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New Phytoecdysteroids from Roots of *Ajuga reptans* varieties.

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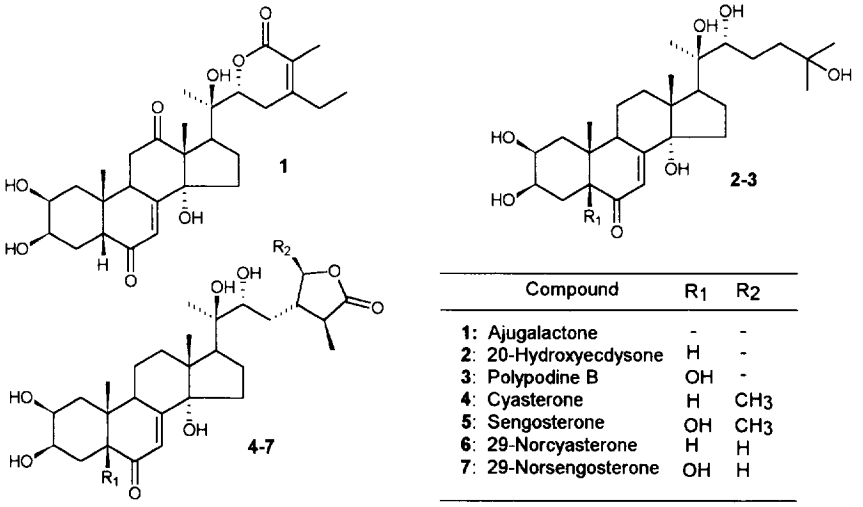
Abstract: Reptansterone (**8**), a new C-29 phytoecdysteroid with a δ-lactone in the side chain, was isolated from roots of a green variety of *Ajuga reptans*. Likewise, other unprecedented members of this polyhydroxysteroid family namely 28-*epi*-sengosterone (**9**), 5,29-dihydroxycapitasterone (**10**) 2- and 3-dehydroajugalactone (**11** and **12**) were isolated from *A. reptans* var. *atropurpurea*. The structures of all these new compounds were inferred from the corresponding ^{1}H and ^{13}C-NMR homo- and heterocorrelations and IR and HPLC-MS(TSP) spectral data.

Many plants contain ecdysteroids, polyhydroxysteroids with a *cis*-fused A/B ring junction and 14α-hydroxy-7-ene-6-one system[1], structurally related with ecdysone, the biosynthetic precursor of the insect moulting hormone. These compounds exhibit physiological activities in insects and also in mammals. Regulation of moulting is the best established function of ecdysteroids in insects, but other hormonal functions involve regeneration, metamorphosis, reproduction and differentiation in all arthropod taxa[2]. Furthermore, these compounds also exhibit interesting activities in mammals, such as antiulcer and antirheumatic, insulin regulation and diuretic or tonic effects[3-5].

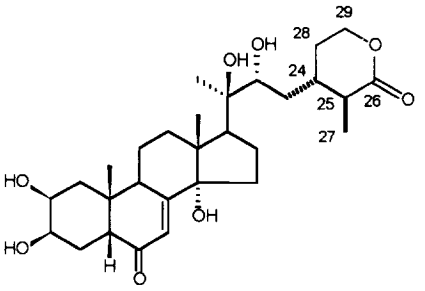


Compound	R_1	R_2
1: Ajugalactone	-	-
2: 20-Hydroxyecdysone	H	-
3: Polypodine B	OH	-
4: Cyasterone	H	CH_3
5: Sengosterone	OH	CH_3
6: 29-Norcyasterone	H	H
7: 29-Norsengosterone	OH	H

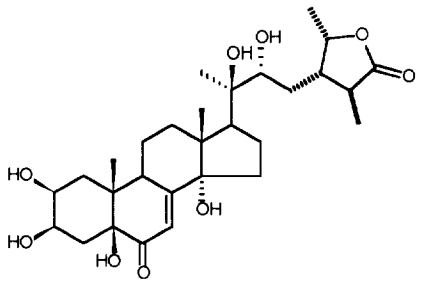
Figure 1.- Structures of most abundant ecdysteroids previously isolated from *Ajuga reptans* plants.

Over 100 different related structures of ecdysteroids have so far been isolated from plants[6]. Nevertheless, the role of these compounds in plants is still an open question, a defensive activity against insect or nematode attack has been suggested, but other functions can not be ruled out. On the other hand, biosynthesis, metabolism and tissue distribution of ecdysteroids is only partially known and the presence of well characterized minor compounds could provide information about those processes.

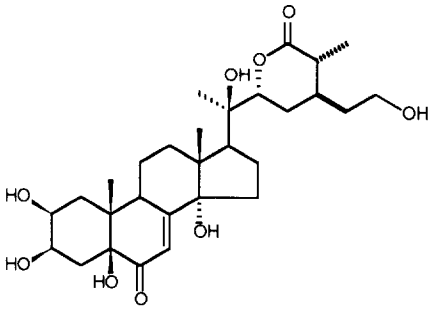
Among the numerous plants containing phytoecdysteroids, those pertaining to *Ajuga* genus are unique for the great variety of such compounds produced[7]. It is worth to mention that in an earlier work we isolated from wild specimens and *in vivo* and *in vitro* cultures of a green variety of *Ajuga reptans* the C-27, C-28 and C-29 phytoecdysteroids depicted in Figure 1 that account for the most part of the contents in these compounds[8-11]. More recently, we characterized in extracts from aerial parts of *A. reptans* var. *atropurpurea*[12], a new family of related structures with a 12-hydroxy-22-oxo functionalization, and different acetylated ecdysteroids (at position 2 or 3) were isolated from dried roots of this variety[13]. Continuing these studies, in the present communication, we report on the isolation of five new phytoecdysteroids (see Figure 2 for structures) from roots of green and purple varieties of this plant.



