Isolation and Structural Elucidation of Two Plant Ecdysteroids, Gerardiasterone and 22-Epi-20-hydroxyecdysone

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Two minor plant ecdysteroids, 22-epi-20-hydroxyecdysone (**1**) and gerardiasterone (**2**), were isolated from *Serratula tinctoria* L. (Compositae). The first compound, a new natural product, was characterized by an unusual stereochemistry at C-22 (i.e., 22*S*). The second compound was identified as (20*R*,23*S*)-20,23-dihydroxyecdysone, a compound previously isolated from the Zooanthid *Gerardia savaglia*.

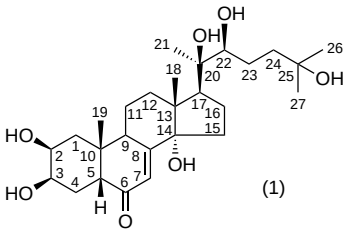
Serratula tinctoria L. (Compositae) is characterized by its high content of 20-hydroxyecdysone and polypodine B and by its broad ecdysteroid spectrum.[1−4] We have isolated 15 ecdysteroids from the roots of *S. tinctoria*[3] and three others from its aerial parts.[4] Ecdysteroid analysis of *S. tinctoria* has shown the occurrence of the four common ecdysteroid skeletal types, namely C19, C21, C27, and C29 systems. The C19 and C21 ecdysteroids deserve special attention because of their limited number.[5] Five of the seven members of these groups have been isolated from *S. tinctoria*.

This paper describes the isolation, structural elucidation, and/or identification of two minor ecdysteroids (**1**, 22-epi-20-hydroxyecdysone, and **2**, gerardiasterone) from the aerial parts of *S. tinctoria*. One of them, 22-epi-20-hydroxyecdysone (**1**), is a new natural product, while **2**, gerardiasterone, has been previously described as an animal metabolite from the Mediterranean Zooanthid *Gerardia savaglia*,[6] and more recently, its structure was confirmed by chemical synthesis.[7,8]

TLC analyses of the MeOH extract of *S. tinctoria* indicated the presence of two ecdysteroids having chromatographic behavior on silica similar to that of 20-hydroxyecdysone. At the same time, their chromatographic behavior (R_f values) suggested that they may not have been isolated before from this plant. Separation using reversed-phase preparative thin-layer chromatography was done to give two fractions.

Fraction 1 was separated by normal-phase HPLC to give **1** and 20-hydroxyecdysone (**3**), which was identified by comparison with an authentic sample.

Positive CI/D MS analysis of **1** gave ions at *m*/*z* 498 ($M + H + NH_3$), 481 ($M + H$), 463, 455, 427, and 409 (losses of one, two, three, or four water molecules), 380 ($M + NH_3 - C_6H_{13}O_2$), 363 ($M - C_6H_{13}O_2$), 345, all of which are the same as those given by **3**. A molecular weight of 480 and the same distribution of OH groups between the nucleus and the side chain as **3** were indicated. Both the molecular weight of these two compounds (**1** and **3**) and their mass fragments derived either from their nucleus or side chain were the same. This fact proves that both the number of hydroxyls and their distributions are the same in compounds **1** and **3**. However, the TLC and HPLC characteristics of these two compounds were different. Comparison of the ^{1}H NMR spectrum of **1** (Table 1) with that of **3** shows that the only significant difference is the H-17 signal, which shows a large downfield shift of +0.2 ppm. Like **3**, compound **1** bears a 25-OH group and a 20,22-diol moiety, so differences between the two compounds concern only the stereochemistry at C-20 and/or C-22. Compound **1** coelutes and has a superimposable ^{1}H



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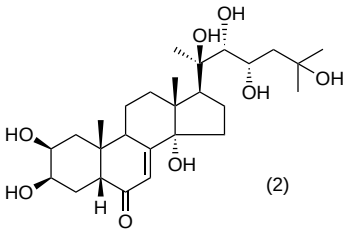

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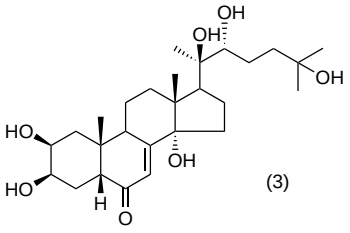

