesses a profile (where 20E, polB and 2d20E predominate) which is similar to those for *S. antirrhina* and *S. chlorifolia*. The profile in *S. echinata* (Lasiocalycinae) is most similar to that of *S. radicosa*.

Thus, no clear patterns are discernible in the relationship between ecdysteroid profiles and the taxonomy of *Silene*, at least at the sectional level. Further studies of a wider range of species are required to assess the chemotaxonomic value of ecdysteroids in this genus at the subsectional and species levels.

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References

[1] R. Lafont, A. Bouthier, I.D. Wilson, in: I. Hrdy (Ed.), Insect Chemical Ecology, Academia, Prague, 1991, p. 197.
[2] L. Dinan, Eur. J. Entomol. 92 (1995) 271.
[3] M. Báthori, J.P. Girault, H. Kalasz, I. Mathé, R. Lafont, J. Pharm. Biomed. Anal. 16 (1997) 327.
[4] J.-P. Girault, M. Báthori, H. Kalasz, I. Mathé, R. Lafont, J. Nat. Prod. 59 (1996) 522.
[5] J.-P. Girault, M. Báthori, E. Varga, K. Szendrei, R. Lafont, J. Nat. Prod. 53 (1990) 279.
[6] M. Báthori, J.-P. Girault, H. Kalasz, I. Mathé, L.N. Dinan, R. Lafont, Arch. Insect Biochem. Physiol. 41 (1999) 1.
[7] L. Dinan, Phytochem. Anal. 3 (1992) 132.
[8] C.Y. Clément, D.A. Bradbrook, R. Lafont, L. Dinan, Insect Biochem. Mol. Biol. 23 (1993) 187.
[9] R. Lafont, I. Wilson (Eds.), The Ecdysone Handbook, Chromatographic Society, Nottingham, 2nd ed., 1996, pp. 398, 262, 336, 100, 84.
[10] D.H.S. Horn, R. Bergamasco, in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 7, Pergamon, Oxford, 1985, p. 185.
[11] W. Greuter, Taxon 44 (1995) 543.
[12] Z. Saatov, M.B. Gorovits, N.K. Abubakirov, Khim Prir Soedin (1993) 627.

[13] M. Báthori, I. Mathé, P. Solymosi, K. Szendrei, Acta Bot. Hung. 33 (1987) 125.
[14] M. Báthori, K. Szendrei, H. Kalasz, R. Ohmacht, J. High Resolut. Chromatogr. Chromatogr. Commun. 9 (1986) 539.
[15] L. Zibareva, Arch. Insect Biochem. Physiol. 43 (2000) 1.
[16] P.K. Chowdhury, Notes R. Bot. Gard. Edinburgh 22 (1957) 221.
[17] Flora Europaea, Vol. 1, Cambridge University Press, 1993, p. 192.
[18] M. Báthori, J.-P. Girault, I. Mathé, R. Lafont, Biomed. Chromatogr. 14 (2000) 464.