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Louis Guibout [a,b], Nilufar Mamadalieva [c], Christine Balducci [b], Jean-Pierre Girault [d] and René Lafont [a]*

[a] *Sorbonne Universités, UPMC Univ Paris 06, IBPS-BIOSIPE, CNRS FR 3631, 7 Quai Saint Bernard, Case Courrier 29, 75252 Paris Cedex 05, France*
[b] *Institut Biophytis, F-93230 Romainville, France*
[c] *Institute of the Chemistry of Plant Substances, Mirzo Ulugbek Str. 77, Tashkent 100170, Uzbekistan*
[d] *Université Paris Descartes, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS UMR 8601, 45 rue des Saints-Pères, F-75270 Paris Cedex 06, France*

**rene.lafont@upmc.fr*

ABSTRACT

Introduction — *Ajuga turkestanica* is a plant used in traditional medicine for its high ecdysteroid content, including the presence of the particularly active turkesterone, which possess efficient anabolic activity.

Objectives —To isolate and identify minor ecdysteroids present in a semi-purified plant fraction containing ca. 70% turkesterone.

Material and Methods — Multi-step preparative HPLC (combining RP- and NP-HPLC systems) was used to purify the different components present in the turkesterone fraction. Isolated compounds were identified by high-resolution mass spectrometry and 2D-NMR.

Results — Fourteen ecdysteroids (including turkesterone and 20-hydroxyecdysone) were isolated. Seven of these, all bearing an 11α-hydroxy group, were previously unreported..

Conclusion — *Ajuga turkestanica* ecdysteroids are characterized by the abundance of 11α-hydroxylated compounds and by the simultaneous presence of 24C, 27C, 28C and 29C ecdysteroids. It is expected that even more ecdysteroids are to be found in this plant since the starting material for this study lacked the less polar ecdysteroids. The simultaneous presence of 20-hydroxyecdysone and turkesterone (its 11α-hydroxy analogue) as the two major ecdysteroids suggests that every ecdysteroid is probably present in both 11α-hydroxy and 11-deoxy forms.

Keywords: *Ajuga turkestanica*; Lamiaceae; phytoecdysteroid:

Introduction

Among the >300 known species of the genus *Ajuga* (Lamiaceae), ≥45 have been chemically investigated. A few of these have been studied in the context of their medical properties, owing to the presence of active molecules such as phytoecdysteroids, diterpenoids, sterols, iridoids, neoclerodanes, anthocyanins, other flavonoids and ionones (Israili and Lyoussi, 2009;

Mamadalieva *et al*., 2013). *Ajuga turkestanica,* is naturally growing in Uzbekistan and Tadzhikistan (Vvedenskiy, 1961; Ganiev *et al*., 1990), and it has been valued for a long time by the local population for its beneficial effects on muscle strength, muscular and stomach aches and its protective action against heart diseases (Cheng *et al*., 2008; Grace *et al*., 2008). Many effects have been documented concerning either extracts or pure compounds isolated from *A. turkestanica* aerial parts or roots. Thus, extracts were shown to increase lactation in female rats (Khalitova *et al*., 1998), to reduce hyperglycaemia in alloxan-induced diabetic rats (Kutepova *et al*., 2001), to help skin wound healing (Syrov *et al*., 1994) or to display antiproliferative, antimicrobial and antioxidant effects (Mamadalieva *et al*., 2013). Most of these effects are probably related to the presence of ecdysteroids (Dinan, 2009). Ecdysteroid-enriched extracts of *A. turkestanica* (or pure turkesterone) display anabolic effects on muscles (Syrov *et al*., 2001; Zubeldia *et al*., 2012), even in a sarcopenia context (Lawrence, 2012). In addition, turkesterone and 20-hydroxyecdysone have anti-stress and immunostimulating effects (Shakhmurova *et al*., 2010). Extracts of *Ajuga turkestanica* are already marketed for all these indications (e.g. AYUSTAN - http://5741.uz.all.biz/preparat-ayustan-g22268), and turkesterone-enriched extracts are sold on the internet for bodybuilders.

Ajuga turkestanica was one of the early plant species investigated for the presence of ecdysteroids. Firstly, 20-hydroxyecdysone and cyasterone were isolated (Usmanov *et al*., 1971), then turkesterone (Usmanov *et al*., 1973, 1975) and several minor components i.e., ajugalactone (Saatov *et al*., 1977), cyasterone 22-acetate (Usmanov *et al*., 1978a), ajugasterone B (Usmanov *et al*., 1978b). More recently, ecdysone and 20-hydroxyecdysone 2,3-acetonide were also indentified (Saatov *et al*., 1999). Turkesterone and 20-hydroxyecdysone (figure 1) are the two major ecdysteroids, accounting each for ca. 0.2-0.4% of dry weight of aerial parts (Abdukadirov *et al*., 2005)

It is well-established that ecdysteroid-rich plants such as *Silene otites* (Báthori *et al*., 1999), or *Rhaponticum carthamoides* (Kokoska and Janovska, 2009; Zhang *et al*., 2010) contain a complex cocktail of various ecdysteroids. Given the high concentrations of ecdysteroids in *A. turkestanica* (*ca.* 1% of dry weight), this plant was expected to contain a much larger set of minor ecdysteroids than already described, and the present work has taken advantage of a medium-scale extraction experiment to isolate and identify additional minor ecdysteroids from this plant.

Materials and methods

Plant material

Roots of *A. turkestanica* were collected in the Surkhan-Darya region of Uzbekistan and identified by Dr. O.A. Nigmatullaev at the Department of Herbal Plants (Institute of the Chemistry of Plant Substances, Uzbekistan) (voucher specimen number 20077092).

