Journal of Natural Products
Vol. 53, No. 2, pp. 279-293 Mar-Apr 1990

ISOLATION AND IDENTIFICATION OF NEW ECDYSTEROIDS FROM THE CARYOPHYLLACEAE

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ABSTRACT.—Several new phytoecdysteroids have been isolated from various plant species of the Caryophyllaceae, i.e., *Lychnis flos-cuculi*, *Silene nutans*, *Silene otites*, and *Silene tatarica*. Some of them (compounds **18**, **22**, and **25**) had previously been described in animals. These compounds were isolated as minor components accompanying larger amounts of more classical compounds like 20-hydroxyecdysone, polypodine B, and/or 2-deoxy(-20-hydroxy)-ecdysone.

These newly isolated ecdysteroids include dihydrorubrosterone [**2**] and 26-hydroxypolypodine B [**26**] from *L. flos-cuculi*; 22-deoxyintegristerone A [**24**] and 5α-22-deoxyintegristerone A [**27**] from *S. nutans*; dihydropoststerone [**1**], 2-deoxyecdysone 22-acetate [**11**], 2-deoxyintegristerone A [**12**], 2-deoxy-20-hydroxyecdysone 22-acetate [**14**], and 20-hydroxyecdysone 22-benzoate 25-glucoside [**20**] from *S. otites*; and 2-deoxy-20-hydroxyecdysone 22-benzoate [**15**] from *S. tatarica*.

Ecdysteroids are widespread biomolecules found in both the animal and plant kingdoms. Ecdysteroids represent hormones in arthropods and possibly also in lower invertebrates (1,2). Their function in plants is still conjectural, and it is believed that they might provide some protection against non-adapted phytophagous insects (3).

Many plants contain ecdysteroids (3–5), and within the Angiospermae, Caryophyllaceae have appeared very suitable for such studies, as many species from this family contain large amounts of phytoecdysteroids (6–8) and because more than ten percent of the already known phytoecdysteroids were isolated for the first time from this family (9–21). This favorable situation has prompted us to investigate several easily available species for minor ecdysteroids, some of which might prove useful either as reference compounds for the identification of ecdysteroids present in animals or for structure/activity relationship studies.

In the present study, plant extracts were processed by a multistep procedure (22–25), the last step being preparative hplc. Pure compounds generally isolated in the milligram (or less) range were identified by spectrometric methods and particularly 2D ^{1}H nmr, which was previously shown to be extremely powerful for the structural elucidation of ecdysteroids isolated in minute amounts, i.e., between 50 and 100 μg (26,27).

EXPERIMENTAL

BIOLOGICAL MATERIALS.—*Silene otites* (L.) Wib. was collected in the vicinity of Szeged, Hungary, *Silene nutans* in the vicinity of Budapest, and *Lychnis flos-cuculi* in the vicinity of Debrecen. *Silene tatarica* (L.) Pres. was cultured in the botanical garden of Vacratot. Voucher species are deposited in the Herbarium of the Jozsef Attila University of Szeged.

Ecdysteroids have been isolated by multi-step procedures from the epigeous parts of *S. otites* (23,25), *S. nutans*, and *L. flos-cuculi* (24,28) and from *S. tatarica* by means of dccc and preparative hplc.

REFERENCE ECDYSTEROIDS.—Reference ecdysteroids were from various sources. 20-Hydroxyecdysone [**16**] and makisterone A [**4**] were purchased from Simes (Milan, Italy). Reference 2-deoxyecdysone

[**10**] and 2-deoxy-20-hydroxyecdysone [**13**] were gifts from Dr. Horn (Acheron, Australia). Synthetic dihydrorubrosterone [**2**] (=2β,3β,14α,17β-tetrahydroxy-5β-androst-7-en-6-one) (29) was a gift from Dr. Hanson (Brighton, U.K.). Reference 20-hydroxyecdysone monoacetates (2-, 3-, 22-, or 25-acetate) were prepared from 20-hydroxyecdysone by extended acetylation (to a mixture of 2,3,22-triacetate and 2,3,22,25-tetraacetate) followed by a short (10 min) hydrolysis with 0.7% K_2CO_3 in 70% aqueous MeOH. Reference poststerone [**6**] was prepared from 20-hydroxyecdysone using oxidation with pyridinium chlorochromate (30). Its reduced forms (2 isomers) were prepared by reduction with a solution of $NaBH_4$ (2 mg/ml) in pyridine for 20 min, then purified by preparative hplc. Reference 5α-epimer of 20-hydroxyecdysone was prepared according to Horn (31).

EXTRACTION AND PREPURIFICATION.—Dry (*S. otites* and *S. nutans*) or fresh plant materials (*L. flos-cuculi*) were crushed, milled, and extracted either with MeOH (24,28) as detailed in Scheme 1, a, or with 30% aqueous MeOH (23) as shown in Scheme 1, b, employing the so-called "exhaustive extraction procedure." In the latter method, the extract was evaporated, and the residue was powdered and repeatedly extracted with 16 volumes of MeOH.

Extracts prepared by either version were evaporated in vacuo. The prepurification was performed by solvent-solvent distribution and fractional precipitation (24) or by fractional precipitation alone (28). In solvent-solvent distribution, the residue was taken up in H_2O, and the solution was extracted with cyclohexane to remove chlorophyll and other apolar contaminating substances. In fractional precipitation, the dry residue was dissolved in MeOH, and the contaminating substances (polar substances such as carbohydrates and proteins) were removed by precipitation with Me_2CO (the ratio of MeOH to Me_2CO was 2:1). The precipitate was filtered and washed with MeOH-Me_2CO (2:1). The filtrate and the washing solution were combined and evaporated. The procedure of dissolution, precipitation, and washing was repeated, with a ratio of MeOH to Me_2CO of 1:1, then 1:2.

FRACTIONATION OF THE CRUDE EXTRACT.—The substances present in the extract were purified by cc. The extract was adsorbed on alumina (Brockman II neutral), the ratio of sorbent to dry material being 3:1. Subsequently twice as much sorbent was used to prepare the column. The components were eluted with a step-wise gradient of CH_2Cl_2 and 96% EtOH to yield partially purified fractions. Further purification was performed as follows.

