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Acetylated ecdysteroids from *Ajuga reptans* var. *atropurpurea* (Lamiales: Lamiaceae)

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Ecdysteroid acetates, *Ajuga reptans* var. *atropurpurea*, Lamiaceae

Abstract. The structural elucidation of acetylated ecdysteroids isolated from dried roots of *Ajuga reptans* var. *atropurpurea*, based on spectroscopic procedures, is reported. Among them 2-O-acetyl- and 3-O-acetyl-20-hydroxyecdysone (20E2Ac, 20E3Ac) and 3-O-acetyl-29-norcyasterone (29NCY3Ac) had previously been only partially described and 3-O-acetylcyasterone (CY3Ac) was heretofore unreported.

INTRODUCTION

Studies on ecdysteroid composition of *Ajuga reptans* have shown so far the presence of several representatives of the three common skeletal types, namely C_{27}, C_{28} and C_{29} structures (Camps et al., 1981, 1982, 1985; Kubo et al., 1983; Matsumoto & Tanaka, 1991; Tomás et al., 1992, 1993). The search for the biosynthetic origin of such an array of compounds is quite an interesting challenge, which adds to the general interest in ecdysteroids and their multiple physiological and ecological effects (Camps, 1991).

The HPLC analysis of *Ajuga reptans* methanolic extracts of different tissues of plants from various origins has shown the presence of several peaks which were considered as putative ecdysteroids on the basis of their UV spectra. However, their retention indices were different from those of known reference compounds previously isolated in our laboratory and, thus, we performed the isolation and structural elucidation of these compounds.

MATERIAL AND METHODS

Plants

Plants were collected in the area of Cabrils (Catalunya, Spain) in September 1993 and were cleaned with water and air-dried. Only roots were used for isolation of the new ecdysteroids, after analytical HPLC showed this organ to have the highest content of these compounds.

HPLC analysis and isolation

The HPLC system (Applied Biosystems Inc.) consisted of two pumps (model 400) for solvent delivery, a dynamic mixer/injector for sample introduction (model 491) and a diode-array detector (model 1000S) for UV spectra acquisition. Chromatograms were monitored at 242 nm and recorded with an integrator (Hewlett-Packard, model 3396A) or a data module (Waters, model 740). Analyses were carried out under reversed-phase conditions, controlled temperature and isocratic conditions [System 1: LiChroCART 125 × 4 mm, packed with LiChrospher 100 RP-18, 5 μm, protected with a guard column (Waters, 24 × 4 mm, packed with Bondapak C-18, 10 μm) at 55°C; i-PrOH : H_2O 1 : 10 at 1 mL/min]. Micropreparative HPLC isolations were carried out under similar conditions (System 2: Spherisorb ODS-2, 10 μm; 300 × 7.8 mm, at 55°C; *i*-PrOH : H_2O 1.1 : 10 at 3 mL/min. System 3: the same column, at 23°C; *i*-PrOH : H_2O 1.5 : 10 at 3 mL/min).