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Table 1. ^{1}H and ^{13}C NMR Data of 22-Epi-20-hydroxyecdysone (**1**)

carbon	multiplicity	δ (ppm)	proton	δ (ppm) and multiplicity[a]
C-1	CH_2	36.3	1-Hax	1.4 (t, 13)
			1-He	1.85
C-2	CH	68.3	2-Hax	3.99 (m, $w_{1/2} = 22$)
C-3	CH	68.2	3-He	4.07 (m, $w_{1/2} = 8$)
C-4	CH_2	32.4	4-Hax	1.75
			4-He	1.75
C-5	CH	51.4	5-H	2.34 (t*)
C-6	C	not observed		
C-7	CH	122.1	7-H	5.98 (d, 2.5)
C-8	C			
C-9	CH	35.1	9-Hax	3.10 (m, $w_{1/2} = 22$)
C-10	C	39.1		
C-11	CH_2	not observed	11-Hax	1.75
			11-He	1.85
C-12	CH_2	32.1	12-Hax	1.75
			12-He	1.95
C-13	C	48.0		
C-14	C	86.3		
C-15	CH_2	not observed	15-Hα	1.65
			15-Hβ	2.05 (m)
C-16	CH_2	not observed	16-Hα	1.85
			16-Hβ	1.9
C-17	CH	50.2	17-H	2.55 (t, 9.2, 9.2)
C-18	CH_3	18.1	18-Me	0.85 (s)
C-19	CH_3	24.2	19-Me	1.00 (s)
C-20	CH	78.6		
C-21	CH_3	21.3	21-Me	1.27 (s)
C-22	CH	78.6	22-H	3.35 (d, 10)
C-23	CH_2	not observed	23-Ha	1.38
			23-Hb	1.88
C-24	CH_2	41.7	24-Ha	1.76
			24-Hb	1.48
C-25	C	73.0		
C-26	CH_3	28.3	26-Me	1.238 (s)
C-27	CH_3	29.3	27-Me	1.243 (s)

[a] Multiplicity of signals: s, singlet; d, doublet; t, triplet; t*, triplet-like structure (because 4-Hax and 4-He are isochronous); m, multiplet; b, broad signal. $w_{1/2}$: width at half-height in Hertz. ax: axial. e: equatorial.

NMR spectrum with synthetic 22-epi-20-hydroxyecdysone ((20*R*,22*S*)-20-hydroxyecdysone), prepared by reduction of 22-oxo-20-hydroxyecdysone[3]—in the form of a mixture of 20-hydroxyecdysone and 22-epi-20-hydroxyecdysone. Therefore, compound **1** is identified as 22-epi-20-hydroxyecdysone. The ^{13}C NMR assignment of **1** is presented in Table 1.

Fraction 2 from the preparative TLC was chromatographed on normal-phase HPLC to give **2**. Chemical ionization/desorption mass spectrometry gave evidence that compound **2** bears an additional OH group on the side chain in comparison to **3**. The ^{1}H NMR data indicated that the location of this additional OH is at position 23. This compound was determined to be gerardiasterone.[6−8] Finally, comparison with an authentic sample (kindly provided by Profs. Guerriero and Honda) showed that both compounds have the same retention time on normal-phase HPLC and superimposable ^{1}H NMR and ^{13}C NMR spectra.

Recently, the configuration of the side chain of gerardiasterone has been elucidated by stereospecific synthesis as 20*R*,22*R*,23*S*.[7,8] The ^{1}H NMR data are very similar between **1** and **2** in regard to the H-17 signal (both chemical shift and coupling constants). As the C-22-configuration is clearly established for **1** as 22-epi (22*S*) and the 22*R*-configuration has been shown for gerardiasterone, this can only be explained if the (22*R*)-OH-(23*S*)-OH moiety has exactly the same effect on the H-17 ^{1}H NMR resonance as the single (22*S*)-OH group in **1**. 22-Epi-20-hydroxyecdysone (**1**) is a new natural product. We previously isolated 22-oxo-20-hydroxyecdysone from the same plant,[3] and it is therefore tempting to regard the latter as a possible biosynthetic intermediate in an oxidation−reduction sequence. A 22-epi (22*S*) configuration has already been described for an ecdysteroid molecule (22-epi-14α-hydroxyacetylpinnasterol) from the red alga *Laurencia pinnata*,[9] and this configuration is also the rule for brassinosteroids.[10]

Experimental Section

Plant Material. *S. tinctoria* L. was grown in the field of the Department of Medicinal Plant Production, University of Horticulture and Food Industry (Budapest, Hungary), and collected in June 1990. A herbarium specimen is deposited in the herbarium of the University of Horticulture and Food Industry. The aerial parts were cleaned, air-dried, and milled.

General Experimental Procedures. Mass spectra were performed with a Riber 10-10B (Nermag SA) using a direct inlet probe. Mass spectra were obtained using a CI/D mode with ammonia as a reagent gas. NMR spectra were recorded on a Bruker AMX-500 apparatus (i.e., 500 MHz for ^{1}H, 125 MHz for ^{13}C) using TSP-d_4 as reference for both carbon and proton.

