Withanolides from *Withania adpressa*

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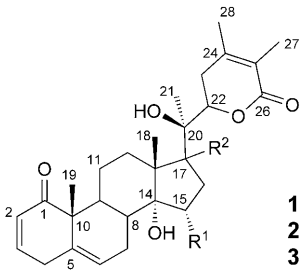
From the leaves of *Withania adpressa*, a plant endemic to Sahara of Morocco and Algeria, the novel steroidal lactone (22*R*)-14α,15α,17β,20β-tetrahydroxy-1-oxowitha-2,5,24-trien-26,22-olide (=(15*S*,17*S*)-14,15,17,20-tetrahydroxy-22,26-epoxyergosta-2,5,24-triene-1,26-dione; **1**), was isolated, along with three known compounds, withanolides F (**2**), J (**3**), and oleanolic acid. Their structures were mainly solved by in-depth 1D- and 2D-NMR (including ADEQUATE) experiments, as well as by HR-MS analyses and chemical evidence.

Introduction. – The genus *Withania* (Solanaceae) is known for elaborating withanolides, which are steroidal lactones characterized by a C_{28} basic skeleton. Several of these substances have displayed various biological activities, such as cytotoxic [1], anticancer [2], immunosuppressive [3], adaptogenic [4], and antifeedant [5] properties. In Morocco, the genus *Withania* is represented by three species: *W. somnifera* (L.) DUNAL, *W. adpressa* COSS., and *W. frutescens* (L.) PAUQUY [6]. *W. adpressa* is a green-grayish shrub, with coriaceous, glaucous leaves; it is endemic to Sahara of Morocco and Algeria [7].

According to the literature, *W. adpressa* has not previously been studied phytochemically. The present investigation describes the isolation and structural elucidation of a new withanolide, identified as (22*R*)-14α,15α,17β,20β-tetrahydroxy-1-oxowitha-2,5,24-trien-26,22-olide (=(15*S*,17*S*)-22,26-epoxy-14,15,17,20-tetrahydroxyergosta-2,5,24-triene-1,26-dione; **1**). This new compound was isolated together with withanolide F (**2**), withanolide J (**3**), and oleanolic acid from the leaves of the title plant.

Results and Discussion. – Compound **1** was obtained as a colorless solid. Its molecular formula, $C_{28}H_{38}O_7$, was deduced from the quasi-molecular ion peak at *m*/*z* 509.2517 ($[M+Na]^+$) by means of high-resolution electrospray-ionization time-of-flight mass spectrometry (HR-ESI-TOF-MS). Liquid-chromatography ion-trap mass spectrometry (LC/IT-MS) showed the pseudo-molecular ion at *m*/*z* 504.0



	R^1	R^2
1	OH	β-OH
2	H	β-OH
3	H	α-OH

([$M+H_2O$]$^+$), with a fragment ion at 169.2 ($C_9H_{13}O_3$) due to cleavage of the C(17)–C(20) bond, confirming the presence of one OH group as well as a six-membered lactone substituted at C(20) of the steroidal skeleton. The second moiety of the molecule, after loosing an OH group, gave rise to the base peak at *m*/*z* 300.9. The most-prominent ions were due to the gradual loss of four molecules of H_2O: *m*/*z* 486.0, 468.8, 451.4, 433.1, and 283.3, confirming the presence of four OH groups.

The UV spectrum of **1** showed a strong absorption at λ_{max} 222 nm, characteristic of α,β-unsaturated C=O and α,β-unsaturated lactone chromophores [8]. The IR absorbances at 3416, 1685, and 1660 cm^{-1} suggested the presence of OH groups, a six-membered cyclic ketone, and an α,β-unsaturated lactone, respectively [9].

The ^{1}H-NMR spectrum of **1** (*Table*) showed five Me *singlets* at δ(H) 1.15, 1.22 (2 Me), 1.75, and 1.88. The appearance of Me(21) as a *singlet* was a strong indication that the neighboring C(20) was a quaternary C-atom, and the appearance of C(21) at low field (δ(C) 1.22) confirmed that an OH group was present at C(20). The downfield shift of Me(27) and Me(28) in the ^{1}H-NMR spectrum suggested the presence of a C=C bond between C(24) and C(25), *i.e.*, the presence of an α,β-unsaturated lactone [10]. H–C(22) coupled only with CH_2(23), and appeared at δ(H) 4.68 (*dd*, *J*(22,23a) = 12.7, *J*(22,23b) = 3.2 Hz). H_a–C(23) coupled with H_b–C(23) (16.0 Hz) and with H–C(22) (12.7 Hz), and appeared as a broad *doublet*; and H_b–C(23), coupling with H_a–C(23) (16.0 Hz) and H–C(22) (3.2 Hz), appeared as a *multiplet*.