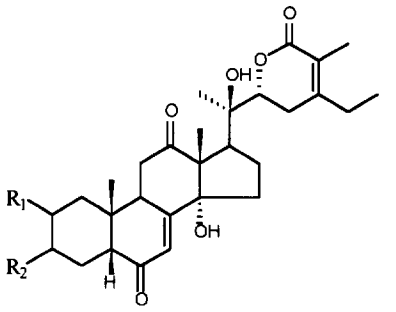
8: Reptansterone (REP)



9: 28-*Epi*-sengosterone (28*epi*5CY)



10: 5,29-Dihydroxycapitasterone (5,29CAP)



11: 3-Dehydroajugalactone (3DAJL) R_1=OH, R_2=O

12: 2-Dehydroajugalactone (2DAJL) R_1=O, R_2=OH

Figure 2.- Structure of the new phytoecdysteroids isolated from *Ajuga reptans* plants.

Results and Discussion

From a methanol extract of dried roots of green wild *Ajuga reptans* cultured in greenhouse we isolated, as described below in the Experimental Part, different known compounds: **7** (29N5CY), **6** (29NCY), **2** (20E), **3** (5,20E), **4** (CY), **5** (5CY) and **1** (AJL) and a new minor component with the spectral features of a

phytoecdysteroid. The structure **8** was assigned to this new compound named reptansterone (REP) by combined information from different spectroscopic methods (IR, MS-TSP and mainly NMR: ^{1}H, ^{13}C, Selective TOCSY, COSY and HETCOR). The ^{1}H NMR and ^{13}C NMR data, summarized in Tables 1 and 2, were consistent with the tetracyclic system of **2** (20E), with a six membered lactone ring in the side chain as confirmed by IR CO absorption at 1718 cm^{-1}. In the ^{1}H-NMR spectrum only one doublet signal appears at δ=1.25 attributable to a CH_3-CH moiety as in capitasterone, but differences in the chemical shifts of H-22 (3.89 ppm) and C-22 (75.7 ppm) point out to the occurrence of a free OH group at this site like in **2** (20E), **5** (5CY) and other related structures[14]. Taking into account the above data and that in the ^{13}C NMR spectrum the CH_2O signal appears at 67.2 ppm it is concluded that C-29 must be enclosed in the lactone ring. The ^{1}H-NMR COSY spectra did not allow to establish definite correlations among the different protons due to superposition of the corresponding absorptions.

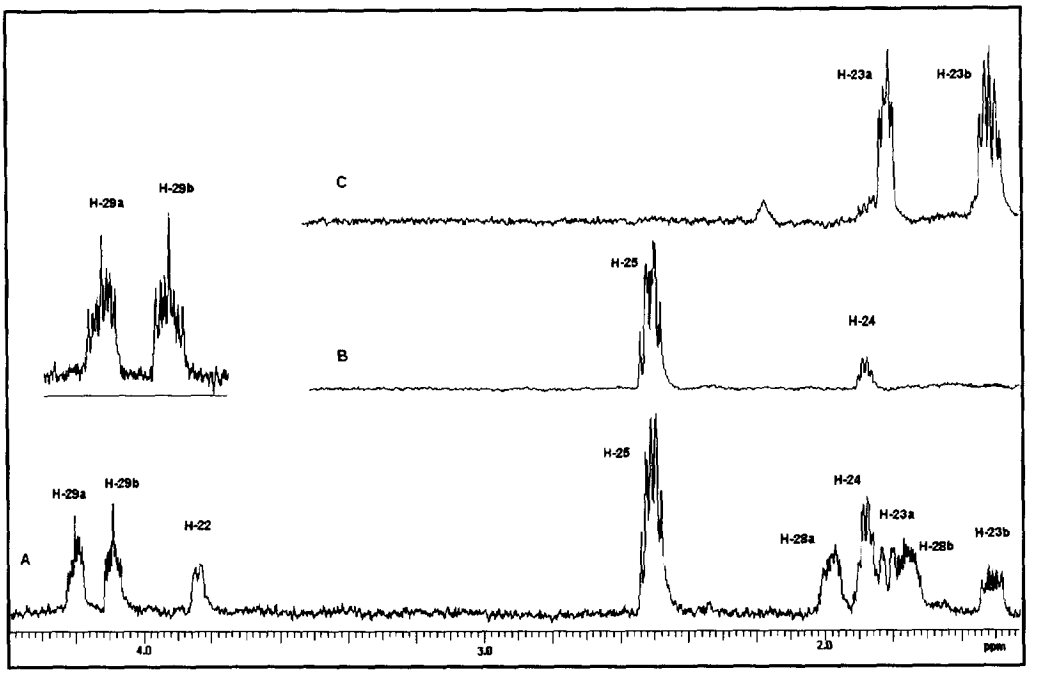
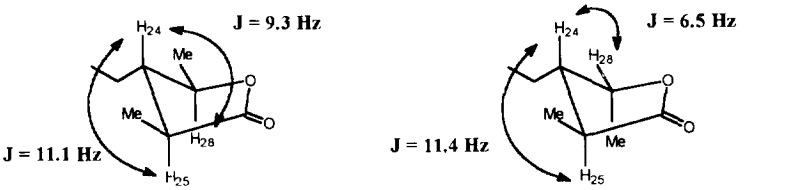


Figure 3.- Selective TOCSY spectra of **8**. Excited signal: A) Me-27 (1.39 ppm) mixing time 75 ms; B) Me-27 (1.39 ppm), mixing time 25 ms; C) H-22 (3.82ppm) mixing time 25 ms. The inset corresponds to a H-29 region expansion of spectra A.