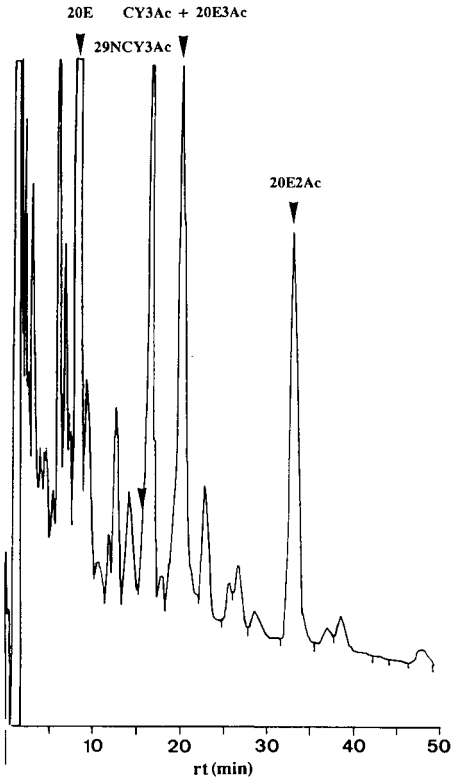


Fig. 1. C_{18} reversed-phase HPLC separation of a methanolic extract of *Ajuga reptans* var. *atropurpurea* roots. LiChroCART 125 × 4 mm, packed with LiChrospher 100 RP-18, 5 µm, at 55°C; i-PrOH : H_2O 1 : 10 at 1 mL/min.

HPLC analysis of crude root extract

A sample (200 mg) of finely powdered dry roots of *Ajuga reptans* var. *atropurpurea* was extracted with methanol and the residue obtained after solvent removal was treated according to a previously described procedure (Tomás et al., 1992). Retention times for the pure newly isolated single compounds under system 1 operating conditions were: 15.68, 19.45, 20.35 and 31.67 min. According to the corresponding spectroscopic data (vide infra) structures 3, 2, 4 and 1 respectively (Fig. 2) were assigned to the peaks in Fig. 1.

Extraction and isolation of phytoecdysteroids

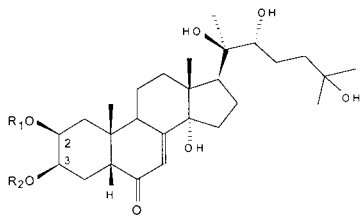
Dried roots of *Ajuga reptans* var. *atropurpurea*, (200 g) were extracted with methanol (5 × 750 mL, 60 min with sonication). The combined extracts were concentrated under vacuum, the volume adjusted to 250 mL, water added (250 mL), and the mixture partitioned with hexane (4 × 200 mL) to remove sterols and other apolar substances. The aqueous methanol layer was then partitioned with chloroform (280 mL). Each layer was back-extracted with the complementary solvent (110 mL). Organic solvents were then evaporated from the aqueous layer, which was afterwards adjusted with methanol (16 mL per each 100 mL of aqueous residue).

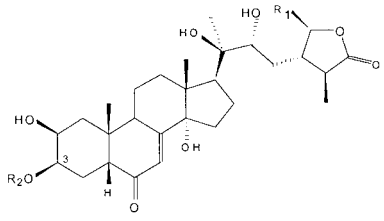
Highly polar substances were removed by filtration of this solution through a C-18 reversed-phase cartridge (10 g), previously conditioned by vacuum filtration of 50 mL of methanol followed by 50 mL of water. Then, a 1/6 aliquot was filtered through, and the retained material eluted with 30 mL of methanol. The cartridge was reconditioned and used again for the next aliquot. The pooled methanolic filtrates were evaporated under vacuum, and the residue diluted with a solution of 4% isopropanol in water (100 mL). The solution was similarly filtered through a C-18 cartridge conditioned as above and eluted by an isopropanol-water system using a 2% gradient increment from 6% to 20% (volume of fractions = 40 mL), and the fractions checked by TLC (Merck Si60F254; $CHCl_3$: MeOH : H_2O 44 : 9 : 1) and HPLC (system 1).

Fractions containing the putative ecdysteroid acetates were dried, and the residue separated by silica gel chromatography using an ethyl acetate-methanol gradient system, the ratio of sorbent to sample being 50 : 1. The final isolation of individual compounds was carried out by reversed-phase HPLC using C_{18}-bonded silica columns [system 2 for compounds 3 and 4 (retention times: 39.1 and 51.2 min respectively); system 3 for 2 and 1 (retention times: 38.5 and 77.5 min respectively)].