Three downfield signals at δ(H) 5.76 (*dd*, *J*(2,3) = 9.8, *J*(2,4a) = 2.0 Hz), 6.88 (*ddd*, *J*(3,2) = 9.8, *J*(3,4b) = 4.7, *J*(3,4a) = 2.4 Hz), and 5.57 (*d*, *J*(6,7a) = 5.2 Hz) represented three vinylic H-atoms, H–C(2), H–C(3), and H–C(6), respectively. The chemical shift of Me(18) (δ(H) 1.22) was consistent with the presence of OH groups at positions 17β and/or 14β. The quaternary ^{13}C-NMR carbon signals at δ(C) 82.1 and 88.0, assigned to C(14) and C(17), respectively, confirmed a 14β,17β-diol structure. The ^{13}C-NMR (DEPT) spectrum of **1** indicated the presence of 28 C-atoms, including five Me, six CH_2, and seven CH groups, as well as ten quaternary C-atoms. The lowfield signals at δ(C) 203.1 and 165.9 were due to ketone and lactone C=O groups, respectively. The signals at δ(C) 127.0, 146.4, and 125.1 were attributed to three vinylic C-atoms, C(2), C(3), and C(6), respectively. The peaks at δ(C) 135.1, 150.7, and 120.2 were assigned to quaternary vinylic C-atoms, C(5), C(24), and C(25), respectively, and the signal at δ(C) 80.9 was attributed to C(22). The resonance at δ(C) 78.2 was assigned to an oxygenated quaternary C-atom, C(20). The signals appearing at δ(C) 20.0, 18.2, 19.4, 20.2, and 12.1 were assigned to Me(18), Me(19), Me(21), Me(27), and Me(28), respectively.

Table. *^{1}H- and ^{13}C-NMR Data of* **1**–**3**. In (D_6)DMSO (**1**, **2**) or (D_6)DMSO/CD_3OD 9 : 1 Solution (**3**). δ in ppm, J in Hz; ergostane atom numbering.