Extraction

Roots of *A. turkestanica* were air-dried in windy place at 23°C during one week after collection and them ground to a powder with a Waring blender. After grinding, plant material (2 kg) was triturated with methanol (3×6 L) during 3 days at room temperature (23°C). The solvent was evaporated (in a rotary vacuum evaporator at 40 °C) and concentrated, and 102 g of dried extract was obtained (yield was 5.1% of the dried plant material). Methanol (60 mL) was added to the dried methanol extract to fluidize it before diluting with water (1 L) and hydrophobic compounds were removed upon partition with chloroform (4x1 L) which was discarded. The water layer (1 L) was then extracted with *n*-butanol (5×1 L). The combined butanol extracts were evaporated to dryness under reduced pressure (at 46 °C).

Isolation of phytoecdysteroids Column chromatography

The *n*-butanol fraction (42 g) was chromatographed over a silica gel column (silica particle size: 63 – 100 µm; Chemapol, Praha, Czech Republic). The column was packed using a simple dry-pack method. The n-butanol extract applied in dried form mixed with silica gel and carefully added to the top of the column. The column (1.6 kg silica, size 10×60 cm) was eluted with $CHCl_3/CH_3OH$ (9:1 v/v, 4 L), and fraction X was obtained containing a mix of less polar ecdysteroids. Continuous elution with $CHCl_3/CH_3OH$ (6:1 v/v, 3 L) gave of fraction Y contained a mixture of 20-hydroxyecdysone, turkesterone and some less polar ecdysteroids. Further elution with $CHCl_3/CH_3OH$ (4:1 v/v, 4 L) gave a polar fraction Z, which contained a small amount of turkesterone and iridoids. Eluates were checked by TLC. TLC was performed on aluminium foil-backed plates (Fluka, Sigma-Aldrich, Germany). Developing solvent was chloroform–methanol–water at 4:1:0.1 (v/v/v). Spots were visualized under UV light (254 nm) and by spraying with vanillin/H_2SO_4 reagent followed by heating to 120 °C for 10 min (Báthori and Kalász, 2001). Fractions X and Y were combined and after evaporating the solvent under reduced pressure, an ecdysteroid-containing fraction (turkesterone + 20-hydroxyecdysone + less polar ecdysteroids) of 8.3 g was obtained.

HPLC separations

An aliquot (700 mg) of the above extract was purified by preparative reversed-phase HPLC (System 1).

Preparative RP-HPLC step (System 1): LC-MS Prep Agilent 1100, Column Sunfire Prep C18 OBD 100 x19 mm (5 µm) from Waters; Linear gradient: A = ACN, B = H_2O+0.1%HCOOH (T=0 10% A - 90% B; T=25 min 35% A - 65% B; T=26 min 10% A - 90% B; T=30 min 10% A - 90% B); Flow-rate = 20 mL.min^{-1}; detection: UV, wavelength = 254 nm. The extract was dissolved in 35 mL of ACN/H_2O (10/90,v/v). 14 aliquots of this solution were injected into the LC-MS Prep. Fractions were collected using an automatic collector (Agilent Prep-FC G1346B) with a time-based trigger mode. Collected peaks were dried using a HT4-Genevac without heating.

The purity of the above fractions was checked by analytical NP-HPLC (column ACE 5 SII, 150 x 4.6 mm) eluted at 1 mL.min^{-1} using either dichloromethane-isopropanol-water (125:40:2.5 v/v/v) or cyclohexane-isopropanol-water (100:40:2.5 v/v/v). Those giving a single peak with both systems were considered as pure enough. Those giving several peaks were further fractionated using the most appropriate NP-HPLC solvent with a semi-preparative Zorbax-SIL column (250 x 9.4 mm) eluted at 4 mL.min^{-1} using three different solvent systems: dichloromethane-isopropanol-water (125:40:2.5 v/v/v) (system 2a), dichloromethane-isopropanol-water (125:30:2 v/v/v) (system 2b), and cyclohexane-isopropanol-water (100:40:2.5 v/v/v) (system 3); detection: UV, wavelength = 254 nm. In some cases, two NP-HPLC steps were required to obtain pure compounds.

HPLC-MS analyses

Analytical HPLC-MS used a LC1100 Agilent system with a Sunfire column (50 x 4.6 mm). Elution was performed in the gradient mode: acetonitrile-0.1% TFA in H_2O, linear gradient 10 to 35% acetonitrile in 25 min, flow-rate = 0.5 mL.min^{-1}. The mass spectrometer was an API 365 equipped with a Turbo Ion Spray source.

HRMS analyses

High-resolution p-ESI (probe electrospray ionization) mass spectra were acquired with an ultra-high resolution mass spectrometer, a hybrid linear ion trap LTQ-Orbitrap (Thermo Fisher Scientific, Les Ulis, France). Individual compounds dissolved in methanol were directly infused in the mass spectrometer. For each compound, we observed ions corresponding to $[M+Na]^+$ (major), $[M+K]^+$ (medium) and $[M+H]^+$ (minor).

NMR analyses

All NMR spectroscopy experiments were recorded on a Bruker AVANCE II 500 Spectrometer in D_2O (500 MHz for ^{1}H and 125 MHz for ^{13}C), at 300K (Girault, 1998). Presaturation of the solvent was used for all 1D and homonuclear 2D ^{1}H experiments. 2D-experiments (^{1}H, TOCSY [spin lock time 15ms and 35ms], NOESY [mixing time 500 ms], HSQC [one bond correlation $^{1}J^{1}H$-^{13}C] and HMBC [^{1}H-^{13}C correlation via two or three bonds via $^{2}J^{1}H$-^{13}C or $^{3}J^{1}H$-^{13}C, long-range delay 80 ms]) were performed by Pulse Field Gradient (PFG) methods using standard Bruker software. The samples were lyophilized and dissolved in D_2O. The sodium salt of 3-(trimethylsilyl)[2,2,3,3-d_4] propionic acid (TSP-d_4) was used as internal reference for the proton and carbon shifts. For ^{1}H 1D measurements, spectral width of 6009.6 Hz and 32K data points were used to yield the Fid corresponding to a digital resolution of 0.0004 ppm/point (0.18 Hz/point). So for signals assigned directly from ^{1}H 1D measurements accurate values of the chemical shifts could be given ± 0.001 ppm. However, for ^{1}H 2D measurements (TOCSY or NOESY), the same spectral width in F2 (^{1}H) of 5000 Hz was used and all data points (t2 x t1) were acquired with 4K x 256. For F1, linear prediction to 4 times t1 was applied to enhance the resolution. With these conditions, the digital resolutions are 0.005 ppm/point and 0.01 ppm/point for F2 and F1, respectively. In the case of heteronuclear experiments (HSQC and HMBC), the same spectral width in F2 (^{1}H) of 5000 Hz was used for both. Spectral widths in F1 (^{13}C) of 20120 Hz and 28923 Hz for HSQC and HMBC, respectively were used. All data points (t2 x t1) were acquired with 4K x 256 (or 160), and linear prediction to 4 times t1 was applied to enhance the resolution in F1. In these conditions, the digital resolutions for F2 (^{1}H) are 0.005 ppm/point for HSQC and HMBC, 0.1 ppm/point for F1 (^{13}C) HSQC and 0.2 ppm/point for F1 (^{13}C) HMBC.