This drawback was overcome by a series of selective TOCSY spectra that confirm the proposed structure (Fig.2). The irradiation of the H-22 signals allow the easy identification of H-23_a and H-23_b absorptions at 1.88 and 1.57 ppm and the irradiation of the Me-27 absorption (δ=1.39 ppm) with a long mixing time (70 ms) allowed observation of all absorptions in the lactone system which permits the assignment of H-28 (at 2.04 and 1.82 ppm) and H-29 (at 4.14 and 4.26 ppm) multiplet signals and the corresponding coupling constants ($J_{29,28a}$=4.0, $J_{29,28b}$=7.0 and $J_{29a,29b}$=11.0 Hz). Finally, the irradiation of the Me-27 absorption with short mixing time (25 ms) allowed the clean observation of the signals of H-25 at 2.6 ppm and H-24 at 1.95 ppm and the determination of coupling constants for H-25 (dq, $J_{25,24}$ = 9 Hz and $J_{25,27}$ = 7Hz) indicating a trans-diaxial arrangement of both ring protons. It is

worth of note that this γ-lactone ring of reptansterone, linking C-24 and C-29 of the side chain, is unique among all phytoecdysteroids structures heretofore reported in the literature.

From methanol extracts of dried roots of *Ajuga reptans* var. *atropurpurea* treated as described below in the Experimental Part four new phytoecdysteroids were isolated, namely, 28-*epi*-sengosterone (28*epi*5CY) **9**, 5,29-dihydroxycapitasterone (5,29CAP) **10**, 2-dehydroajugalactone (2DAJL) **11** and 3-dehydroajugalactone (3DAJL) **12** (see Figure 2 for structures).



A) Sengosterone (5CY) **B) 28-*Epi*-sengosterone (28*epi*5CY)**

Figure 4.- H,H vicinal coupling constants values for methines in the side chain ring system of sengosterone **5** and its 28 epimer **9**

As shown in Tables 1 and 2, the ^{1}H and ^{13}CNMR spectra for compound **9** showed a high analogy with those of sengosterone. Thus, the chemical shifts of Me-21 and H-22 are identical in both compounds, but the absorptions for H-28 and one of the CH_3 groups are shifted + 0.99 and -0.20 ppm, respectively, suggesting that the new compound might be one epimer at C-24, C-25 or C-28. The final assignment was carried out by selective TOCSY and ROESY experiments. Selective TOCSY spectra were performed at three different mixing times by selecting the low field CH_3 doublet at 1.17 ppm which led us to observe the full correlation frame for the side chain and the relative bond separation (with respect to the selected methyl). Thus, the H-28 signal masked in the original spectrum by the absorption of H_2O appears as a quintuplet with a coupling constant $J_{24\text{-}28}$=$J_{28\text{-}29}$=6.5 Hz. When comparing the $J_{24\text{-}28}$ and $J_{24\text{-}25}$ of CY and 5CY (9.3 and 11.1 Hz) with those of the new compound (6.5 and 11.4 Hz, respectively), it is confirmed that the inversion of configuration is at C-28, thereby H-24 and H-28 are in a quasiaxial-quasiequatorial relative position. This arrangement is confirmed in the ROESY spectrum where a correlation of the absorption of H-28 with those of H-24 and Me-29 can be established indicating a spatial interaction among these moieties. Likewise, in the ^{13}C-NMR a high field shift of the C-29 absorption is observed when compared with that of sengosterone **5** due to the change of arrangement from quasiequatorial to quasiaxial. This stereochemical arrangement in the butyrolactone moiety is unprecedented in this family of natural compounds

As shown in Tables 1 and 2 the ^{1}H and ^{13}C-NMR spectra of compounds **11** and **12** exhibit similitudes with those of ajugalactone (**1**), except in the presence of an extra carbonyl group and differences in the chemical shifts in the absorptions corresponding to ring A. For 2DAJL **12**, the absence of the H-2 absorption signal and the changes of chemical shifts for H-1e, H-3e and H-4a (+0.48, +0.39 and +0.24 ppm, respectively), suggest the presence of the extra carbonyl group at C-2 that gives the ^{13}C NMR signal at 209.4 ppm. It is noteworthy the magnitude 11.1 Hz of the $J_{3,4a}$ that could be rationalized by a conformation change in the ring A, possibly due to the formation of a hydrogen bond between the C=O at the 2 position and the OH at the 3 site, originating an eclipsed arrangement between H-3 and H-4a. In the case of compound **11**, the changes observed in the chemical shifts of H-1, H-2, H-5 and H-9 in 3 DAJL when compared to the corresponding absorptions in AJL (+0.65, +0.87, -0.22 and +0.41 ppm)

are similar to those reported in the literature for 3-dehydroecdysone when compared to those for ecdysone[14] which allow easily the corresponding structural assignations.

Table I. ^{1}H-NMR chemical shifts for reference compounds **1**, **2**, **5**[12] and for the new isolated ecdysteroids **8-12** obtained from ^{1}H-NMR, COSY and HETCOR. na means not assigned chemical shift.