	1		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
C(1)	–	203.1	–	203.5	–	204.1
H–C(2)	5.76 (*dd*, *J*(2,3) = 9.8, *J*(2,4a) = 2.0)	127.0	5.74–5.79 (*m*)	126.9	5.75 (*dd*, *J*(2,3) = 9.5, *J*(2,4a) = 2.5)	127.4
H–C(3)	6.88 (*ddd*, *J*(3,2) = 9.8, *J*(3,4b) = 4.7, *J*(3,4a) = 2.4)	146.4	6.88 (*ddd*, *J*(3,2) = 9.8, *J*(3,4b) = 4.9, *J*(3,4a) = 2.2)	146.5	6.86 (*ddd*, *J*(3,2) = 10.1, *J*(3,4b) = 5.0, *J*(3,4a) = 2.5)	146.8
H_a–C(4)	3.28 (br. *d*, *J*(4a,4b) = 21.4)	32.9	2.23–3.31 (*m*)	32.8	2.22–3.31 (*m*)	33.3
H_b–C(4)	2.83 (*dd*, *J*(4b,4a) = 21.4, *J*(4b,3) = 4.8)	–	2.83 (*dd*, *J*(4b,4a) = 21.6, *J*(4b,3) = 4.7)	–	2.82 (*dd*, *J*(4b,4a) = 21.8, *J*(4b,3) = 5.0)	–
C(5)	–	135.1	–	135.2	–	135.8
H–C(6)	5.57 (*d*, *J*(6,7a) = 5.2)	125.1	5.56–5.60 (*m*)	124.7	5.56–5.59 (*m*)	124.8
H_a–C(7)	1.99–2.04 (*m*)	25.5	2.00–2.07 (*m*)	25.1	1.96–2.01 (*m*)	24.8
H_b–C(7)	1.91–1.99 (*m*)	–	1.70–1.75 (*m*)	–	1.67–1.74 (*m*)	–
H–C(8)	1.98–2.06 (*m*)	32.9	1.68–1.74 (*m*)	36.6	2.02–2.10 (*m*)	35.9
H–C(9)	2.09–2.17 (*m*)	35.2	2.02–2.15 (*m*)	35.2	1.71–1.78 (*m*)	36.2
C(10)	–	50.3	–	50.1	–	51.5
H_a–C(11)	2.06 (*qd*, *J*(11b,9) = *J*(11b,11a) = *J*(11b,12a) = 13.4, *J*(11b,12b) = 4.7)	22.7	2.00–2.07 (*m*)	22.8	2.02–2.08 (*m*)	22.2
H_b–C(11)	1.53 (*d*, *J*(11a,11b) = 15.6)	–	1.47–1.53 (*m*)	–	1.49–1.54 (*m*)	–
H_a–C(12)	2.23 (*td*, *J*(12a,12b) = *J*(12a,11b) = 11.8, *J*(12a,11a) = 4.0)	31.7	1.13–1.19 (*td*, *J*(12a,12b) = *J*(12a,11b) = 12.0, *J*(12a,11a) = 4.5)	30.1	2.13–2.19 (*m*)	27.1
H_b–C(12)	1.10–1.17 (*m*)	–	1.13–1.21 (*m*)	–	1.39–1.44 (*m*)	–
C(13)	–	53.1	–	53.7	–	50.6
14-OH	5.77 (*s*)	82.1	5.76 (*s*)	81.4	[a])	85.3
H_a–C(15)	3.65 (*d*, *J*(15,16a) = 5.8)	74.5	1.32–1.40 (*m*)	31.9	1.64–1.71 (*m*)	32.9
H_b–C(15)[b])	4.60 (br. *s*)	–	1.52–1.60 (*m*)	–	1.44–1.51 (*m*)	–
H_a–C(16)	2.72 (*dd*, *J*(16a,16b) = 15.5, *J*(16a,15) = 6.6)	46.4	2.32–2.39 (*m*)	35.7	2.67 (*ddd*, *J*(16a,16b) = 14.2, *J*(16a,15a) = 11.7, *J*(16a,15b) = 1.9)	33.6
H_b–C(16)	1.56 (*d*, *J*(16b,16a) = 15.6)	–	1.49–1.55 (*m*)	–	1.74–1.81 (*m*)	–
17-OH	4.60 (br. *s*)	88.0	4.64 (*s*)	87.3	[a])	87.8
Me(18)	1.22 (*s*)	20.0	1.00 (*s*)	20.3	1.02 (*s*)	18.3
Me(19)	1.15 (*s*)	18.2	1.14 (*s*)	18.3	1.15 (*s*)	18.7
20-OH	6.65 (*s*)	78.2	6.85 (*s*)	78.1	[a])	77.0
Me(21)	1.22 (*s*)	19.4	1.20–1.26 (*m*)	19.1	1.16 (*s*)	19.7
H–C(22)	4.68 (*dd*, *J*(22,23a) = 12.7, *J*(22,23b) = 3.2)	80.9	4.65 (*dd*, *J*(22,23a) = 12.7, *J*(22,23b) = 3.6)	81.0	4.53 (*dd*, *J*(22,23a) = 12.9, *J*(22,23b) = 3.5)	80.1

Table (cont.)

	1		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
H_a–C(23)	2.49 (br. *d*, *J*(23a,23b) = 16.0)	34.3	2.35–2.50 (*m*)	34.3	2.28–2.35 (*m*)	31.6
H_b–C(23)	2.35–2.42 (*m*)	–	2.35–2.50 (*m*)	–	2.41–2.49 (*m*)	–
C(24)	–	150.7	–	150.7	–	151.1
C(25)	–	120.2	–	120.1	–	120.3
C(26)	–	165.9	–	165.9	–	166.4
Me(27)	1.88 (*s*)	20.2	1.85 (*s*)	20.2	1.88 (*s*)	20.2
Me(28)	1.75 (*s*)	12.1	1.72 (*s*)	12.1	1.73 (*s*)	12.1

[a]) OH Group exchanges in this solvent. [b]) 15-OH for **1**.