Results and discussion

Isolation of ecdysteroids

Preparative HPLC (system 1) allowed the separation and collection of 8 fractions (Figure 2 and Table 1). The purity of each fraction was checked by analytical NP-HPLC-UV. Fractions 1 (= turkesterone), 4 (= 20-hydroxyecdysone), 5, 6 and 8 contained a single major compound. The three remaining fractions (2, 3 and 7) were mixtures (see supplementary figure 1) and were further fractionated using one or two additional semi-preparative NP-HPLC steps as described in Table 1. NP-HPLC was also used to improve the purity of fractions 5 and 6 and remove minor contaminants. Finally, 14 pure compounds were isolated as amorphous powders and identified by HRESIMS and NMR.

Seven of the 14 have already been described in the Ecdybase (Lafont *et al*., 2002) i.e., turkesterone (compound 1), atrotosterone C (compound 2.3.1), abutasterone (compound 2.3.2), 20-hydroxyecdysone (compounds 3.2 and 4), 25-hydroxydacryhainansterone (compound 3.3), cyasterone (compound 6) and ajugasterone C (compound 7.1). The corresponding data are provided as supplementary material. Seven other ecdysteroids were isolated for the first time, and their identification is described below.

Identification of the newly isolated ecdysteroids

The structures of the new ecdysteroids are given in figure 3. Their ^{1}H and ^{13}C NMR data are given in Tables 2 and 3. Full NMR spectra are provided in the supplementary materials for turkesterone and the 7 new ecdysteroids. All the new compounds isolated in this work present a 5-Hβ steroid nucleus with an 11α-hydroxy (equatorial) substituent identical with turkesterone, which result in typical signal characteristics as described for turkesterone (see supplementary

material and figure s3). Thus the modifications concern only their side-chain with respect to turkesterone.

25-Hydroxyatrotosterone A (Compound 2.1 - 1.6 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 511 $(M+H)^+$, 493 $(MH-H_2O)^+$, 475 $(MH-2H_2O)^+$, 457 $(MH-3H_2O)^+$ and 439 $(MH-4H_2O)^+$. HR-ESIMS positive ion was observed for $M+Na^+$ at *m/z* 533.30800 (*m/z* expected for $C_{28}H_{46}O_8Na$ 533.30849, Δ = - 0.92 ppm). The compound formula is $C_{28}H_{46}O_8$, which indicates that it bears one additional carbon and two extra hydrogen atoms with respect to turkesterone. Examination of the 1D ^1H NMR spectrum shows a new methyl signal doublet at 0.96 ppm, (d, 6.8 Hz) bound to CH-24 (figure s4). This CH-24 is assigned from HMBC correlations observed with ^1H of 26-Me and 27-Me methyl signals. ^1H and ^{13}C NMR signals of methyls 26 and 27 correspond to values in agreement with 25 hydroxylated ecdysteroids. This is confirmed from HMBC correlations observed with ^1H of 26, 27, 28 methyl signals with the same ^{13}C NMR signal at 76.4 ppm thus assigned unambiguously to C-25. Moreover, ^1H and ^{13}C NMR signals of the side-chain are identical with those of makisterone A, both recorded in D_2O (Bakrim *et al*., 2014), suggesting that the configuration of the asymmetric center at C-24 is equivalent for these two compounds. Thus, the structure was assigned as 25-hydroxyatrotosterone A.

11-Hydroxycyasterone (Compound 2.2 - 0.9 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 537 $(M+H)^+$, 519 $(MH-H_2O)^+$, 501 $(MH-2H_2O)^+$, 483 $(MH-3H_2O)^+$ and 465 $(MH-4H_2O)^+$. An HR-ESIMS positive ion was observed for $M+Na^+$ at *m/z* 559.28768 (*m/z* expected for $C_{29}H_{44}O_9Na$ 559.28775, Δ = – 0.13 ppm). The compound formula corresponds therefore to $C_{29}H_{44}O_9$, thus it has two additional carbons and one extra-oxygen with respect to turkesterone. Examination of 1D and 2D ^1H NMR spectra (figure s5) shows two methyl signal doublets, one at 1.32 ppm, (d, 7.2 Hz) J coupled to the oxymethine group CH-25, with its ^1H signal 25-H at 2.67 ppm, (dq, 10.8, 7.1 Hz) and the other one at 1.45 ppm, (d, 6.1 Hz), J-coupled to oxymethine group CH-28, with its ^1H signal H-28 at 4.34 ppm, (dq, 9.4, 6.1 Hz). HMBC show that these two methyl signals are correlated to the same carbon, assigned unambiguously as CH-24 from HMBC correlation 22-H => C-24. Moreover, 24-H presents the reverse HMBC correlation 24-H => C-27 and C-29 to the ^{13}C signals of these two methyl groups. The ^1H methyl signal at 1.32 ppm presents an HMBC correlation with the ^{13}C signal of an ester carbonyl group at 185.9 ppm. All these elements lead us to propose a side-chain with a lactone group as in cyasterone. Moreover, the ^1H and ^{13}C NMR data, when compared with those of cyasterone in D_2O, show that these two compounds have identical side-chains. Side-chain ^1H and ^{13}C chemical shifts and both HMBC and NOESY experiments show identical values and correlations to those observed for cyasterone, suggesting equivalent stereochemistry of the lactone ring. Thus, the structure was assigned as 11-hydroxycyasterone.