	1	**2**	**5**	**8**	**9**	**10**	**11**	**12**
H-1	1.96	2.14, 1.91	2.22, 2.08	2.13, 1.91	na	2.24, 2.08	na	2.44, 1.88
H-2	4.00	4.17	4.25	4.19	4.27	4.24	4.87	-
H-3	4.20	4.21	4.16	4.25	4.18	4.18	-	4.59
H-4	2.02, 1.62	2.01, 1.80	2.06, 1.95	2.05, 1.81	na	2.04, 1.95	na	2.46, 1.87
H-5	3.09	3.01	-	3.03	-	-	2.78	3.00
H-7	6.40	6.25	6.31	6.27	6.31	6.26	6.41	6.39
H-9	4.00	3.58	3.65	3.60	3.66	3.62	4.41	3.73
H-11	2.86	1.88, 1.71	1.93, 1.80	1.89, 1.74	na	1.87, 1.75	3.01	2.88
H-12	-	2.58, 1.95	2.64;2.05	2.61, 2.04	2.62, 2.08	2.58, 1.90	-	-
H-15	2.46, 2.04	2.14, 1.89	2.21, 1.97	na	na	2.20, 1.85	na	na
H-16	2.62, 2.08	2.44, 2.08	2.47, 2.05	2.40, 1.98	na	2.35, 2.07	na	2.58, 2.04
H-17	3.50	3.00	2.85	2.86	2.88	2.90	3.51	3.43
H-22	4.46	3.87	3.94	3.89	3.90	4.78	4.46	4.44
H-23	2.42, 2.23	2.14, 1.85	1.72, 1.70	1.88, 1.57	1.81, 1.69	2.22, 1.85	2.44, 2.23	2.47, 2.22
H-24	-	2.28, 1.81	2.22	1.94	2.48	2.22	-	-
H-25	-	-	2.36	2.56	2.33	2.62	-	-
H-28	1.87	-	4.02	2.04, 1.82	5.01	1.83, 1.55	1.87	1.86
H-29	-	-	-	4.26, 4.14	-	3.86	-	-
Me-18	1.54	1.21	1.24	1.22	1.24	1.11	1.55	1.50
Me-19	1.16	1.06	1.17	1.06	1.18	1.16	1.19	1.24
Me-21	1.70	1.58	1.57	1.56	1.61	1.44	1.70	1.66
Me-26	-	1.36	-	-	-	-	-	-
Me-27	1.87	1.36	1.36	1.39	1.17	1.25	1.87	1.87
Me-29	0.68	-	1.31	-	1.31	-	0.67	0.68

Finally, the last compound isolated **10** (5,29 CAP) exhibited in the ^{13}C NMR spectrum 29 signals, 21 of them coincident with those of sengosterone. The occurrence of a δ-lactone ring in the side chain was evidenced by the carbonyl absorptions at 1714 cm^{-1} in the IR and 174 ppm in the ^{13}C NMR spectrum, as well as the signal at 83.5 ppm assigned to C-22, coincident with that of ajugalactone, indicating that this carbon is enclosed in the lactone ring. The COSY spectrum allowed the unambiguous assignment of all the signals that were coincident with the 5β-hydroxy systems of 5CY and 5,20E and the confirmation of the lactone ring by similitude with ajugalactone signals. Thus, low field signal (d,d) at 4.78 ppm of one deshielded CH was assigned to H-22 and the adjacent side chain signals were assigned by the corresponding COSY correlations to H-23 (1.85 and 2.22 ppm), Me-27 (1.25 ppm), H-25 (2.62 ppm) and H-24 (2.22 ppm). The coupling constants of H-22 with H-23a and H-23e (11.5 Hz and 4.5 Hz, respectively) suggest an axial arrangement of this hydrogen, maintaining a *R* configuration at C-22. Likewise, the multiplet at 3.86 ppm is attributable to a -CH_2-O moiety, bound to a methylene group CH_aH_b, since there is a correlation of that moiety with two protons at 1.55 and 1.83 ppm, being structurally related these protons among them and with a signal at 2.22 ppm (H-24). These data are consistent with the occurrence of a saturated six membered lactone ring with a hydroxy group at C-29. Closely related structures such as capitasterone[15] and precyasterone[16] have been reported in the literature, but scarce ^{1}H-NMR data are given in these cases, although

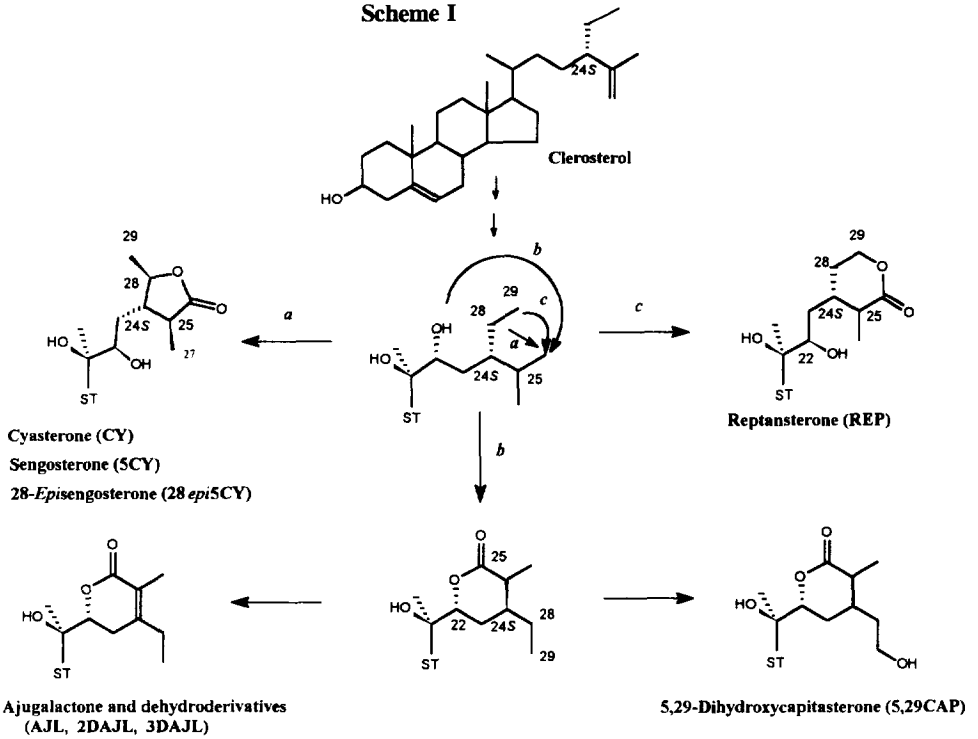
there is coincidence in the absorptions of C-18, C-21 and C-27 methyl groups. Finally, the HETCOR spectrum allowed to complete the assignment of the chemical shifts of C-28 and C-29 methylene chain carbons.