The ^{1}H,^{1}H-COSY, NOESY, HSQC, and HMBC spectra allowed us to determine the molecular structure of compound **1**. However, because HMBC data are often compatible with structures where carbon pairs are inverted, ADEQUATE experiments were carried out (*Fig.*) [11], which are based on the low natural abundance of directly-bound pairs of ^{13}C, to unequivocally confirm the structure. The close resemblance of the ^{13}C-NMR chemical shifts of rings *C* and *D* in **1** with those of coagulin H [12] was a strong indication that, indeed, the two products differed only in ring *B*. The β-configuration of the OH group at C(14) was excluded since the ^{13}C-NMR chemical shifts of known compounds [13–15] with β-configuration were significantly different at C(8), C(14), C(16), and C(18). The α-orientation of the 14-OH group was also supported by an NOE observed with H_a–C(12). Similarly, the α-configuration of the 17-OH group could be excluded because the ^{13}C-NMR chemical shifts for C(12), C(13), C(18), C(21), and C(22) were not well-correlated with those reported in the literature [16][17]. Finally, NOE due to contacts between CH_2(12), H–C(22), and CH_2(23) would not be possible if the side chain was β-oriented.

All known 15-hydroxylated withanolides are α-configured [12][14][15]. The scalar coupling constant ^3J(15,16), with a value of 6 Hz, was taken as a clear indication of the

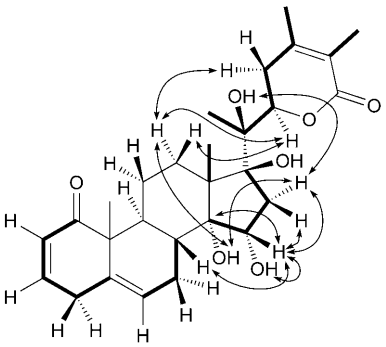


Figure. *Key 1,1-ADEQUATE* (▬) *and NOE* (↔) *correlations of* **1**

α-configuration of the 15-OH group [12][13]. Further, an NOE between H–C(8) and H–C(15), both lying above the molecular plane (β), confirmed that the 15-OH function was α-oriented. The configuration at C(22) was determined as (*R*), *i.e.*, the natural configuration in ergostanes, since H–C(22) was found to be a double *doublet* with two coupling constants, which is characteristic for axial–axial and axial–equatorial interactions with CH_2(23) [18].

The IR, UV, ^{1}H-NMR, and ^{13}C-NMR data of compound **2** were very similar to those of **1**, the only difference being the absence of the 15-OH group. The molecular skeleton was easily assembled by means of COSY and HMBC spectra. The configuration of **2** was based on the very close match of ^{13}C-NMR chemical shifts with literature data of compounds carrying α- and β-OH groups at C(14) and C(17), respectively [12][16–20]. Compound **2** had been isolated previously from *W. somnifera* growing in Israel, and named withanolide F [19]. However, this is the first report of its isolation from *W. adpressa*.

A set of classical NMR experiments, including ^{1}H-NMR, DQF-COSY, {^{1}H}^{13}C, DEPT-135, HSQC, and HMBC, allowed us to identify compound **3** as withanolide J [17]. The ^{1}H,^{1}H-COSY data and ^{1}H,^{13}C-NMR correlations for **3** were identical with those of withanolide F (**2**). The pattern of differences in ^{13}C-NMR chemical shifts between the two withanolides demonstrated that they were 17-epimers [21]. Moreover, both the ^{1}H- and ^{13}C-NMR data of compound **3** successfully passed the test for the determination of the chirality at C(17) developed by *Kirson* and *Gottlieb* [16] for a pair of molecules that only differ from ours in a 5β,6β-epoxide instead of a C(5)=C(6) function. Finally, compound **3** was identified as withanolide J by comparison of its ^{13}C-NMR data with those published earlier [17].

Oleanolic acid, a pentacyclic triterpenoid sapogenin, was isolated for the first time from the genus *Withania*. It was characterized by comparison of its physical and spectroscopic (^{1}H-, ^{13}C-NMR, DQF-COSY, DEPT-135, HSQC, HMBC) data with those published in the literature [22], and by comparison with an authentic sample.

We thank *André Pinto* for performing most NMR experiments and for spectrometer management. The ADEQUATE experiment was run on the 600-MHz spectrometer located at EPFL, Lausanne, in the frame of the inter-university program 'spectromètre à haut champ d'intérêt romand'.