11-Hydroxysidisterone (Compound 3.1 - 1.0 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 433 $(M+H)^+$, 415 $(MH-H_2O)^+$, 397 $(MH-2H_2O)^+$, 379 $(MH-3H_2O)^+$ and 361 $(MH-4H_2O)^+$. An HR-ESIMS positive ion is observed for $M+Na^+$ at *m/z* 455.20418 (expected for $C_{24}H_{32}O_7Na$ 455.20402, Δ = + 0.35 ppm), thus the compound formula is $C_{24}H_{32}O_7$. The compound presents the same steroid nucleus as turkesterone. It is a C_{24} ecdysteroid lacking 3 carbon atoms, 1 oxygen atom and 12 hydrogen atoms when compared to turkesterone. Again, examination of ^1H and ^{13}C NMR data (figure s6) show that side-chain modifications with respect to turkesterone correspond to 3 supplementary unsaturations (or equivalents). Examination of the 1D ^1H NMR spectrum shows two new ethylenic ^1H signals at 7.92 ppm, (d, 5.7 Hz) and 6.15 ppm, (d, 5.7 Hz) which are J-coupled together. HMBC shows that 17-H at 2.75 ppm (t, 9.5) is correlated to carbon signals of the side-chain corresponding to C-20 (95.4 ppm), C-21 (25.8 ppm) and C-22 (167.2 ppm), this last one corresponding to the chemical shift of an ethylenic carbon bond. From a HSQC, the ^1H signal at 7.92 ppm could be assigned as 22-H. From HMBC, 22-H is correlated to carbon signals of the end of the side-chain corresponding to C-23 (120.9 ppm), an ethylenic carbon, and C-24 (179.0 ppm), a typical

value for a carbonyl ester or a lactone group. These elements suggest that a cyclic structure is present in the side-chain, corresponding to a five-membered lactone ring with a double bond between C-22 and C-23, similar to that of sidisterone (Girault *et al*., 1996). Comparison with 1D and 2D ^{1}H and ^{13}C NMR data for the side-chain of sidisterone in D_2O (Bakrim *et al*., 2014) shows perfect agreement indicating that the compound is 11-hydroxysidisterone and suggesting that the configuration of the asymmetric centre at C-20 is equivalent for these two compounds.

Turkesterone 22-acetate (Compound 3.4 - 0.7 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 539 $(M+H)^+$, 503 $(MH-2H_2O)^+$, 485 $(MH-3H_2O)^+$, 479 $(MH-CH_3COOH)^+$, 443 $(MH-2H_2O-CH_3COOH)^+$, 425 $(MH-3H_2O-CH_3COOH)^+$ and 407 $(MH-4H_2O-CH_3COOH)^+$. An HR-ESIMS positive ion is observed for $M+Na^+$ at *m/z* 561.30318 (*m/z* expected for $C_{29}H_{46}O_9Na$ 561.30340, $\Delta = -0.39$ ppm). The compound formula is therefore $C_{29}H_{46}O_9$. It is a C_{29} ecdysteroid with two carbons, one oxygen atom and two hydrogen atoms more than turkesterone. Again, ^{1}H and ^{13}C NMR data (figure s7) show only side-chain modifications with respect to turkesterone. Examination of the 1D ^{1}H NMR spectrum shows a new methyl signal singlet at 2.17 ppm (s), typical of an acetate group. This is confirmed by HMBC, which shows that this methyl is correlated to a quaternary ester carbonyl at 177.2 ppm. The presence of this acetate group at C-22 is confirmed by the large high frequency (downfield) shift of the 22-H signal (4.85 ppm, dd, 10.2, 1.5 Hz) with respect to turkesterone or 20-hydroxyecdysone. This leads to the conclusion that the compound is turkesterone 22-acetate.

22-Oxo-turkesterone (Compound 5 - 1.4 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 495 $(M+H)^+$, 477 $(MH-H_2O)^+$, 459 $(MH-2H_2O)^+$, 441 $(MH-3H_2O)^+$ and 423 $(MH-4H_2O)^+$. An HR-ESIMS positive ion is observed for $M+Na^+$ at *m/z* 517.27681 (*m/z* expected for $C_{27}H_{42}O_8Na$ 517.27719, $\Delta = -0.73$ ppm). The compound formula is $C_{27}H_{42}O_8$, with two missing hydrogen atoms with respect to turkesterone. The side-chain modification with respect to turkesterone corresponds to one supplementary unsaturation. Examination of the 1D ^{1}H NMR spectrum (figure s8) shows disappearance of the ^{1}H signal for 22-H and large high frequencies (downfield) shifts of both 23-Ha,b of the side-chain (2.79 ppm) with respect to turkesterone. Moreover, HMBC shows that the 21-methyl is correlated to a quaternary carbonyl at 220.4 ppm, a typical value for a ketonic carbonyl, which leads us to conclude that it is a 22-oxo derivative. So, the structure corresponds to 22-oxo-turkesterone.