Table II. ^{13}C-NMR chemical shifts for reference compounds **1**, **2**, **5**[12] and new phytoecdysteroids **8-11**. Data obtained from ^{13}C, DEPT and HETCOR spectra.

	1	**2**	**5**	**8**	**9**	**10**	**11**	**12**
C-1	37.8	38.1	34.8	37.9	34.8	34.8	38.5	48.9
C-2	67.9	68.3	67.8	68.1	67.9	67.9	71.7	209.4
C-3	67.6	68.2	69.8	68.0	69.8	69.9	209.7	74.6
C-4	32.1	32.5	35.9	32.4	35.9	36.0	45.4	35.7
C-5	50.8	51.5	79.8	51.4	79.8	79.9	57.5	55.1
C-6	202.7	203.6	200.9	203.5	200.9	200.9	198.2	199.6
C-7	123.3	121.8	120.1	121.6	120.0	120.0	122.5	122.9
C-8	166.8	166.1	166.4	166.0	166.6	166.6	166.8	166.8
C-9	36.8	34.7	38.2	34.4	38.2	38.2	37.4	38.3
C-10	39.8	38.8	44.8	38.6	44.8	44.8	39.9	44.2
C-11	36.8	21.3	22.0	21.0	22.0	22.0	36.8	36.4
C-12	210.2	32.2	32.2	32.0	32.1	32.0	208.4	209.2
C-13	61.5	48.3	48.2	48.1	48.2	47.9	61.7	61.6
C-14	89.1	84.4	83.9	84.0	83.9	83.9	89.1	88.8
C-15	31.9	31.9	31.9	31.6	31.6	31.6	32.0	31.9
C-16	21.0	21.6	21.2	21.4	21.3	21.1	21.0	21.0
C-17	43.8	50.3	49.8	49.8	50.0	49.7	43.9	43.9
C-18	17.4	18.0	17.8	17.8	17.9	18.0	17.4	17.4
C-19	23.7	24.6	17.1	24.4	17.1	17.1	22.1	22.3
C-20	74.9	77.1	76.7	76.8	76.7	76.3	74.9	74.9
C-21	22.1	21.8	20.9	21.1	21.1	20.7	22.1	22.1
C-22	83.1	77.7	73.9	75.7	76.4	83.5	83.1	83.1
C-23	30.1	27.6	34.4	36.9	30.8	28.6	30.1	30.0
C-24	154.2	42.6	48.6	37.8	46.0	33.5	154.2	154.3
C-25	121.2	69.9	42.4	41.2	38.7	41.0	121.2	121.1
C-26	162.1	30.1	179.2	174.8	179.1	174.5	163.1	161.2
C-27	12.2	30.2	15.8	16.5	16.3	13.7	12.2	12.2
C-28	27.0	-	79.8	29.6	78.7	31.1	27.1	27.0
C-29	11.5	-	19.3	67.2	14.3	60.2	11.5	11.5

At this point, only the relative stereochemistries at the new chiral centers in the side chain could be assessed. Absolute stereochemistry in phytoecdysteroids has been usually determined by X-ray diffraction in only a few cases due to the difficulty of obtaining good crystals of these compounds. On the other hand, recent studies on phytoecdysteroids biosynthesis using *in vitro* roots cultures of *Ajuga reptans* showed that, whereas incubation with labeled acetate promoted label incorporation into C-27, C-28 and C-29 structures, labeled cholesterol only afforded this incorporation into C-27 phytoecdysteroids suggesting that C-24 alkylsterols might be the biosynthetic precursors of C-28 and C-29 compounds[17]. In this context, as depicted in Scheme 1, a biosynthetic route could be tentatively proposed for all the different C-29 phytoecdysteroids isolated from *A. reptans* starting from clerosterol, a 24-ethylsterol, occuring together with the corresponding 22,23-didehydroderivative, in this plant[18]. As all the C-28 and C-29 phytoecdysteroids isolated from *Ajuga reptans* had a 24*S* stereochemistry it is acceptable to assign tentatively this stereochemistry to the new isolated compounds. Work is currently under way in our laboratory to confirm this stereochemistry using alternative methodologies to X-ray diffraction and to study the different biosynthetic pathways involved in the phytoecdysteroid production in *Ajuga reptans*.

Scheme I



Experimental section

Plants. *Ajuga reptans* green and purple varieties were obtained by propagation from wild plant in greenhouse and cultured under optimal conditions. The green variety was obtained in the area of Cassà de la Selva (Girona, Spain) and the purple one in Cabrils (Barcelona, Spain). Both plant varieties were harvested at the end of September 1993. Plant samples were water cleaned and air dried and after that, the roots were separated, dried, powdered and used in analytical and preparative work.

HPLC analysis. The chromatographic system used consisted of two pumps (Applied Biosystems-400) injector provided with dynamic mixer (Applied Biosystems-491), diode-UV detector programmer (Applied Biosystems-10005), oven (Spark-Holland SPH-99) and integrator (Hewlett-Packard 3396A). Analytical HPLC (system 1) were carried out using a Merck LiChroCART 125x4 mm column packed with 5 μm LiChrospher 100-RP reversed-phase. Micropreparative HPLC isolations (systems 3-5) were carried out in a Tracer 30x0.78 cm column packed with 10 μm Spherisorb ODS-2 reversed-phase, and for system 2 in a Tracer 15x1 cm column packed with 5 μm Spherisorb ODS-2 reversed-phase.