The main ecdysteroid component, 20-hydroxyecdysone [**16**] was obtained in pure form by stepwise recrystallization [in EtOAc-MeOH (2:1)] (23,24).

The additional components—2-deoxyecdysone [**10**], 2-deoxy-20-hydroxyecdysone [**13**], and 20-hydroxyecdysone 22-acetate [**18**] from *S. otites* (23); rubrosterone [**7**] and polypodine B [**5**] from *L. flos-cuculi* (24)—were purified by repeated cc (on alumina or silica columns) followed by stepwise crystallization as described above.

All the other ecdysteroids were minor components of the plants. Their isolation required the use of combined chromatographic procedures, and additional cc (Sephadex LH 20), preparative tlc, dccc, or hplc served for the final purification (24,25). If a high ratio of contaminating flavonoids was present, cc on polyamide was required. Ecdysteroids were eluted by H_2O and H_2O-MeOH (1:1) from the polyamide column (28).

A detailed description of the isolation scheme of ecdysteroids from *S. otites* is given in Figure 1.

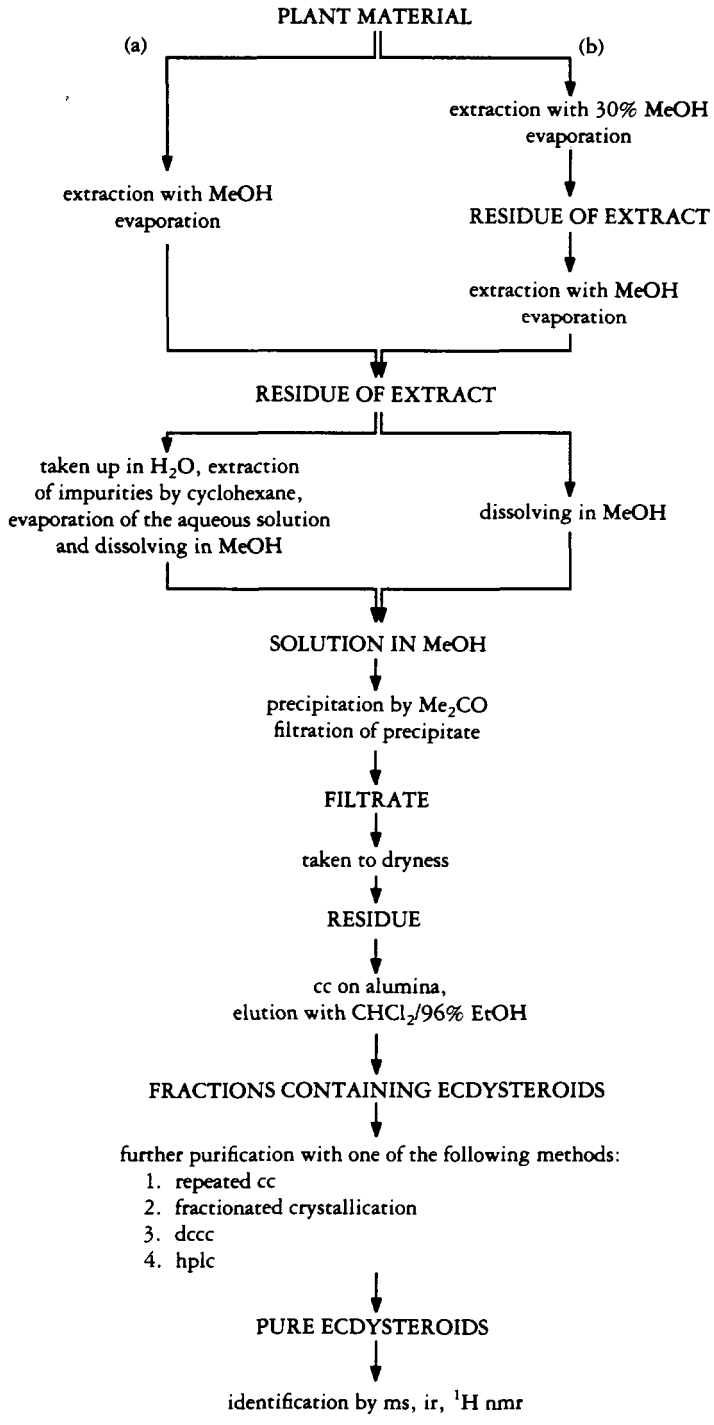
In the case of *S. tatarica*, the supply of the plant material was limited (10 g), and the highest possible yield of the isolated substances was sought. To this end the crushed material was extracted with MeOH in a Soxhlet apparatus for 20 min, and this was followed by precipitation with Me_2CO. The filtrate was evaporated and subjected to dccc separation (system 1, see below). The pure ecdysteroids were obtained from the dccc fractions by preparative hplc.

Dccc was done using a EYELA DCC-A instrument (Rikakikai Co. Ltd, Tokyo, Japan) with the following systems: (1) $CHCl_3$-MeOH-H_2O (65:20:20), (2) $CHCl_3$-C_6H_6-EtOAc-MeOH-H_2O (45:2:3:60:40), and (3) $CHCl_3$-MeOH-H_2O (13:7:4).

Samples were dissolved in the upper phase. Flow rates were 15 ml/h in systems 1 and 2 and 7.5 ml/h in system 3, and fractions of 5 ml (systems 1 and 2) or 2.5 ml (system 3) were collected.

Tlc silica plates GF254 were from E. Merck (Darmstadt, F.R.G). They were developed with EtOAc-MeOH-NH_3 (85:10:5) (solvent system A) or with $CHCl_3$-EtOH (85:15) (solvent system B), EtOAc-EtOH-H_2O (8:2:1) (solvent system C), and $CHCl_3$-MeOH-Me_2CO (6:2:1) (solvent system D). Solvents of analytical grade were purchased from Reanal (Budapest, Hungary). After development, ecdysteroids were detected either directly by fluorescence quenching (uv 254 nm) or using the vanillin-H_2SO_4 spray reagent and observation either in daylight or uv light (356 nm) (32).