Precoated silica gel 60 F_{254} TLC plates and RP-18 F_{254}S TLC plates, 0.25 mm thick (E. Merck, Darmstadt, Germany), were used. Normal phase TLC systems were as follows: (1) CH_2Cl_2−EtOH (96%) 85:15 (v/v); (2) EtOAc−MeOH−NH_3 concentrated 85:10:5 (v/v/v); (3) EtOAc−EtOH−water 8:2:1 (v/v/v). R_f values (in TLC system 2) of compounds **1**, **2**, and **3** were 0.14, 0.12, and 0.18, respectively. Each of compounds **1**, **2**, and **3** gave a turquoise color after vanillin−sulfuric acid spray reagent. Reversed-phase TLC system were as follows: (4) MeOH−water 65:35 (v/v).

Ecdysteroids were detected after development, either directly by fluorescence quenching at 254 nm (in the case of reversed-phase TLC, only this type of detection was used) or by using the vanillin−sulfuric acid spray reagent[4] and observing the spots both in daylight and also under UV light at 356 nm. Preparative HPLC separations were performed using Waters HPLC equipment (Millford, MA) comprising two 6000A pumps, an M720 programmer, a WISP automatic injector, and an M440UV detector set at 254 nm. A Zorbax-SIL (250 × 9.4 mm) column (DuPont) was used with a flow rate of 4 mL min^{-1}. The mobile phases were as follows: (1) CH_2Cl_2−2-PrOH−water (125:40:3, v/v/v); (2) CH_2Cl_2−2-PrOH−water (125:25:2, v/v/v); (3) cyclohexane−2-PrOH−water (80:40:3, v/v/v). Retention data (t_R, min) of the compounds were in the mobile phases 1, 2, and 3; 26.4, n.o. (not observed), 16.4 for compound **1**; n.o., 45.2, and 17.0 for compound **2**; 17.6, 35.2, and 13.4 for compound **3**, respectively.

Extraction and Purification. The dried and milled plant materials (864.9 g) were macerated with MeOH in a percolator at room temperature for 24 h and extracted with 15.5 L of MeOH. After evaporation of the solvent, the crude extract (161.4 g) was dissolved in 700 mL of boiling 50% (v/v) aqueous MeOH, which after cooling was extracted six times with 350 mL of C_6H_6. The C_6H_6 phase was extracted three times with

500 mL of 50% aqueous MeOH. The aqueous MeOH extracts were combined with the aqueous phase and concentrated to dryness. The residue (129.9 g) was dissolved in 300 mL of hot MeOH. The MeOH solution was mixed with 600 mL of acetone and the precipitate removed by decantation and washed twice with 150 mL of MeOH−acetone (1:2, v/v). The washings and supernatant were combined, concentrated to dryness, and redissolved in 200 mL of MeOH to which 200 mL of acetone was added. The precipitate was washed twice with 150 mL MeOH−acetone, 1:1; the washings and the filtrate were combined and concentrated to dryness. Each of the purification steps was monitored by TLC using systems 1, 2, and 3.

Isolation of Ecdysteroids. The purified extract (72.1 g) was dissolved in 200 mL of MeOH, adsorbed onto 300 g of silica gel (silica gel for column chromatography, particle size 0.063−0.2 mm from E. Merck), and packed on the top of a 150 cm × 5 cm column containing 1400 g of silica gel. Ecdysteroids were eluted stepwise with 9:1, 8:2, 7:3, and 5:5 (v/v) CH_2Cl_2−EtOH; the individual steps consisted of 15.9, 13.8, 10.2, and 4.2 L eluent, respectively, with 300 mL fractions being collected. Fractions 56−61 were combined, concentrated to dryness (3 g), and further purified by column chromatography. The residue of fractions 56−61 was dissolved in 100 mL of MeOH, adsorbed on 200 g of alumina, and chromatographed on a 70.5 × 5.5 cm column containing 1100 g of alumina. The eluent was EtOAc−MeOH−water (85:10:5, v/v/v), and 30 mL fractions were collected. Fractions 52−75 were combined, concentrated to dryness (0.72 g), redissolved in MeOH, adsorbed on 5 g of silica gel, and further purified using 50 g of silica gel in a 3 × 50 cm column, eluting with 85:15 (v/v) CH_2Cl_2−EtOH and collecting 20 mL fractions. Fractions 73−96 were combined, concentrated to dryness (dry residue: 0.021 g), and redissolved in MeOH. Preparative separation using reversed-phase TLC was done on RP-18 $F_{254}S$ TLC plates with MeOH−water (65:35) to give two fractions. Zones of ecdysteroids were located under UV light. Zones at R_f 0.51 (**1**, fraction 1), 0.55 (**2**, fraction 2), and 0.48 (**3**) were scraped and harvested. Ecdysteroids were eluted using methanol and taken to dryness.

Final purification of **1**−**3** was accomplished by normal-phase HPLC using solvent systems 1 and 2 (fractions 1 and 2). The yield was 110, 132, and 72 μg for **1**, **2**, and **3**, respectively.

^{13}C NMR assignments for **1** were confirmed by PFG-HMQC and HMBC spectra.

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