NMR spectroscopy

^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) were recorded on a Varian Unity 300 spectrometer. Chemical shifts are given in ppm. The ^{13}C-NMR multiplicities were determined by DEPT experiments. The coupling constants and width at half height ($w_{1/2}$) are given in Hz. 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 plugs with magnetic susceptibility





1	20E2Ac	R_1 = Ac	R_2 = H	3	29NCY3Ac	R_1 = H	R_2 = Ac
2	20E3Ac	R_1 = H	R_2 = Ac	4	CY3Ac	R_1 = Me	R_2 = Ac
5	20E	R_1 = H	R_2 = H	6	29NCY	R_1 = H	R_2 = H
				7	CY	R_1 = Me	R_2 = H

Fig. 2. Ecdysteroids isolated from *Ajuga reptans* var. *atropurpurea* and parent structures.

similar to the value of pyridine to avoid heterogeneities of magnetic field inside the sample volume (Shigemi Inc., BMS-05 microtube).

Mass spectrometry

Spectra were obtained by high-performance liquid chromatography-thermospray mass spectrometry (HPLC-TSP-MS) with a HP-5988A quadrupole apparatus (direct flow injection with HCO_2NH_4 50 mM/ CH_3CN (50 : 50) at 1 mL/min, positive mode, TSP tip 180°C, stem 96°C and ion source 260°C).

Analytical data of isolated compounds

^1H and ^{13}C-NMR data of all four derivatives are presented in Tables 1 and 2.

20-Hydroxyecdysone 2-acetate (1). 11.9 mg of the pure compound were obtained. MS(TSP) m/z: 540 $[M+NH_4]^+$ (7%), 523 $[M+1]^+$ (44%), 522 $[M]^+$ (51%), 504 $[M-H_2O]^+$ (81%), 487 $[M+1-2H_2O]^+$ (100%), 469 $[M+1-3H_2O]^+$ (55%), 463 $[M+1-CH_3CO_2H]^+$ (50%), 462 $[M-CH_3CO_2H]^+$ (30%), 445 $[M+1-H_2O-CH_3CO_2H]^+$ (56%), 427 $[M+1-2H_2O-CH_3CO_2H]^+$ (98%), 389 $[M+2-H_2O-(C\text{-}20/C\text{-}22)]^+$ (side-chain cleavage between C-20 and C-22) (44%), 362 $[M+1-(C\text{-}17/C\text{-}20)]^+$ (44%), 346 $[M\text{-}15-(C\text{-}17/C\text{-}20)]^+$ (46%).

20-Hydroxyecdysone 3-acetate (2). 39.1 mg were obtained of this compound. MS(TSP) m/z: 540 $[M+NH_4]^+$ (60%), 523 $[M+1]^+$ (100%), 522 $[M]^+$ (37%), 505 $[M+1-H_2O]^+$ (65%), 487 $[M+1-2H_2O]^+$ (48%), 469 $[M+1-3H_2O]^+$ (32%), 463 $[M+1-CH_3CO_2H]^+$ (22%), 462 $[M-CH_3CO_2H]^+$ (17%), 445 $[M+1-H_2O-CH_3CO_2H]^+$ (21%), 427 $[M+1-2H_2O-CH_3CO_2H]^+$ (28%), 362 $[M+1-(C\text{-}17/C\text{-}20)]^+$ (21%), 328 $[M\text{-}15-H_2O-(C\text{-}17/C\text{-}20)]^+$ (21%).

29-Norcyasterone 3-acetate (3). 3.4 mg were obtained. MS(TSP) m/z: 566 $[M+NH_4]^+$ (100%), 549 $[M+1]^+$ (41%), 548 $[M]^+$ (19%), 531 $[M+1-H_2O]^+$ (61%), 506 $[M+NH_4-CH_3CO_2H]^+$ (4%), 489 $[M+1-CH_3CO_2H]^+$ (3%).

Cyasterone 3-acetate (4). 1.3 mg were obtained. MS(TSP) m/z: 580 $[M+NH_4]^+$ (88%), 563 $[M+1]^+$ (51%), 562 $[M]^+$ (100%), 545 $[M+1-H_2O]^+$ (69%), 520 $[M+NH_4-CH_3CO_2H]^+$ (27%), 502 $[M-CH_3CO_2H]^+$ (38%).