Polar ecdysteroids (glucosides) and specific compound pairs were purified by reversed-phase hplc using C-18-bonded silica columns: Spherisorb-5ODS2 (Phase Sep.) (250 mm long, 4.6 mm i.d.) and Zorbax-ODS® (DuPont) (250 mm long, 9.4 mm i.d.) eluted with MeCN/0.1% TFA mixtures. With the



SCHEME 1

isocratic solvent mode, the percentage of MeCN was 23% for nonpolar fractions (solvent system 1) and 15% for polar ecdysteroids (solvent system 2).

Nonpolar ecdysteroids were purified by silica hplc using Zorbax-Sil* columns (DuPont) (250 mm long, 4.6 or 9.4 mm i.d. according with sample size) eluted at 1 (or 4) ml/min with $CHCl_2$-iPrOH-H_2O (125:40:3) (solvent system 3), $CHCl_2$-iPrOH-H_2O (125:25:2) (solvent system 4), or $CHCl_2$-iPrOH-H_2O

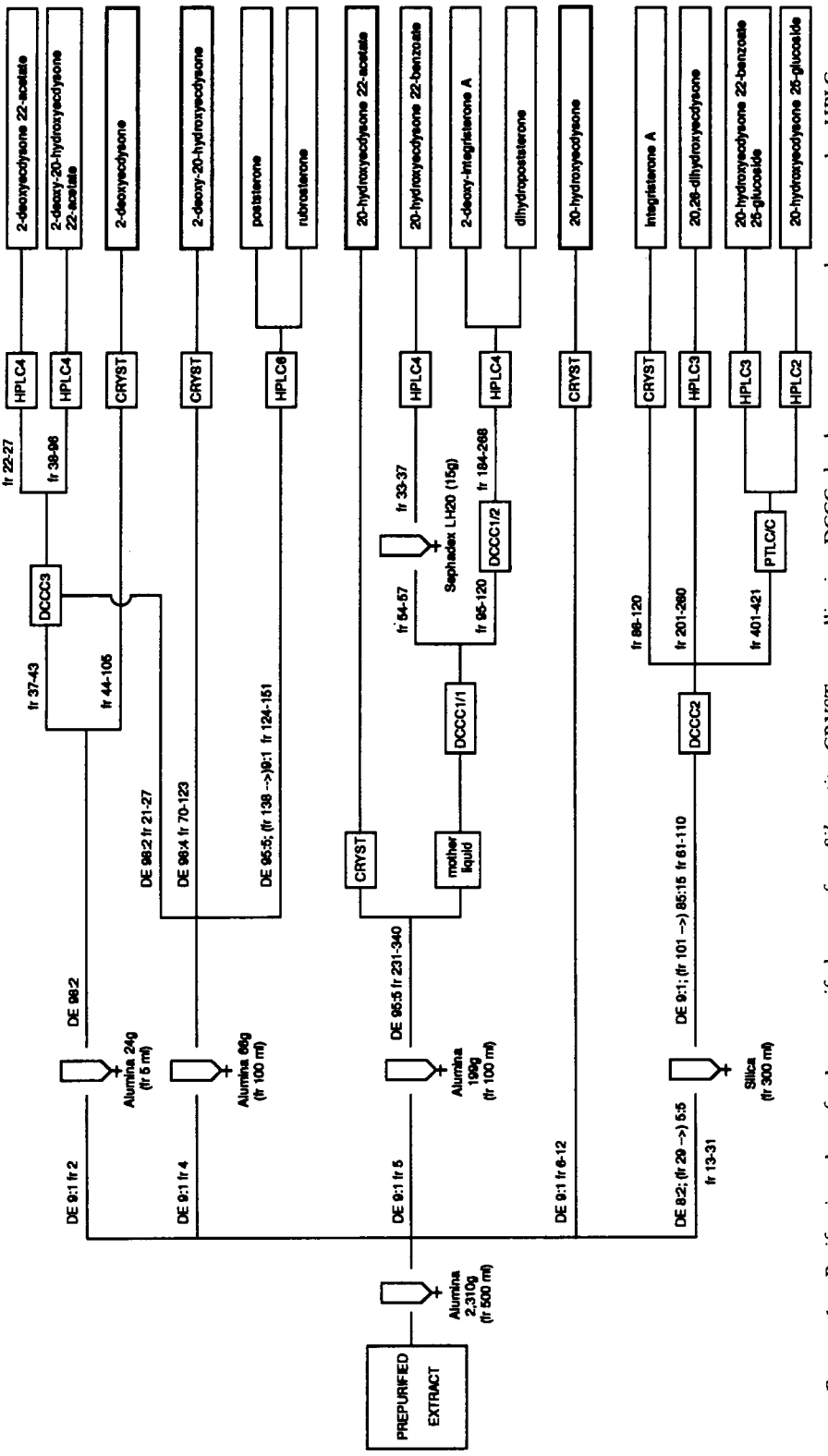


FIGURE 1. Purification scheme for the prepurified extract from *Silene otites*. CRYST: crystallization. DCCC: droplet counter-current chromatography. HPLC: high-performance liquid chromatography (numbers refer to solvent systems used; see Experimental). PTLC: preparative thin-layer chromatography. DE: CH_2Cl_2/EtOH mixture. fr: fraction number. ↦ refers to solvent change at the indicated fraction number (step-gradient).

(125:20:1.5) (solvent system 5) according to sample polarity (33,34). These solvent systems proved, however, unable to resolve satisfactorily certain pairs of compounds (e.g., rubrosterone/poststerone), which had to be further run with an EtOH-containing solvent [$CHCl_2$-EtOH-H_2O (840:145:15) (solvent system 6)] or a reversed-phase system.

Purified ecdysteroids were dissolved in a small volume of MeOH, 1 μl of which (ca. 1 μg) was used for mass spectrum determination with a Riber 10-10B apparatus (Nermag S.A.) using a chemical ionization/desorption (ci/d) mode with NH_3 as the reagent gas (35). Hrms was performed on some of the newly isolated ecdysteroids using a VG Analytical model 7070EQ and a ci/d technique with CH_4 as the reagent gas and PFK (perfluorokerosene) as internal standard for calibration.