11-Hydroxy-Δ^{24}-capitasterone (Compound 7.2 - 1.9 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 519 $(M+H)^+$, 501 $(MH-H_2O)^+$, 483 $(MH-2H_2O)^+$, 465 $(MH-3H_2O)^+$, 447 $(MH-4H_2O)^+$ and 429 $(MH-5H_2O)^+$. An HR-ESIMS positive ion was observed for $M+Na^+$ at *m/z* 541.27651 (*m/z* expected for $C_{27}H_{42}O_8Na$ 541.27719, $\Delta = -1.27$ ppm). The compound formula is therefore $C_{29}H_{42}O_8$. The modifications with respect to turkesterone correspond to 3 supplementary unsaturations or equivalents. Examination of 1D and 2D ^{1}H NMR spectra (figure s9) shows 2 new ^{1}H methyl signals. One is a triplet (overlapping the 19-Me singlet) at 1.10 ppm, (t, 7.2) J-coupled to a CH_2 group at 2.38 ppm (q, 7.2). This chemical shift corresponds to a CH_2 group linked to an ethylenic double bond in agreement with a CH_3-CH_2-C=C< motif. The other methyl signal is a singlet at 1.87 ppm (sb), and such a chemical shift corresponds to a methyl linked to an ethylenic double bond. Moreover, HMBC shows that this methyl signal (1.87 ppm) is correlated to two ethylenic carbon signals at 122.2 and 161.7 ppm and with the ^{13}C signal of an ester carbonyl group at 173.5 ppm. The CH_2 group at 2.38 ppm is also correlated to the same two ethylenic carbon signals. All this leads us to propose a structure for the side-chain of an α,β-unsaturated lactone. The 22-H signal shows a large high frequency (downfield) shift at 4.38 ppm (dd, 13.4, 3.5) with its corresponding CH-22 signal at 84.4 ppm, and these values are in agreement with a lactonization involving the 22-hydroxyl group to obtain a six-membered α,β-ethylenic lactone. This lactone structure is confirmed by all the data obtained from HMBC, HSQC and NOESY and TOCSY experiments. So, the structure of this compound is related to that of capitasterone, a C_{29} ecdysteroid bearing

a six-membered lactone ring (Takemoto *et al*., 1968). Thus, its structure corresponds to 11-hydroxy-Δ^{24}-capitasterone.

Turkesterone 20,22-acetonide (Compound 8 - 1.0 mg)
Turbo Ion Spray-MS gives positive ions at *m/z* 537 $(M+H)^+$, 519 $(MH-H_2O)^+$, 483 $(MH-3H_2O)^+$, 479 $(MH-(CH_3)_2CO)^+$, 461 $(MH-H_2O-(CH_3)_2CO)^+$, 443 $(MH-2H_2O-(CH_3)_2CO)^+$, 425 $(MH-3H_2O-(CH_3)_2CO)^+$ and 407 $(MH-4H_2O-(CH_3)_2CO)^+$. An HR-ESIMS positive ion is observed for $M+Na^+$ at *m/z* 559.32346 (*m/z* expected for $C_{30}H_{48}O_8Na$ 559.32414, Δ = – 1.22 ppm). ^1H and ^{13}C NMR data show a single side-chain modification with respect to turkesterone, corresponding to one supplementary unsaturation or equivalent. Examination of 1D ^{1}and 2D ^1H NMR spectra (figure s10) shows 2 new ^1H singlet methyl signals at 1.41 ppm (s) and 1.49 ppm (s). Moreover, HMBC shows that these two ^1H methyl signals are correlated to the same quaternary carbon atom at 109.8 ppm, a typical value for a quaternary carbon of an acetonide group. This acetal group was easily located as bridging 20-O and 22-O, as we observe a shift of both C-20 and C-22 to higher frequencies (downfield) at 87.1 and 84.4 ppm, respectively, and similarly for the ^1H-22 signal at 3.87 ppm (dd, 6.4, 4 Hz). These findings lead to the straightforward conclusion that it is a 20-22 acetonide derivative. Therefore, the compound corresponds to turkesterone 20,22-acetonide.

Thus *A. turkestanica* contains a complex cocktail of ecdysteroids, and additional studies on less polar fractions will probably allow the isolation of even more compounds.

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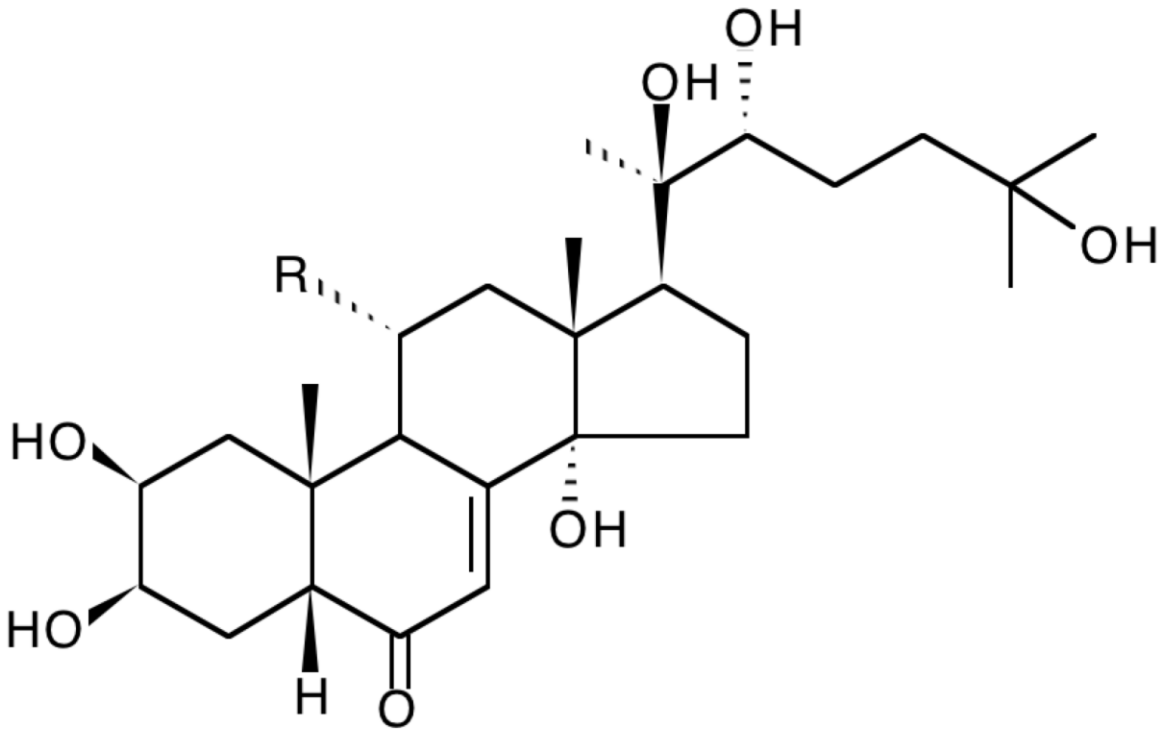


Figure 1. Structure of the two major ecdysteroids from *Ajuga turkestanica*. R = H : 20-hydroxyecdysone; R = OH : turkesterone.