Experimental Part

General. Thin-layer chromatography (TLC): pre-coated silica gel *60 F_{254}* plates (*Merck*), visualization under UV light and by spraying with *Dragendorff*'s reagent. Column chromatography (CC): regular silica gel (63–200 μm) or reverse-phase (RP) silica gel (40–63 μm), *Lobar Lichroprep RP-18* (*Merck*) and *SymetryPrep RP-18* (150×19 mm i.d.; *Waters*). Melting points (m.p.): *Mettler FP800* apparatus; uncorrected. Optical rotations: *Perkin-Elmer 241-MC* polarimeter. IR Spectra: *Perkin-Elmer Spectrum-One-B FT-IR* spectrometer, with KBr pellets; in cm^{-1}. UV Spectra: λ_{max} (log ε) in nm. NMR Spectra: *Bruker DRX*-500 apparatus, at 500 (^{1}H) or 125 MHz (^{13}C), or *Bruker DRX-600* apparatus equipped with a low-temperature detection coil optimized for ^{1}H detection, at 600 (^{1}H) or 150 MHz (^{13}C); δ in ppm rel. to Me_4Si, *J* in Hz. HR-ESI- and APCI-MS: *QStar XL-TOF* and *MAT-LCQ* mass spectrometers, resp., in *m*/*z*.

Plant Material. The leaves of *Withania adpressa* Coss. were collected in the South of Morocco, April 2003. The plant was authenticated by Dr. *Aziz Abbad*, Department of Biology, Cadi Ayyad University, Morocco. A voucher specimen (No Mar4223) was deposited at the Herbarium of the Botany Department, Faculty of Sciences-Semlalia, Marrakech, Morocco.

Extraction and Isolation. The dried and powdered leaves (3 kg) of *W. adpressa* were successively extracted with hexane and MeOH (12.5 l each) in a *Soxhlet* apparatus. After solvent evaporation, the methanolic residue (390 g) was suspended in H_2O (500 ml) and extracted with CH_2Cl_2 (5×500 ml). The CH_2Cl_2-soluble fraction (15 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:0 → 0:100) to afford nine fractions: *Fr. 1–9*. *Fr. 3* (800 mg) was further subjected to CC (1. *LichroPrep*; MeCN/H_2O 10:90 → 100:0; 2. *SymetryPrep*; MeCN/H_2O 4:6) to afford **1** (80 mg). *Fr. 2* (350 mg) was purified by CC (*Lichroprep*; MeCN/H_2O 5:95 → 100:0) to afford eight fractions: *Fr. 2.1* to *2.8*. *Fr. 2.7* afforded **2** (57 mg). *Fr. 1* (1.5 g) was resubjected to CC (*Lichroprep*; MeCN/H_2O 10:90 → 100:0), which gave 62 fractions (*Fr. 1.1* to *1.62*). *Fr. 1.12* yielded withanolide J (180 mg), and *Fr. 1.61* afforded oleanolic acid (210 mg).

*(15*S*,17*S*)-14,15,17,20-Tetrahydroxy-22,26-epoxyergosta-2,5,24-triene-1,26-dione* (**1**). Colorless solid. M.p. 182–185°. $[\alpha]_D^{25} = +103.3$ ($c = 1$, MeOH). UV (MeOH): 222 (4.24). IR (KBr): 3416, 2978, 2942, 1685, 1660, 1450, 1389, 1296, 1143, 1093, 1026, 1001. ^{1}H- and ^{13}C-NMR: see *Table*. HR-ESI-MS: 509.2517 ($[M+Na]^+$, $C_{28}H_{38}NaO_7^+$; calc. 509.2516).

Withanolide F (*=(17*S*)-14,17,20-Trihydroxy-22,26-epoxyergosta-2,5,24-triene-1,26-dione*; **2**). Colorless solid. M.p. 159–160°. $[\alpha]_D^{25} = +205.1$ ($c = 1$, MeOH). UV (MeOH): 226 (4.23). IR (KBr): 3424, 2955, 2877, 1686, 1452, 1388, 1322, 1283, 1141, 1092, 1024, 1001. ^{1}H- and ^{13}C-NMR: see *Table*. HR-ESI-MS: 493.2583 ($[M+Na]^+$, $C_{28}H_{38}NaO_6^+$; calc. 493.2567).

Withanolide J (*=(17*R*)-14,17,20-Trihydroxy-22,26-epoxyergosta-2,5,24-triene-1,26-dione*; **3**). Colorless solid. M.p. 180°. $[\alpha]_D^{25} = +316.6$ ($c = 1$, MeOH). UV (MeOH): 226 (4.26). IR (KBr): 3426, 2970, 2876, 1683, 1663, 1456, 1410, 1385, 1321, 1139. ^{1}H- and ^{13}C-NMR: see *Table*. HR-ESI-MS: 493.2599 ($[M+Na]^+$, $C_{28}H_{38}NaO_6^+$; calc. 493.2567).

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