^{1}H-nmr spectra were obtained with a Bruker WM250 apparatus. Purified compounds were first lyophilized in D_2O (99.95%) before being dissolved in CD_3OD, $CDCl_3$, or D_2O, reference TSP-d_4 [sodium 3-(trimethylsilyl)-1-propionate-2,2,3,3-d_4]. The ^{1}H-nmr spectra were recorded using standard Ft techniques at 20°. Analyses of spectra were obtained by homonuclear 2D correlation spectroscopy (2D-COSY) as described earlier (27,36). NOe difference experiments have been performed with vacuum-degassed samples and with saturation alternatively on and off resonance (delays of saturation 1.5 sec) and with a relaxation time of 3 sec.

RESULTS

Both already known and new ecdysteroids were identified in the present study. They are listed in Tables 1 and 2. Their occurrence within the four plant species analyzed and their chromatographic properties (hplc, tlc) are reported. Their nmr spectral data are given in Table 3. Only new compounds will be discussed in detail.

HPLC ANALYSIS OF PLANT EXTRACTS.—Some examples of plant extract analysis by normal phase hplc are given in Figure 2. It is apparent that each plant contains a few major components (which are indicated in the figure), including 20-hydroxyecdysone, together with several minor compounds. Almost all the peaks from these chromatograms are ecdysteroids, some of which are not resolved with the solvent system used. Based on the data from crude extracts and hplc analyses, the 20-hydroxyecdysone content relative to dry wt could be calculated as 0.98% for *S. otites*, 0.27% for *S. nutans*, and 0.167% for *L. flos-coculi*.

GENERAL RULES FOR THE ANALYSIS OF ^{1}H-NMR DATA.—The various structural modifications of the ecdysone or 20-hydroxyecdysone molecules give specific modifications in the ^{1}H-nmr spectra which can be easily used for structure assessment.

(a) Compounds of the ecdysone versus 20-hydroxyecdysone series can be easily distinguished by the H-21 signal which undergoes changes both in its chemical shift (27) and shape: a singlet for 20-OH compounds, a doublet in 20-deoxycompounds.

(b) 2-Deoxyecdysteroids share the main features already known for 2-deoxyecdysone [**10**] and 2-deoxy-20-hydroxyecdysone [**13**], i.e., the lack of H-2 signal in the >C*H*-OH zone, broadening of the H_{eq}-3 signal ($w_{1/2} \approx 12$ Hz) and correlation of this signal in the 2D COSY experiment with four signals in the upfield part of the spectrum (H_{ax}-2, H_{eq}-2, H_{ax}-4, H_{eq}-4), and a small downfield shift (+0.06 ppm) of the H-9 signal with respect to ecdysone or 20-hydroxyecdysone [**16**].

(c) 22-Deoxyecdysteroids show specific characteristics similar to those of 22-deoxy-20-hydroxyecdysone (37): the lack of an H-22 signal in the >C*H*-OH zone, a downfield shift (+0.07 ppm) of the H-21 signal, and small upfield shifts (+0.04 ppm) of the H-18 signal and H-17 signals.

(d) 26-Hydroxyecdysteroids are characterized by the appearance of a -CH_2-OH signal (two if the diastereoisomers 25*R*,25*S* are present) as a singlet at $\delta = 3.45$ ppm and the loss of the H-26 signal, an upfield shift of the H-27 signal (−0.04 ppm in CD_3OD, −0.08 ppm in D_2O), and a downfield shift of the H-21 signal (+0.02 ppm in CD_3OD, +0.03 ppm in D_2O).

(e) 22-OH Esterification is established thanks to the large downfield shift of the H-

TABLE 1. Nomenclature, Occurrence, and Some Chromatographic Properties (dccc, tlc) of the Phytoecdysteroids Isolated in the Present Study.

Ecdysteroid	Species[a]	Dccc (fraction number)			R_f(tlc)			
		System 1	System 2	System 3	System A	System B	System C	System D
Dihydropoststerone [1]	So							
Dihydrorubrosterone [2]	Lfc							
2-Deoxyecdysone 22-acetate [11]	So			22–27	0.59	0.57		
2-Deoxy-integristerone A [12]	So	95–120						
2-Deoxy-20-hydroxyecdysone 22-acetate [14]	So	69–77		38–96	0.40	0.51		
2-Deoxy-20-hydroxyecdysone 22-benzoate [15]	St							
20-Hydroxyecdysone 22-acetate [18]	So	74–75			0.19	0.23		
20-Hydroxyecdysone 22-benzoate 25-glucoside [20]	So		201–260				0.48	0.25
20-Hydroxyecdysone 3-acetate [22]	Lfc							
22-Deoxy-integristerone A [24]	Sn				0.19	0.13	0.60	0.41
22-Deoxy-20,26-dihydroxyecdysone [25]	Sn						0.49	0.30
26-Hydroxy-polypodine B [26]	Lfc,Sn						0.50	0.29
5α-22-Deoxy-integristerone A [27]	Sn							

[a]So = *Silene otites*; Lfc = *Lychnis flos-cuculi*; St = *Silene tatarica*; Sn = *Silene nutans*.

TABLE 2. Chromatographic (hplc) and Ms Data of the Phytoecdysteroids Isolated in the Present Study.