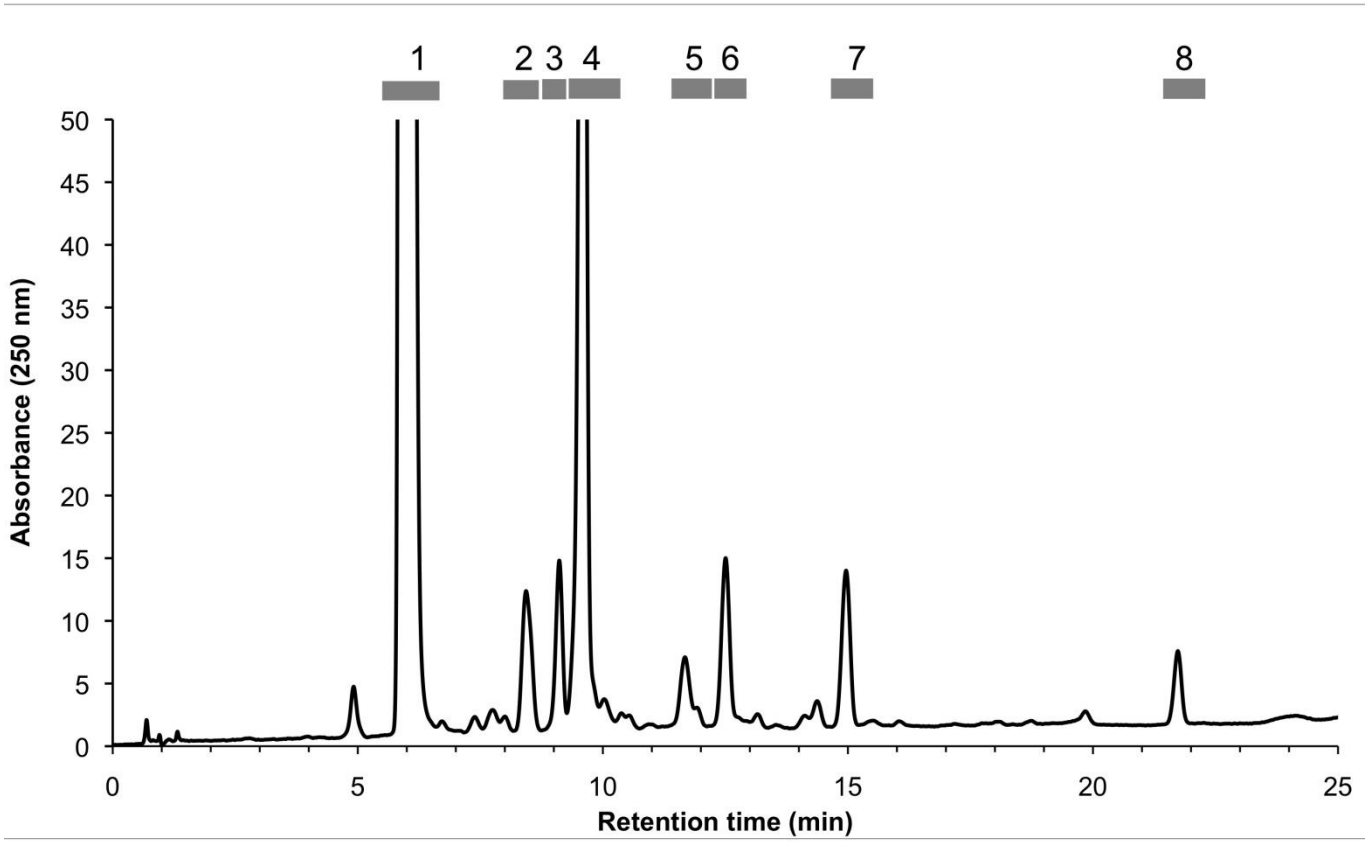
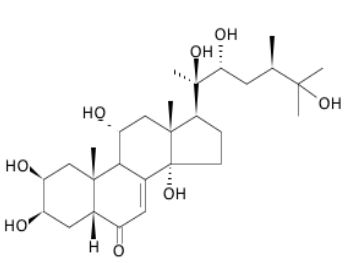
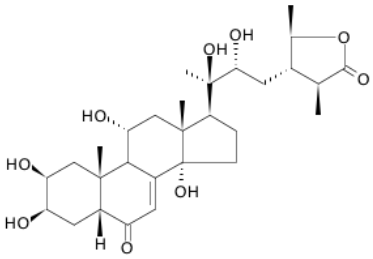


Figure 2. Preparative RP-HPLC chromatogram of the semi-purified *A. turkestanica* extract. Operating conditions: see text. Eight fractions were collected (1 = turkesterone, 4 = 20-hydroxyecdysone; for other peaks, see Table 1 and text).



Compound 2.1



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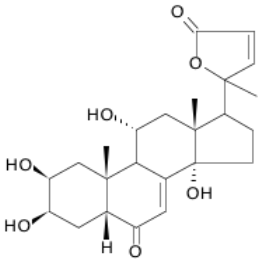
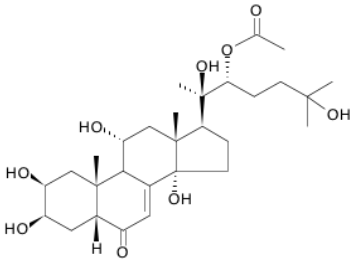