The following eluent and temperature systems were used:

System 1: i-PrOH:H_2O (9:91); 1.0 mL/min; 55°C

System 2: i-PrOH: H_2O (10:90); 3.0 mL/min; 35°C

System 3: i-PrOH: H_2O (13.87); 3.0 mL/min; 23°C

System 4: i-PrOH: H_2O (12.88); 3.0 mL/min: 55°C

System 5: i-PrOH: H_2O (13.87); 3.0 mL/min; 55 C

System 6: CH_3CN: H_2O (20.80): 3.0 mL/min; 23°C

NMR spectroscopy. ^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz), H,H-COSY[19] and H,C-HETCOR[20] were recorded on a Varian Unity 300 spectrometer under standard conditions. Selective TOCSY[21] (Top-Hat excitation) and ROESY[22] were recorded in a Varian Unity 500 MHz. Chemical shifts are given in ppm, the coupling constants and width at half height ($w_{1/2}$) are given in Hz and the ^{13}C-NMR multiplicities were determined by DEPT experiments. For small samples, spectra were recorded by dissolving in 250 µL (sample concentration *ca.* 25 mM) of dry deuteropyridine and using special low volume NMR tubes with reduction and antivortex glass pluls (Shigemi Inc., BMS-05 microtube).

Mass spectrometry. Spectra were obtained by HPLC-MS using thermospray ion source in a HP-5988A quadrupole instrument with the following conditions: direct flow injection of the sample dissolved in acetonitrile, mobile phase of buffered water (ammonium formate 50 mM pH=6): acetonitrile (50:50) at 1 ml/min. TSP temperatures: tip=180°C, stem=96°C and ion source=260°C.

Isolation and characterization of phytoecdysteroids from green variety. The vegetal material (500 g) was extracted with methanol (4 x 4 L, 48 h). The joined extracts were evaporated under reduced pressure and the residue was treated with a 1:1 H_2O:MeOH mixture (500 mL). The resulting suspension was centrifugated and the supernatant extracted with chloroform (3 x 200 mL). the chloroform layer was dried with Na_2SO_4 and evaporated under reduced pressure to give a residue (1.1 g). Column chromatography of this residue was carried out on silica gel (70 g; deactivated with 10% H_2O) eluting with a $CHCl_3$:MeOH gradient elution system [95:5, 150 mL (fr 1-5), 93:7, 450 mL (fr 6-20), 91:9, 300 mL (fr 21-30); 89:11, 300 mL (fr 31-40)]. HPLC analyses revealed the occurrence of the previously isolated phytoecdysteroids (estimated contents in ppm referred to dry roots weight): 29N5CY (198±16), 29NCY (1494±116), 5,20E (144±10), 5CY (66±8), 20E (69±13), CY (125±14), AJL (358±37), 29NCY3Ac (118±6) and CY3Ac (47±9). Likewise, HPLC analysis of residue of fractions 26-32 (30 mg) revealed the presence of a new phytoecdysteroid, that could be isolated by preparative thin layer chromatography on silica gel by succesive elution with 100:8 and 8:1.2 AcOEt:MeOH to give 29NCY (4.6 mg), CY (6.5 mg) and a mixture of two compounds (5 mg). Final micropreparative HPLC (system 2) of this mixture afforded **reptansterone** (retention time = 25.21 min) as amorphous white powder (1 mg) with the following spectral features.

Reptansterone (REP), [(20R,22R,24ζ)-2β,3β,14α,20,22-pentahydroxy-6-oxo-5β-stigmast-7-ene-26,24^2-lactone].
IR(KBr) (ν cm^{-1}): 3416 (OH), 1718 (δ–lactone), 1654 (cyclohexenone).
MS(TSP) m/z: 521 $[M+H]^+$ (38%), 503 $[M+H-H_2O]^+$ (100%). m/z (negative ions): 565 $[M+HCOO]^-$ (100%), 547 $[M+HCOO-H_2O]^-$ (55%).
^{1}H-NMR (300 MHz) (δ ppm): 6.27(d, 2.1 Hz, 1H, H-7), 4.30-4.20(cs, 2H, H-3e H-29a), 4.20-4.10(cs, 2H, H-2a H-29b), 3.89($w_{1/2}$= 18 Hz, 1H, H-22), 3.60($w_{1/2}$ = 22 Hz, 1H, H-9), 3.03($w_{1/2}$ = 23 Hz, 1H, H-5), 2.86(t, 9.1 Hz, 1H, H-17), 2.68-2.53(cs, 2H, H-12a, H-25), 2.40(m, 1H, H-16a), 2.30-1.65(cs, 14H, H-1 H-4 H-11 H-12b H-15 H-16b H-23a H-24 H-28), 1.56(br s, 4H, Me-21 H-23b), 1.39(d, 6.9 Hz, 3H, Me-27), 1.22(s, 3H, Me-18), 1.06(s, 3H, Me-19).

Isolation and characterization of phytoecdysteroids from purple variety. Dried roots of *Ajuga reptans* var. *atropurpurea*, (200 g) were extracted with methanol (5 x 750 mL, 60 min. with sonication) and procesed as described previously[12] by partition with hexane and chloroform. The joined chloroform washings were evaporated under vacuum and the residue (7.08 g) was separated by silica gel column chromatography (1:50 substrate:silica) with a $CHCl_3$:MeOH gradient elution system, collecting 20 mL fractions [95:5, 240 mL (fr 1-12); 93:7, 680 mL (fr 13-47)]. Analytical HPLC revealed the presence of new phytoecdysteroids in fr 7-10. The residue of these joined fractions (69 mg) was separated by successive micropreparative HPLC (systems 5 and 6) to give **3DAJL** (rt =36.9 min; 2 mg) and **2DAJL** (rt = 42.7 min; 2 mg).