Ecdysteroid	hplc (Retention time, min)			Molecular mass	Major ions *m/z* (Chemical ionization/Desorption)
	System 1	System 3	System 4		
Dihydropoststerone [1]		14.7	26.8	364	382,365,347,345
Dihydrorubrosterone [2]		13.4	22.0	336	354,337,319,303,301
2-Deoxyecdysone 22-acetate [11]		5.4	8.2	490	508,491,473,455,431,313,397,396
2-Deoxy-integristerone A [12]		13.5	32.1	480	498,481,463,445,427,380,363,347,345
2-Deoxy-20-hydroxyecdysone 22-acetate [14]		6.4	10.1	506	507,489,473,471,447,429,413,411,347,329
2-Deoxy-20-hydroxyecdysone 22-benzoate [15]		6.9	11.1	568	569,551,533,447,429,411,347,329
20-Hydroxyecdysone 22-acetate [18]	12.4	10.9	22.4	522	540,523,505,487,463,445,427,409,363,345
20-Hydroxyecdysone 22-benzoate 25-glucoside [20]		24.6		746	747,729,643,625,607,463,445,427,363,345
20-Hydroxyecdysone 3-acetate [22]	10.8	8.7	13.8	522	540,523,505,487,469,405,387,345,329,327
22-Deoxy-integristerone A [24]		13.8	32.8	480	498,481,463,445,427,409,391,380,361
22-Deoxy-20,26-dihydroxyecdysone [25]		32.3	87.6	480	498,481,463,445,427,391,363,345
26-Hydroxy-polypodine B [26]	4.7	30.9	77.2	512	513,495,477,459,441,396,379,363,361,345
5α-22-Deoxy-integristerone A [27]		17.6	40.4	480	498,481,463,445,427,409,380,363,347

TABLE 3. ^{1}H-nmr data for newly isolated phytoecdysteroids.[a]

Proton	Compound					
	1 (CD_3OD)	**2** (CD_3OD)	**11** (CD_3OD)	**12** (D_2O)	**14** (CD_3OD)	**15** (CD_3OD)
Ha-1	1.42 (dd,13,12)	1.42 (dd,13,12)		—		
He-1				3.68 (mb,20°) (dd,4,8,80°)		
Ha-2	3.84 (m)	3.80 (m)				
He-3	3.95 (m,$w_{1/2}$ = 8)	3.93 (m,$w_{1/2}$ = 8)	3.98 (m,$w_{1/2}$ = 12)	3.70 (mb,20°) 3.87 (mb,80°)	3.98 (m,$w_{1/2}$ = 12)	3.98 (mb,$w_{1/2}$ = 12)
Ha-4						
He-4						
H-5	2.38 (dd,12,5)	2.38 (dd,12,5)	2.33 (dd,12,3)	2.76 (mb,20°) (dd,4,8,80°)	2.43 (dd,12,4)	2.38 (dd,12,5)
H-7	5.82 (d,2.5)	5.76 (d,2.5)	5.80 (d,2.5)	5.94 (d,2.5,80°)	5.79 (d,2.5)	5.81 (d,2.5)
Ha-9	3.14 (m,$w_{1/2}$ = 22)	3.14 (m,$w_{1/2}$ = 22)	3.20	3.23 (mb,20°) 3.19 (m,$w_{1/2}$ = 22,80°)	3.20 (m,$w_{1/2}$ = 22)	3.20 (m,$w_{1/2}$ = 22)
Ha-11						
He-11						
Ha-12						
He-12						
Ha-16						
Hb-16						
H-17		4.29 (dd,9.3,6.5)		2.36 (b,20°) 2.34 (t,8.5,80°)	2.39 (m)	2.47 (t,8.5)
Hb-22	—	—	4.87 (d,)[b]	3.43 (dd,11,2,80°)	4.87 (d,)[b]	5.15 (dd,12,2)
Ha-23	—	—				
Hb-23	—	—				
Me-18	0.69 (s)	0.70 (s)	0.70 (s)	0.85 (s)	0.87 (s)	0.89 (s)
Me-19	0.96 (s)	0.96 (s)	0.96 (s)	1.10 (s)	0.95 (s)	0.95 (s)
Me-21	1.22 (d,6.5)	—	0.98 (d,6.5)	1.22 (s)	1.28 (s)	1.42 (s)
Me-26	—	—	1.18 (s)	1.23 (s)	1.15 (s)	1.15 (s)
Me-27	—	—	1.19 (s)	1.24 (s)	1.16 (s)	1.16 (s)
Other	>C*H*-OH(20) ca. 0.65(m)	—	CH_3CO- 2.04(s)	—	CH_3CO- 2.08(s)	C_6H_5CO- 7.48(t,7) 7.60(t,7) 8.07(t,7)

[a]Multiplicity of signals: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad signal. $w_{1/2}$ indicates width at half-height in Hertz; δ in ppm.

[b]Indicates signal overlapping with the CD_3OH signal.

22 signal: ca. +1.3–1.4 ppm for acetylated compounds with the appearance of a CH_3CO- singlet signal at ca. 2.0–2.1 ppm and downfield shift of the H-21 signal (+0.03 ppm in the ecdysone series, 0.1 ppm in the 20-hydroxyecdysone series); +1.7 ppm for benzoate esters with the appearance of three signals in the aromatic zone, 8.08 ppm (d, 7 Hz) for two H_{ortho}, 7.61 ppm (t, 7 Hz) for one H_{para}, and 7.49 ppm (t, 7 Hz) for two H_{meta}; and a large downfield shift of the H-21 signal (+0.23 ppm in the 20-hydroxyecdysone series in CD_3OD) and a smaller one (ca. +0.1 ppm) for the H-17 signal.

(f) 1β-OH Ecdysteroids are characterized by: the appearance of a new signal in the

TABLE 3. Continued.