Compound 3.1



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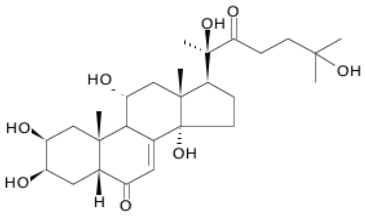
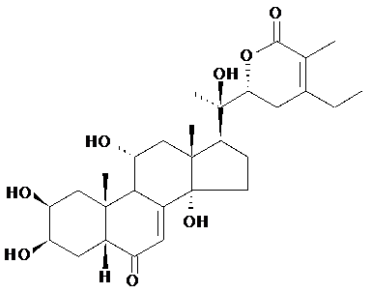
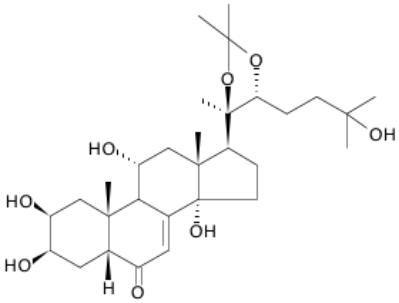


Compound 5



Compound 7.2



Compound 8

Figure 3. Structural formulae of the 7 new ecdysteroids isolated from *Ajuga turkestanica* (see names in Table 1).

Table 1 - HPLC separation of ecdysteroids present in the crude turkesterone extract from *A. turkestanica*.

System 1		System 2a	System 2b	System 3	Compound number	Identification	Amount isolated (mg)
Fraction	Retention interval (min)	Ret (min)	Ret (min)	Ret (min)			
1	5.10 – 6.60				1	Turkesterone	442.3
2	7.90 – 8.70	12.83 15.76 25.98		20.00 24.70	2.1 2.2 2.3.1 2.3.2	25-Hydroxyatrotosterone A* 11-Hydroxycyasterone* Atrotosterone C Abutasterone	1.6 0.9 0.1 0.6
3	8.80 – 9.40		25.58 35.50 40.65 49.75		3.1 3.2 3.3 3.4	11-Hydroxysidisterone* 20-Hydroxyecdysone 25-Hydroxydacryhainansterone Turkesterone 22-acetate*	1.0 1.1 4.6 0.7
4	9.50 – 10.50				4	20-Hydroxyecdysone	38.8
5	11.40 – 12.20	26.80			5	22-Oxo-turkesterone*	1.4
6	12.30 – 13.00	6.76			6	Cyasterone	3.4
7	14.60 – 15.50			10.99 19.45	7.1 7.2	Ajugasterone C 11-Hydroxy-Δ^{24}-capitasterone*	0.8 1.9
8	21.40 – 22.20	13.95			8	Turkesterone 22-acetonide*	1.0

* New ecdysteroid

Table 2. ^{1}H NMR data of the newly isolated ecdysteroids.

^{1}H	Compound 2.1	Compound 2.2	Compound 3.1	Compound 3.4	Compound 5	Compound 7.2	Compound 8
1–H_{ax} 1–H_{eq}	1.39 (t, 13) 2.48 (dd, 13, 3.7)	1.39 (t, 13) 2.48 (dd, 13.2, 4)	1.41 (t, 13) 2.50 (dd, 13, 3.9)	1.40 (t, 13) 2.48 (dd, 13.1, 4)	1.40 (t, 12.8) 2.48 (dd, 13, 3.9)	1.40 (t, 13) 2.48 (dd, 13, 3.7)	1.40 (t, 13) 2.48 (dd, 13.1, 4)
2–H_{ax}	4.09 (m, $w_{1/2}$=11)	4.09 (m, $w_{1/2}$=11)	4.10 (m, $w_{1/2}$=12)	4.10 (m, $w_{1/2}$=11)	4.10 (m, $w_{1/2}$=12)	4.10 (m, $w_{1/2}$=10)	4.09 (m, $w_{1/2}$=10)
3–H_{eq}	4.09 (m, $w_{1/2}$=11)	4.09 (m, $w_{1/2}$=11)	4.10 (m, $w_{1/2}$=12)	4.10 (m, $w_{1/2}$=11)	4.10 (m, $w_{1/2}$=12)	4.10 (m, $w_{1/2}$=10)	4.09 (m, $w_{1/2}$=10)
4–H_{ax} 4–H_{eq}	1.74 1.79	1.77 1.77	1.74 1.79	1.74 1.79	1.74 1.79	1.75 1.79	1.74 1.79
5–H	2.31 (dd, 12.3, 5.3)	2.31	2.33(dd, 12.1, 5.4)	2.31 (dd, 12.3, 5.3)	2.31 (dd, 12.3, 5.3)	2.32 (dd, 12.3, 5)	2.31
7–H	5.98 (d, 2.6)	5.99 (d, 2.6)	5.98 (d, 2.6)	5.99 (d, 2.6)	5.97 (d, 2.6)	6.00 (d, 2.5)	5.99 (d, 2.6)
9–H_{ax}	3.13 (dd, 8.8, 2.6)	3.13 (dd, 8.8, 2.6)	3.13 (dd, 8.8, 2.6)	3.13 (dd, 8.8, 2.6)	3.14 (dd, 8.8, 2.6)	3.14 (dd, 8.6, 2.5)	3.13 (dd, 8.8, 2.6)
11–H_{ax} 11–H_{eq}	4.23 (m, $w_{1/2}$=27 ddd, 10.8,9,6.1) –	4.23 (m, $w_{1/2}$=27 ddd, 10.8,9,6.1) –	4.20 (m, 12–H isochronous)	4.22 (m, $w_{1/2}$=27 ddd,10.8,9,6.1) –	4.24 (m, $w_{1/2}$=27) (ddd, 11,9,6.1) –	4.21 (m, $w_{1/2}$=27) –	4.20 (m, $w_{1/2}$=27) –
12–H_{ax} 12–H_{eq}	2.05) 2.28 (dd, 12.7, 6)	2.07 2.28 (dd, 12.7, 6.1)	2.13 2.13	2.07 (dd, 12.3, 12) 2.27 (dd, 12.7, 6)	2.16 (t, 12.3) 2.28 (dd, 12.5, 6.1)	2.10 (m) 2.15 (m)	2.04 2.23 (dd, 12.7, 6.1)
15–H_{β} 15–H_{α}	2.06 1.65	2.06 1.66 (m)	2.05 1.68	2.06 1.69	2.05 (m,$w_{1/2}$=26) 1.65	2.10 1.70	2.05 1.67
16–H_{α}* 16–H_{β}*	1.90 1.84	1.93 1.83	1.87 1.51	1.89 1.95	1.68 1.60	1.94 1.88	1.96 2.03
17–H	2.32 (m)	2.32 (m)	2.75 (t, 9.5)	2.35 (t, 9.7)	2.62 (t, 9.4)	2.53 (t, 9.5)	2.32 (m)
18–Me	0.87 (s)	0.87 (s)	0.76 (s)	0.85 (s)	0.84 (s)	0.84 (s)	0.82(s)
19–Me	1.09 (s)	1.10 (s)	1.10 (s)	1.09 (s)	1.10 (s)	1.10 (s)	1.09 (s)
21–Me	1.26 (s)	1.26 (s)	1.61 (s)	1.36 (s)	1.51 (s)	1.36(s)	1.27 (s)
22–H	3.55 (d, 11)	3.63 (d, 10.8)	7.92 (d, 5.7)	4.85 (dd, 10.2, 1.5)	–	4.38 (dd 13.4, 3.5)	3.87 (dd, 6.4, 4)
23–Ha 23–Hb	1.25 1.52	1.59 (m) 1.76	6.15 (d, 5.7) –	1.54 (m) 1.77 (m)	2.79 (m, $w_{1/2}$=12) 2.79 (m, $w_{1/2}$=12)	2.62 (tb,) 2.46	1.58 1.58
24–Ha 24–Hb	1.73 –	2.08 –	– –	1.46 (m) 1.52 (m)	1.76 1.76	– –	1.58 1.71
25–H	–	2.67 (dq, 10.8, 7.1)	_	_	_	–	–
26–Me	1.18 (s)	–	–	1.22 (s)	1.24 (s)	–	1.247 (s)
27–Me	1.20 (s)	1.32 (d, 7.2)	–	1.22 (s)	1.24 (s)	1.87(s)	1.251 (s)
28–H	28–Me 0.96 (d, 6.8)	28–CH 4.34 (dq, 9.4, 6.1)	_	22 – CH_3CO_2 2.174 (s)	–	28–CH_2 2.38 (q, 7.2)	CH_3 (a) 1.41 (s) CH_3 (b) 1.49 (s)
29–Me	–	1.45 (d, 6.1)	–	–	–	1.10 (t, 7.2)	