RESULTS

The ecdysteroids present in *Ajuga reptans* var. *atropurpurea* have been previously described (Matsumoto & Tanaka, 1991) as being 20-hydroxyecdysone, norcyasterone, cyasterone and isocyasterone, from both plant roots and hairy root cultures. In the present work several compounds not previously described in *Ajuga reptans*, with the common feature of being acetyl derivatives have been isolated and structurally identified as the already known 20-hydroxyecdysone 2- and 3-monoacetates (1 and 2, 20E2Ac and 20E3Ac),

TABLE 1. ^{1}H-NMR (in C_5D_5N) spectral data of 20-hydroxyecdysone 2-acetate (1), 20-hydroxyecdysone 3-acetate (2), 29-norcyasterone 3-acetate (3), cyasterone 3-acetate (4), 20-hydroxyecdysone (5), 29-norcyasterone (6) and cyasterone (7). Chemical shifts are given in ppm.

	1	2	3	4	5 *	6	7 #
1-Ha		1.81 **			1.91		
1-He		2.25 **			2.14		
2-Ha	5.25 ($w_{1/2}$=22.5)	4.30 ($w_{1/2}$=22.0)	4.28 (bd $w_{1/2}$=21.0)	4.29 (ba $w_{1/2}$=22.3)	4.17 (m $w_{1/2}$=22)	4.17 (m $w_{1/2}$=21.0)	4.16 (m $w_{1/2}$=21.0)
3-He	4.29 (ba $w_{1/2}$=7.0)	5.51 (ba $w_{1/2}$=8.0)	5.49 (ba $w_{1/2}$=9.0)	5.50 (ba $w_{1/2}$=9.3)	4.21 (m $w_{1/2}$=8)	4.22 (m $w_{1/2}$=8.5)	4.22 (m $w_{1/2}$=8.8)
4-Ha		1.80 **			1.80		
4-He		1.97 **			2.01		
5-H	3.04	2.67 (dd 13.2,3.9)	2.67 (dd 13.5,4.2)	2.67 (dd 13.2,4.2)	3.01	3.01 (dd 12.9,3.6)	3.01 (dd 13.2,3.7)
7-H	6.25 (d 2.4)	6.24 (d 2.1)	6.26 (d 1.8)	6.28 (d 2.4)	6.25 (d 2.5)	6.27 (d 2.2)	6.31 (d 3.3)
9-Ha	3.65 (bt $w_{1/2}$=20.0)	3.57 (bt $w_{1/2}$=23.0)	3.59 (bt $w_{1/2}$=23.0)	3.59 (bt $w_{1/2}$=23.0)	3.58 (m $w_{1/2}$=22)	3.60	3.59
12-Ha	2.50	2.57			2.58 (ddd 13,13,5)	2.63	2.65 (ddd 12.9,12.9,5.1)
17-H	3.02	3.00 (bt 9.3)	2.87 (bt 9.0)	2.87 (bt 9.0)	3.00	2.87 (bt 8.5)	2.86 (bt 8.8)
22-H	3.86 (bd $w_{1/2}$=15.0)	3.88 (bd $w_{1/2}$=14.7)	3.89 (bd $w_{1/2}$=19.6)	3.95 (ba $w_{1/2}$=17.0)	3.87 (m $w_{1/2}$=16)	3.89	3.94 (m $w_{1/2}$=13)
25-H	—	—	2.22 (dq 10.8,6.9)	2.38 (dq 11.1,6.9)	—	2.22 (dq 10.5,7.2)	2.37 (dq 11.1,6.9)
28-H	—	—	4.65 (dd ca. 9.2,7.6)	4.01 (dq 9.3,6.0)	—	4.65 (dd 9.0,7.8)	4.01 (dq 9.3,6.0)
			3.96 (dd ca. 10.4,8.8)			3.96 (dd ca. 10.4,8.8)	
18-Me	1.20 (s)	1.23 (s)	1.24 (s)	1.25 (s)	1.21 (s)	1.23 (s)	1.24 (s)
19-Me	1.07 (s)	1.08 (s)	1.08 (s)	1.09 (s)	1.06 (s)	1.07 (s)	1.07 (s)
21-Me	1.57 (s)	1.60 (s)	1.57 (s)	1.58 (s)	1.58 (s)	1.57 (s)	1.57 (s)
26-Me	1.38 (s)	1.37 (s)	—	—	1.36 (s)	—	—
27-Me	1.38 (s)	1.37 (s)	1.16(d 6.9)	1.37 (d 6.9)	1.36 (s)	1.16 (d 6.9)	1.36 (d 6.9)
29-Me	—	—	—	1.31 (d 6.0)	—	—	1.31 (d 6.0)
CH_3COO	1.93 (s)	1.98 (s)	1.96 (s)	1.97 (s)	—	—	—