Compound						
18 ($CDCl_3$)	**20** (CD_3OD)	**22** ($CDCl_3$)	**24** (D_2O)	**25** (D_2O)	**26** (CD_3OD)	**27** (D_2O)
1.4		1.3	—			3.73 (d,3.5)
1.85		1.9	3.92 (m,$w_{1/2}$ = 7)			—
3.88 (m,$w_{1/2}$ = 22)	3.82 (m)	4.02 (m,$w_{1/2}$ = 22)	4.03 (t,3.2)	3.99 (m,$w_{1/2}$ = 22)	3.94 (m)	(2-He) 3.94 (m,$w_{1/2}$ = 8)
4.11 (m,$w_{1/2}$ = 8)	3.94 (m,$w_{1/2}$ = 8)	5.21 (m,$w_{1/2}$ = 8)	4.15 (m,$w_{1/2}$ = 8)	4.07 (m,$w_{1/2}$ = 8)	3.97 (m)	3.71 (m,$w_{1/2}$ = 18)
1.6		1.6				1.65
1.85		1.85				1.88
2.42 (dd,13,4)	2.37 (dd,12,5)	2.3	2.62 (t-like)	2.35 (t-like)	—	2.56 (dd,11,7.2)
5.84 (d,2.5)	5.81 (d,2)	5.85 (d,2.5)	6.00 (d,1.5)	5.97 (d,2.5)	5.84 (d,2.5)	6.00 (d,2.5)
2.98 (m,$w_{1/2}$ = 21)	ca. 3.1–3.2	3.01 (m,$w_{1/2}$ = 22)	3.04 (m,$w_{1/2}$ = 22)	3.09 (m,$w_{1/2}$ = 22)	3.14 (m,$w_{1/2}$ = 22)	2.91 (m,$w_{1/2}$ = 21)
1.6		1.70				1.78
1.75		1.80				2.28
						2.26
						1.88
		2.0				
		1.75				
2.35 (m)	2.46 (m)	2.33 (m)	2.30 (m)	2.30 (m)	2.35 (m)	2.28 (m)
4.84 (db,10)	5.14 (db,10)	3.44 (d,10)			3.33 (db,10)	
1.5		1.35				
1.7		1.65				
0.79 (s)	0.88 (s)	0.87 (s)	0.83 (s)	0.82 (s)	0.88 (s)	0.81 (s)
0.97 (s)	0.95 (s)	1.01 (s)	1.09 (s)	1.00 (s)	0.90 (s)	0.93 (s)
1.20 (s)	1.41 (s)	1.21 (s)	1.30 (s)	1.30 (s)	1.20 (s)	1.29 (s)
1.22 (s)	1.22 (s)	1.24 (s)	1.22 (s)	3.45 (s),CH_2OH	3.35 (s),CH_3OH	1.22 (s)
1.26 (s)	1.22 (s)	1.25 (s)	1.22 (s)	1.17 (s)	1.14,1.15 (s)	1.22 (s)
CH_3CO- 2.10(s)	C_6H_5CO- 7.49(t,7) 7.61(t,7) 8.08(t,7) sugar	CH_3CO- 2.12(s)	—	—	—	—

>CH-OH zone ($w_{1/2}$ = 6 Hz, broad); the H_{ax}-2 signal appearing as a narrow triplet $^3J_{1,2} = {}^3J_{2,3}$ = 3.2 Hz; a downfield shift of the H_{eq}-3 signal (+0.08 ppm) with no change in its line shape; a strong downfield shift of the H-5 signal (+0.26 ppm) which results from the axial interaction between 1β-OH and H_{ax}-5; a downfield shift of the H-19 signal (+0.09 ppm); and an upfield shift of the H-9 signal (−0.09 ppm).

(g) 5β-OH Ecdysteroids show the disappearance of the H-5 signal, a modification of the H_{ax}-2 and H_{eq}-3 signals which become nearly isochronous and give a complex multiplet at 4.10–4.15 ppm, a small upfield shift of the 19-methyl signal (−0.06 ppm), a large downfield shift of the H_{ax}-1 signal (resulting from the axial interaction

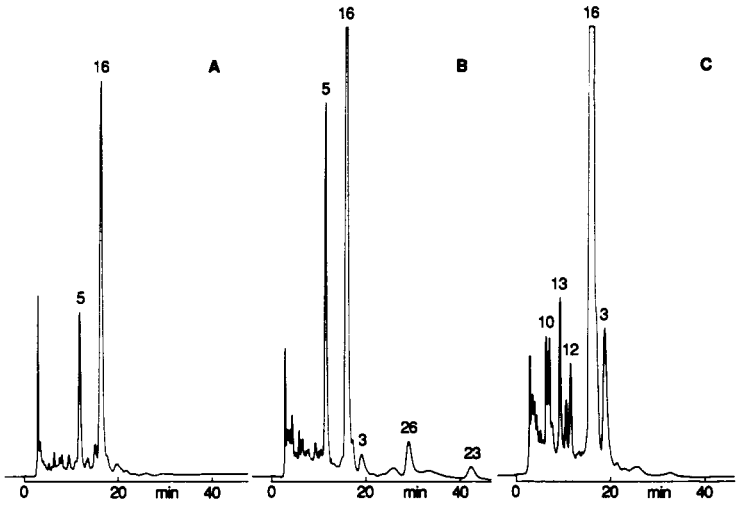


FIGURE 2. Hplc analysis (solvent system 3, analytical column, flow-rate 1 ml/min) of prepurified plant extracts. A: *Lychnis flos-cuculi*. B: *Silene nutans*. C: *Silene otites*. Numbers refer to compounds; see Table 1.

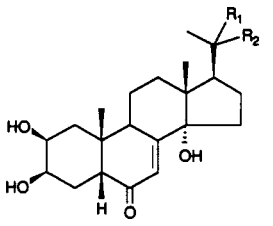
between 5β-OH and H_{ax}-1, as observed in the 2D COSY experiments), and a large downfield shift of the H_{ax}-4 and H_{eq}-4 signals.

(h) Side-chain cleavage products are characterized by very large changes by ms analysis. In addition, nmr spectra are characterized by the disappearance of either C-26 and C-27 methyl signals (C-20–C-22 cleavage as in poststerone [**6**]) or C-20, C-26, and C-27 methyl signals (C-17–C-20 cleavage as in rubrosterone [**7**]).

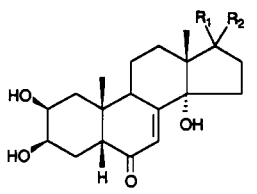
(i) 5α-Ecdysteroids have nmr spectra (in D_2O) which are characterized by downfield shifts (data for 5α,20-hydroxyecdysone with respect to 20-hydroxyecdysone) of the signals for H-5 (ca. +0.3 ppm), H_{eq}-1 (+0.2 ppm), H_{ax}-1 (+0.27 ppm), H_{eq}-4 (+0.1 ppm) and H_{eq}-2 (+0.05 ppm). In contrast, one notes upfield shifts for H_{ax}-9 (−0.38 ppm), H_{ax}-3 (−0.33 ppm), and H_{ax}-4 (−0.1 ppm). In these compounds, H-2 is equatorial (width at half-height $w_{1/2} = 8$ Hz) and H-3 is axial ($w_{1/2} \leq 21$ Hz). Strong nOe could be obtained between H-9 and H-5 and H_{ax}-1.