Solutions in D_2O, T= 300K; referenced to TSP–d4. Chemical shifts δ in ppm, δ□□HDO)= 4.758 ppm,□ δ□19–Me (20E) = 1.00 ppm ; Multiplicity of signals : *s* – singlet; *d* – doublet; *t* – triplet; *m* – multiplet; *br* – broad signal ; $w_{1/2}$: width at half–height in Hertz.

assignments could be reversed. t deceptively simple triplet (4–H_{ax} and 4–H_{eq} isochronous)

Table 3. ^{13}C NMR data of the newly isolated ecdysteroids.

^{13}C	Multiplicity	Compound 2.1	Compound 2.2	Compound 3.1	Compound 3.4	Compound 5	Compound 7.2	Compound 8
C-1	CH_2	39.1	39.2	39.2	39.4	39.2	39.3	39.3
C-2	CH	69.3	69.3	69.4	69.6	69.3	69.6	69.4
C-3	CH	69.3	69.3	69.4	69.6	69.3	69.6	69.4
C-4	CH_2	33.7	33.6	33.7	33.9	33.6	33.8	33.7
C-5	CH	53.4	53.4	53.5	53.6	53.4	53.5	53.6
C-6	C	210.4	210.6	210.5	211.0	210.4	*	*
C-7	CH	123.9	123.9	124.2	124.2	123.9	124.4	124.0
C-8	C	167.6	167.9	166.7	167.9	166.8	*	167.3
C-9	CH	43.4	43.4	43.4	43.7	43.4	43.7	43.4
C-10	C	40.7	40.3	40.8	41.3	40.8	40.7	40.3
C-11	CH_2	70.3	70.3	70.1	70.5	70.2	70.4	70.3
C-12	CH_2	43.9	43.8	43.2	43.9	43.7	43.8	43.6
C-13	C	49.4	49.3	48.9	49.6	49.5	49.1	49.4
C-14	C	86.6	86.6	86.7	87.2	86.8	87.1	86.3
C-15	CH_2	32.4	32.5	32.3	32.7	32.4	32.6	32.3
C-16	CH_2	22.0	22.1	23.3	22.6	22.3	22.5	22.9
C-17	CH	50.8	50.7	52.7	51.5	51.7	51.0	51.3
C-18	CH_3	19.7	19.7	20.4	20.0	19.6	20.1	19.6
C-19	CH_3	25.3	25.3	25.3	25.4	25.3	25.4	25.4
C-20	C	80.2	79.9	95.4	79.3	84.0	78.1	87.1
C-21	CH_3	21.5	21.3	25.8	22.5	25.6	22.2	23.1
C-22	CH	76.1	75.9	167.2	82.3	220.4	84.4	84.4
C-23	CH_2	35.2	35.2	120.9	26.9	34.1	30.7	25.5
C-24	CH_2	42.0	49.7	179.0	41.9	38.6	161.7	42.5
C-25	C	76.4	44.4	–	73.9	73.2	122.2	72.2
C 26	CH_3	27.3	185.9	–	30.0	29.6	173.5	29.7
C-27	CH_3	27.7	17.0	–	30.0	29.6	13.6	30.0
C-28		CH_3-28 15.1	CH-28 84.1	–	22-CH_3CO_2 23.1	–	28-CH_2 29.4	CH_3-a, 28.1
C-29		–	CH_3-29 20.4	–	22-CH_3CO_2 177.2	–	29-CH_3 13.1	CH_3-b, 30.2

Solutions in D_2O, T= 300K referenced to TSP-d_4. Chemical shifts in ppm.
*signal not detected (too low concentration of the sample)