The methanolic layer was filtered through a C-18 reverse phase to remove highly polar substances and fractioned by a second pass through a C-18 Sep-Pak cartridge[12] (namely SP fractions). The occurrence of new

phytoecdysteroids was observed in 9-10 SP fractions. The residue of joined 9-10 SP fractions (0.65 g) was separated by silica gel column chromatography (1:70 substrate:silica) with a CH_2Cl_2:MeOH gradient elution system, collecting 20 mL fractions [94:6, 40 mL (fr 1-2); 93:7, 440 mL (fr 3-24); 91:9, 500 mL (fr 25-50); 90:10, 600 mL (fr 51-80)]. By using micropreparative HPLC (system 3) the following phytoecdysteroids were isolated: from fr 22-29: 29N5CY (rt = 13.8 min; 1 mg), 5CY (rt = 17.5 min; 13 mg), **28epi5CY** (rt = 22.5 min; 1.6 mg), AJL (rt = 39.6 min; 1 mg); from fr.41-44: 29NCY (rt = 16.7 min; 3 mg), CY (rt = 20.2 min; 0.5 mg); from fr 58-64: **5,29CAP** (rt = 21.2 min; 1.5 mg) and by using the HPLC (system 4) from fr 65-70: 5,20 E (rt = 17.9 min; 0.5 mg) and 20-hydroxyecdysone 20E; (rt = 22.5 min; 4 mg).

3-Dehydroajugalactone (3DAJL), [(20R,22R)-2β,14α,20-trihydroxy-3,6,12-trioxo-5β-stigmasta-7,24-diene-26,22 lactone]:

IR (KBr) (ν cm^{-1}): 3427 (OH), 1750-1600 (broad band).

MS(TSP) m/z: 532 $[M+NH_4]^+$ (77%), 515 [M+1] (56%), 514 $[M]^+$ (100%), 497 $[M+1-H_2O]^+$ (63%).

^{1}H-NMR (300 Mhz) (δ ppm): 6.41(d, 2.7 Hz, 1H, H-7), 4.87(partially overlapped with H_2O absorption, 1H, H-2a), 4.46(dd, 12.9, 3.3 Hz, 1H, H-22), 4.41(partially overlapped, 1H, H-9), 3.51(t, 8.6 Hz, 1H, H-17), 3.08-2.92(cs, 2H, H-11), 2.78(dd, 13.4, 5.5 Hz, 1H, H-5), 2.70-2.35(cs, 6H, H-1a, H-1b, H-4a, H-15a, H-16a, H-23a), 2.23(m, 1H, H-23b), 2.15-1.70 (cs, 5H, H-4b, H-15b, H-16b, H-28), 1.87 (s, 3H, Me-27), 1.70 (s, 3H, Me-21), 1.55 (s, 3H, Me-18), 1.19 (s, 3H, Me-19), 0.67 (t, 7.5, 3H, Me-29).

2-Dehydroajugalactone (2DAJL), [(20R,22R)-3β,14α,20-trihydroxy-2,6,12-trioxo-5β-stigmasta-7,24-diene-26,22 lactone]:

IR (KBr) (ν cm^{-1}): 3427 (OH), 1716 (δ–lactone), 1693 (C=O), 1684 (cyclohexenone).

MS (TSP) m/z: 532 $[M+NH_4]^+$ (100%), 515 $[M+1]^+$ (38%), 514 $[M]^+$ (39%), 497 $[M+1-H_2O)^+$ (48%).

^{1}H-NMR (300 MHz) (δ ppm): 6.39(d, 2.7 Hz, 1H, H-7), 4.59(dd, 11.1, 7.5 Hz, 1H, H-3), 4.44(dd, 13.0, 3.5 Hz, 1H, H-22), 3.73(ddd, 9.3, 9.2, 2.7 Hz, 1H, H-9), 3.43 (br t, 9.0 Hz, 1H, H-17), 3.00(dd, 13.5, 4.3 Hz, 1H, H-5), 2.94-2.86(cs, 2H, 11-H), 2.68-2.36(cs, 4H, H-1a H-4a H-16a H-23a), 2.22(dd, 17.4, 3.0 Hz, 1H, H-23b), 2.18-1.80(cs, 7H, H-1b H-4b H-15 H-16b H-28), 1.87(s, 3H, Me-27), 1.66(s, 3H, Me-21), 1.50(s, 3H, Me-18), 1.24(s, 3H, Me-19), 0.68(t, 7.5 Hz, 3H, Me-29).

28-Epi-sengosterone (28epi5CY), [(20R,22R,24ζ)-28-epi-2β,3β,5β,14α,20,22-hexahydroxy-6-oxo-stigmast-7-ene-26,24^1-lactone]:

IR (KBr) (ν cm^{-1}): 3416 (OH), 1749 (γ-lactone), 1670 (cyclohexenone).

MS (TSP) m/z: 554 $[M+NH_4]^+$ (25%), 537 $[M+1]^+$ (100%), 536[M]$^+$ (82%), 519 $[M+1-H_2O]^+$ (34%), 501 $[M+1-2H_2O]^+$ (21%).

^{1}H NMR (300 MHz) (δ ppm): 6.27(d, 2.1 Hz, 1H, H-7), 6.11($w_{1/2}$ = 8 Hz, 1H, H-22OH), 5.88(d, 8.1 Hz, 1H, H-3OH), 5.56($w_{1/2}$ = 15 Hz, 1H, H-2OH), 4.27($w_{1/2}$ = 24 Hz, 1H, H-2a), 4.18($w_{1/2}$ = 15 Hz, 1H, H-3e), 3.90($w_{1/2}$ = 20 Hz, 1H, 22-H), 3.66($w_{1/2}$ = 24 Hz, 1H, H-9), 2.88(t, 8.4 Hz, 1H, H-17), 2.62(m, 1H, H-12a), 2.56-2.40(cs, 2H, H-16a, H-24), 2.33(dq, 11.4, 6.9 Hz, 1H, H-25), 2.26-1.64(cs, 12H, H-1, H-4, H-11, H-12b, H-15, H-16b, H-23), 1.61(s, 3H, Me-21), 1.31(d, 6.0 Hz, 3H, Me-29), 1.24(s, 3H, Me-18), 1.18(s, 3H, Me-19), 1.17(d, 7.0, 3H, Me-27).