COMPOUND **1**.—This compound has a mol wt of 364 (mol wt of poststerone is 362), and it is in all points identical with one of the products obtained by the reduction of poststerone with $NaBH_4$ (see Experimental). This compound presents the main features of the A, B, C rings of ecdysone in CD_3OD; one notes a new >C*H*OH signal coupled to a methyl signal and to H-17. The exact stereochemistry at C-20 was not determined.

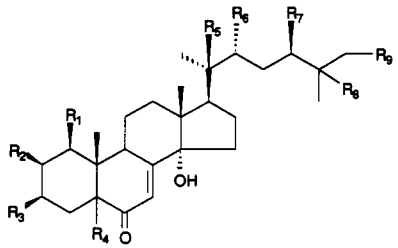
COMPOUND **2**.—Ms data show a mol wt of 336 (mol wt of rubrosterone is 334). Ms and nmr data clearly indicate the complete loss of the side-chain due to C-17–C-20 cleavage. The main signals for rings A, B, and C are similar to those of common ecdysteroids and are characteristic for compounds having 2β-OH, 3β-OH, and 5β A/B-ring junction. One notes the appearance of a new signal in the >C*H*-OH region (δ = 4.29 ppm, dd, 6.5,9). This additional -OH group is located on the D ring. From biogenetic reasons (isolation of rubrosterone [**7**] from the same species) and from nmr data, 17-OH is the most plausible candidate. Moreover, the presence of the additional -OH in positions 15α or 15β would modify the chemical shift of H-7. This compound is identical with the product earlier synthesized by Cochrane and Hanson (29) and described as a putative ecdysone metabolite by these authors. The stereochemistry of the 17-OH in β



1 R_1=H, R_2=OH
6 R_1,R_2=O



2 R_1=H, R_2=OH
7 R_1,R_2=O



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
3	OH	OH	OH	βH	OH	OH	H	OH	H
4	H	OH	OH	βH	OH	OH	Me	OH	H
5	H	OH	OH	βOH	OH	OH	H	OH	H
8	H	OH	OH	βH	OH	H	H	OH	H
9	H	OH	OH	βH	OH	OH	H	OAc	H
10	H	H	OH	βH	H	OH	H	OH	H
11	H	H	OH	βH	H	OAc	H	OH	H
12	OH	H	OH	βH	OH	OH	H	OH	H
13	H	H	OH	βH	OH	OH	H	OH	H
14	H	H	OH	βH	OH	OAc	H	OH	H
15	H	H	OH	βH	OH	OBz	H	OH	H
16	H	OH	OH	βH	OH	OH	H	OH	H
17	H	OAc	OH	βH	OH	OH	H	OH	H
18	H	OH	OH	βH	OH	OAc	H	OH	H
19	H	OH	OH	βH	OH	OBz	H	OH	H
20	H	OH	OH	βH	OH	OBz	H	OGlu	H
21	H	OH	OH	βH	OH	OH	H	OGlu	H
22	H	OH	OAc	βH	OH	OH	H	OH	H
23	H	OH	OH	βH	OH	OH	H	OH	OH
24	H	OH	OH	βH	OH	H	H	OH	H
25	H	OH	OH	βH	OH	H	H	OH	OH
26	H	OH	OH	βOH	OH	OH	H	OH	OH
27	OH	OH	OH	αH	OH	H	H	OH	H

position is confirmed by its coupling pattern (doublet of doublet). Inspection of Dreiding molecular models shows that with a 17α-OH substitution the H-17 signal would be a doublet. The dihedral angle between Hβ-17 and Hα-16 has a value of nearly 90°, and this would lead to a value for $^3J_{17H\beta\text{-}16H\alpha}$ of ca. 0 (38). The values of 6.5 and 9 Hz are in agreement only with an H-17 in the α position and a 17β-OH.

COMPOUND **11**.—This compound has a mol wt of 490 and shows a fragmentation pattern with losses of 60 amu, which are typical of acetates. Hrms give the exact mass for the $[MH]^+$ ion as 491.3289 (calcd for $C_{29}H_{47}O_6$, 491.3372). Its nmr spectrum shows the typical features for the lack of 20-OH (a), lack of 2-OH (b), and 22-acetylation (e). From these considerations the structure for 2-deoxyecdysone 22-acetate can be established.

COMPOUND **12**.—This compound has a mol wt of 480, identical with that of 20-hydroxyecdysone, and its nmr spectrum presents no modifications of the side-chain with respect to 20-hydroxyecdysone. The modifications are located on the A ring. At 20° in D_2O we observe a broadening of the signals, which by contrast become sharp at 80°. The typical features of 1β-OH (f) and 2-deoxy compounds (b) are observed, thus enabling us to assign the structure of 2-deoxy-integristerone A. The broadening of the signals at 20° could be due to a slow conformational equilibrium of the A ring, as a result of hydrogen bonding of 1β-OH and 3β-OH.

COMPOUND **14**.—This compound has a mol wt of 506. Hrms gives the exact mass for the $[MH]^+$ ion as 507.3200 (calcd for $C_{29}H_{47}O_7$, 507.3321). It presents the typical features of 20-OH compounds (a), 2-deoxy series (b), and 22-acetates (e), and this allows the structural elucidation of 2-deoxy-20-hydroxyecdysone 22-acetate.

COMPOUND **15**.—This compound has a mol wt of 568, and it presents the characteristics of 20-OH compounds (a), 2-deoxy series (b), and 22-benzoates (e). All these data are consistent with the with the structure of 2-deoxy-20-hydroxyecdysone 22-benzoate.

COMPOUND **18**.—This compound is identical with the synthetic reference compound 20-hydroxyecdysone 22-acetate, a compound already found in snails (26), pycnogonids (37), and insects (39). Its nmr spectrum has the characteristics of 20-hydroxy compounds (a) and for 22-acetylation (e).