* Lafont & Wilson (1992);
# the spectra of 7 has been partially described (cf. Lafont & Wilson, 1992);
** stereochemical assignments (ax : eq) was uncertain;
ba – broad absorption; bd – broad doublet; bt – broad triplet.

29-norcyasterone 3-acetate (3, 29NCY3Ac) and the new compound cyasterone 3-acetate (4, CY3Ac) from roots of a garden sample of *Ajuga reptans* var. *atropurpurea*. Only 29-norcyasterone 3-acetate (3, 29NCY3Ac) had been previously described as a minor component in *Ajuga reptans* and characterised by X-ray analysis (Camps et al., 1985), whereas only limited spectroscopic data were available for compounds 1 and 2 (Galbraith & Horn, 1969; Isaac et al., 1981; Girault et al., 1990; Lafont & Wilson, 1992, and references cited therein for isolation from other natural sources).

TABLE 2. ^{13}C-NMR (in C_5D_5N) spectral data of 20-hydroxyecdysone 2-acetate (1), 20-hydroxyecdysone 3-acetate (2), 29-norcyasterone 3-acetate (3), cyasterone 3-acetate (4), 20-hydroxyecdysone (5), 29-norcyasterone (6) and cyasterone (7). Chemical shifts are given in ppm.

	1	2	3	4	5 #	6	7
C-1	33.63$	38.64	38.70*	38.74*	38.09	37.96	37.97
C-2	72.50	66.06	66.07	66.10	68.33	68.09	68.10
C-3	65.09	71.20	71.26	71.29	68.23	68.02	68.03
C-4	32.55$	29.62	29.65	29.65	32.53	32.44	32.46
C-5	51.09	51.88	51.93	51.95	51.48	51.38	51.39
C-6	202.81	202.24	202.15	202.13	203.56	203.43	203.44
C-7	121.55	121.36	121.55	121.65	121.79	121.76	121.84
C-8	166.20	166.48	166.28	166.21	166.11	165.90	165.84
C-9	34.25	34.35	34.40	34.41	34.67	34.39	34.48
C-10	38.70	38.48	38.57*	38.62*	38.80	38.67	38.70
C-11	21.06&	21.05	21.07	21.09&	21.29	21.09	21.09
C-12	31.89*	31.85	31.78	31.91"	32.19	32.02	32.04
C-13	47.94	47.99	48.14	48.17	48.27	48.17	48.18
C-14	84.09	84.06	84.05	84.11	84.42	84.09	84.13
C-15	31.64*	31.65	31.94	32.01"	31.88	31.83	31.92
C-16	21.35&	21.38	21.35	21.36&	21.61	21.36	21.34
C-17	49.94	50.00	50.11	49.66	50.28	50.12	50.01
C-18	17.78	17.81	17.88	17.91	17.99	17.89	17.92
C-19	24.19	24.21	24.26	24.28	24.55	24.45	24.47
C-20	76.75	76.77	76.64	76.77	77.09	76.66	76.77
C-21	21.58	21.63	21.26"	21.01	21.77	21.24	20.96
C-22	77.43	77.48	75.97	73.99	77.75	75.97	73.94
C-23	27.40	27.39	34.75	34.52	27.59	34.74	34.38
C-24	42.58	42.56	40.68	48.70	42.64	40.67*	48.64
C-25	69.51	69.49	43.32	42.46	69.86	43.31*	42.43
C-26	29.84	29.88	179.78	179.18	30.10	179.78	179.20
C-27	30.16	30.06	14.44	15.94	30.15	14.44	15.94
C-28	—	—	72.89	79.83	—	72.90	79.82
C-29	—	—	—	19.35	—	—	19.32
CH_3COO	21.10	21.05	21.07"	21.09	—	—	—
CH_3COO	170.40	170.56	170.56	170.57	—	—	—