5,29-Dihydroxycapitasterone (5,29CAP), [(20R,22R,24ζ)-2β,3β,5β,14α,20,29-hexahydroxy-6-oxo-stigmast-7-ene-26,22-lactone]:

IR (KBr) (ν cm^{-1}): 3405 (OH), 1714 (δ-lactone), 1671 (cyclohexenone).

MS (TSP) m/z: 555 $[M+1+NH_4]^+$ (17%), 554 $[M+NH_4]^+$ (42%), 537 $[M+1]^+$ (100%), 536 $[M]^+$ (82%), 519 $[M+1-H_2O]^+$ (54%), 501 $[M+1-2H_2O]^+$ (11%).

^{1}H NMR (300 Mhz) (δ ppm): 6.26(d, 2.7 Hz,1H, H-7), 4.78(dd, 11.5, 4.5 Hz, 1H, H-22), 4.30-4.10(cs, 2H, H-2a H-3e), 3.86($w_{1/2}$) = 19 Hz, 2H, H-29), 3.62($w_{1/2}$ = 23 Hz, 1H, H-9), 2.90(t, 8.8 Hz, 1H, H-17), 2.70-2.55(cs, 2H, H-12a, H-25),

2.35(m, 1H, H-16a), 2.30-1.65 (cs, 14H, H-1 H-4 H-11 H-12b H-15 H-16b H-23 H-24 H-28a), 1.55(m, 1H, H-28b), 1.44(s, 3H, Me-21), 1.25(d, 7.2 Hz, 3H, Me-27), 1.16(s, 3H, Me-18), 1.11(s, 3H, Me-19).

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Notes and references

1. Lafont, R.; Horn, D.H.S. In *Ecdysone, From Chemistry to Mode of Action.*; Koolman, J. Ed.; Georg Thieme: Stuttgart, **1989**, pp 39-64.
2. Rees, H. H. In *Ecdysone, From Chemistry to Mode of Action.*; Koolman, J. Ed.; Georg Thieme: Stuttgart, **1989**, pp 28-38.
3. Abubakirov, N. K.; Sultanov, M. B.; Sirov, V. N.; Kurmukov, A. G.; Baltaev, U.; Novosel'skaya, I. L.; Mamatkhanov, A. U.; Gorovits, M. B.; Shakirov, T. T. *Chem. Abstr.* **1989**, *110*, 121377b.
4. Kosovskii, M. I.; Sirov, V. N.; Mirakhmedov, M. M.; Katkova, S. P.; Khushbaktova, Z. A. *Probl. Endokrinol.* **1989**, *35*, 77.
5. Sirov, V. N.; Khushbaktova, Z. A.; Mirzaev, Y. R.; Baltaev, U. A. *Khim. Farm. Zh.*, **1989**, *23*, 441.
6. Camps, F. In *Ecological Chemistry and Biochemistry of Plant Terpenoids.*; Harborne, J.B. and Tomas-Barberán, F.A. Eds.; Clarendon Press: Oxford, **1991**, pp 331-376.
7. Camps, F.; Coll, J. *Phytochemistry*, **1993**, 32, 1361-1370.
8. Camps, F.; Coll, J.; Cortel, A. *Rev. Latinoam. Quim.*, **1981**, 12, 81-87.
9. Camps, F.; Coll, J.; Cortel, A. *Chem. Letters*, **1982**, 9, 1313-1316.
10. Camps, F.; Coll, J.; Cortel, A.; Miravitlles, C.; Molins, E. *J. Chem. Res. (S)*, **1985**, 14-15.
11. Calcagno, M.P.; Camps, F.; Coll, J.; Melé, E.; Messeguer, J.; Tomàs, J. *An. Quim.*, **1994**, 90, 483-486.
12. Calcagno, M.P.; Camps, F.; Coll, J.; Melé, E.; Sánchez-Baeza, F. *Tetrahedron*, **1995**, 51, 12119-12126.
13. Calcagno, M.P.; Camps, F.; Coll, J.; Melé, E. *Eur. J. Entomol.*, **1995**, 92, 287-294.
14. Lafont, R.D.; Wilson, I.D. The Ecdysone Handbook, The Chromatographic Society, Nottingham, U.K., **1992**.
15. Takemoto, T.; Nomoto, K.; Hikino, Y.; Hikino, H. *Tetrahedron Lett.* , **1968**, 4629-4632.
16. Hikino, H.; Nomoto, K.; Ino, R.; Takemoto, T. *Chem. Pharm. Bull.*, **1970**. 18, 1078-1081.
17. Nagakari, M.; Kushiro, T.; Matsumoto, T.; Tanaka, N.; Kakinuma, K.; Fujimoto, Y. *Phytochemistry, 1994*, 36, 907-914.
18. Camps, F.; Coll, J.; Cortel A., *An. Quim.*, **1983**, 79C, 228-231.
19. Piantini, U.; Sorenson, O.W.; Ernst, R.R. *J. Am. Chem. Soc.*, **1982**, 104, 6800-6802.
20. Reynolds, W.F.; McLean, S.; Perpick-Dumont, M.; Enriquez, R. *Mag. Reson. Chem.*, **1988**, 26, 1068-1069.
21. Bax, A.; Davis, D.G. *J. Mag. Reson.*, **1985**, 65, 355-362.
22. Bax, A.; Sklenar, V.; Summers, M. *J. Mag. Reson.*, **1986**, 70, 327-329.

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