COMPOUND **20**.—This compound has a mol wt of 746. It presents the characteristics of 20-hydroxycompounds (a) and of 22-benzoate derivatives (e). Seven new signals are found in the region expected for sugar resonances. Analysis of the shifts and couplings aided by two-dimensional COSY experiment identified the sugar moiety unambiguously at β-D-glucopyranoside [$\delta H_{1'} = 4.41$ (d, 7.6); $\delta H_{2'} = 3.12$; $\delta H_{6'} = 3.78$ (dd, 12.3, 2.2); $\delta H_{6''} = 3.62$; the other signals overlap in the CD_3OH region]. The unaffected chemical shifts of H-3 and H-2 protons, the benzoate esterification of 22-OH, and the downfield shift of 26-, 27-Me signals (+0.03 ppm) are in agreement with a 25-glucosidation. All these data are consistent with the structure of 20-hydroxyecdysone 22-benzoate 25-*O*-β-D-glucopyranoside.

COMPOUND **22**.—This compound was in all points identical with a synthetic reference compound, 20-hydroxyecdysone 3-acetate. Whether this compound is already present in plants as a native compound or if it results from the already mentioned equilibration between 2- and 3-acetates (40) has not been further investigated.

COMPOUND **24**.—This compound has a mol wt of 480 and the typical features of 20-OH family (a), 22-deoxy series (c), and 1β-OH compounds (f), which allow us to conclude that it is 22-deoxyintegristerone A.

COMPOUND **25**.—This compound has a mol wt of 480, and it is characterized by the presence of a 20-OH (a) and a 26-OH (d) and the lack of a 22-OH (c). From these data the structure of 22-deoxy-20,26-dihydroxyecdysone is established. This compound is identical with that previously found in animals (37).

COMPOUND **26**.—This compound has a mol wt of 512. The mass spectrum indicates the presence of two additional -OH groups with respect to 20-hydroxyecdysone (one on the nucleus and one of the side-chain). It presents the typical features for 20-hydroxylation (a), 26-hydroxylation (d), and 5β-hydroxylation (g). All these data are consistent with the structure of 26-hydroxypolypodine B (or 5,20,26-trihydroxyec-

dysone). As observed for polypodine B, 5β-hydroxylation results in a decreased polarity during normal-phase hplc analyses.

COMPOUND **27**.—This compound has a mol wt of 480. Its nmr spectrum presents the characteristics for the presence of 20-OH (a) and the lack of 22-OH (c). One observes three signals in the >C*H*-OH zone, and from the 2D COSY experiment they were assigned to H-1 (3.73), H-2 (3.94), and H-3 (3.71). From the 1D spectra, coupling constants, and width at half-height they were further identified as H_{ax}-1, H_{eq}-2, and H_{ax}-3. Therefore two structures would be consistent with these data, either 1α-OH, 2α-OH, 3α-OH in the 5β series [**A**] or 1β-OH, 2β-OH, 3β-OH in the 5α series [**B**] (Figure 3).

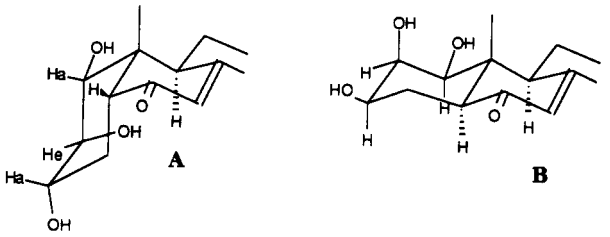


FIGURE 3. Partial structures of compound **27**.

By comparison of the spectrum of compound **27** with 5α-20-hydroxyecdysone prepared by equilibration of 20-hydroxyecdysone (31), it appears that the most plausible structure for compound **27** would be **B**, as the chemical shifts for protons H-5, H-3, H_{ax}-4 and H_{eq}-4 are nearly the same for both compounds. A strong downfield shift of the H_{eq}-11 signal (d = 2.28 ppm) is in agreement with 1-OH_{eq}. The structure **B** (5α series) is definitively established thanks to nOe difference experiments. Saturation of H-9 signal leads to two strong nOe with H-5 and H_{ax}-1 (ca. 10%) in agreement only with that structure.

DISCUSSION

Our present experiments underline (a) the complexity of ecdysteroid patterns in plants and (b) the power of ^{1}H-nmr analysis for structure elucidation of compounds isolated in low amounts (50 μg–1 mg).

Besides major compounds, among which 20-hydroxyecdysone (i.e., the major insect hormone) seems to be the most abundant in most cases, plants contain many minor ecdysteroids which were overlooked in previous studies. Because of the improvement of analytical methods, it has become feasible to identify these compounds. We have in general limited the present study to the major compound from each dccc fraction, thus eliminating many trace compounds which no doubt are also ecdysteroids. A survey of the identified compounds clearly indicates that any combination of individual modifications of the ecdysone molecule may be expected to be found in these plants. These individual changes include hydroxylation (at C-1, C-5, C-20, C-26), combination (at C-2, C-3, C-22) with organic acids (acetates, benzoates) or sugars, and side-chain cleavage (between C-17–C-20 or C-20–C-22). Less hydroxylated compounds also accumulate to some extent, e.g., 2-deoxy- or 22-deoxy- ecdysteroids. Finally, the same plant may contain C_{28} compounds (e.g., makisterone A) together with the more classical C_{27} ones. Because these individual changes may occur in various combinations, we expect that numerous phytoecdysteroids may be found and that the present list is far from complete.

As underlined in the Results section, structure elucidation of new ecdysteroids can be easily performed from ^{1}H-nmr spectral data. Each individual change in the molecule leads to specific modifications of the spectrum. Of course, for obtaining good quality spectra with small amounts a high degree of purity of ecdysteroids is a prerequisite; this can be achieved through the last hplc step. Compounds eluting from the hplc column were collected in glass tubes rinsed several times with hplc quality MeOH and evaporated under N_2, then lyophilized in D_2O prior to nmr analysis. This last step (possibly repeated twice) allowed the complete elimination of any traces of hplc solvent and was absolutely required for minor compounds isolated in the 50-μg range.

The size of the known phytoecdysteroid family of compounds has been continuously increasing since 1966, when the first representative was isolated. It contains now more than 100 compounds (5), and no doubt this number will rapidly increase with the general use of very efficient isolation and identification techniques.

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Received 21 November 1988