Lafont & Wilson (1992).
Assignments in each column marked with the same sign can be exchanged.

DISCUSSION

In Tables 1 and 2, NMR data for 20-hydroxyecdysone 2- and 3-acetates, 29-norcyasterone 3-acetate and cyasterone 3-acetate have been summarised. For comparison, the

corresponding data for 20-hydroxyecdysone (5, 20E), 29-norcyasterone (6, 29NCY) and cyasterone (7, CY) are also included (Lafont & Wilson, 1992). Among these last compounds, 6 had also been only partially described (Camps et al., 1982) prior to the present communication.

^{1}H-NMR data for compounds 1–4 clearly show the presence of an acetate methyl singlet at 1.93–1.98 ppm, and the acetylated position can be straightforwardly recognised by the shift of the proton directly linked to the substituted position (+1.1 to +1.3 ppm) and the signal shape, which is maintained. In 20E2Ac (1), shifts of the H_{eq}-3 (+0.08 ppm), the H_{ax}-9 (+0.07 ppm) and the H_{ax}-12 signals (–0.08 ppm) are also observed.

In 20E3Ac, 29NCY3Ac and CY3Ac (2, 3 and 4, respectively) the presence of the acetyl substituent at position 3 leads to deshielding of H_{ax}-2 (+0.11–0.13 ppm) and shielding of the H-5 signals (–0.34 ppm) owing to the 1,3 diaxial interaction.

^{13}C-NMR data have not been previously reported for the isolated compounds. Comparison of data between the newly isolated compounds and the parent ecdysteroids reveals the expected shifts for the ipso and alpha positions. In 20-hydroxyecdysone 2-acetate (1), the C-2 signal is shifted by +4.2 ppm, whereas those of C-1 and C-3 are shifted –4.5 and –3.1 ppm, respectively. In the 3-acetylated compounds (2, 3 and 4) the C-3 shift is in the range +3.0 to +3.5 ppm, and those of C-2 and C-4 in the range –1.8 to –2.9 ppm.

The MS fragmentations follow the pattern described in the literature (Marco et al., 1992). In all cases several 60 amu losses were observed, that were ascribed to the elimination of acetic acid, as well as 18 amu losses owing to dehydration.

Partial acyl migration from a 2-acetate yielding a mixture of 2- and 3- derivatives upon oxidative treatment with Jones reagent (Lloyd-Jones et al., 1973), and the complementary process from 3 to 2 (Isaac et al., 1981), have been reported. Likewise, slow equilibration of acetates at C2 and C3, upon storage in solution, was put forward as the likely explanation for cross-contamination of two chromatographically well separated fractions (Rudel et al., 1992). Therefore, these precedents would caution the possible establishment of 20-hydroxyecdysone 2-acetate as a true natural compound present in *Ajuga reptans*, although HPLC analysis has shown its presence in crude extracts with a minimum of manipulation.

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