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NEW WITHANOLIDES FROM A CROSS OF A SOUTH AFRICAN CHEMOTYPE BY CHEMOTYPE II (ISRAEL) IN *WITHANIA SOMNIFERA**

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Key Word Index—*Withania somnifera*; Solanaceae; withanolides; steroidal lactones.

Abstract—Three new withanolides have been isolated from hybrids obtained by crossing a chemotype of *Withania somnifera* received from South Africa and chemotype II originating in Israel. The compounds have been characterized as 4β,20α-dihydroxy-1-oxo-5β,6β-epoxy-20*R*,22*R*-witha-24-enolide, 20α-hydroxy-1,4-dioxo-5β,6β-epoxy-20*R*,22*R*-witha-2,24-dienolide, and 20α-hydroxy-1,4-dioxo-5β,6β-epoxy-20*R*,22*R*-witha-2-enolide. The major steroid of the plant is withanolide D, while the other known withanolides present are 4β,20α-dihydroxy-1-oxo-5β,6β-epoxy-20*R*,22*R*,24*S*,25*R*-witha-2-enolide and withaferin A. The structures assigned to the new compounds are based on spectral evidence, analysis of their fragmentation under electron impact, and on chemical correlation with known compounds. The formation of these withanolides in this new hybrid is discussed briefly.

The structures of the withanolides, C_{28} steroidal lactones occurring in plants of the Solanaceae, isolated from *Withania somnifera* chemotype II, and those isolated from a chemotype grown from seeds obtained from South Africa, have been published previously [2, 3]. This paper describes the chemical investigation of the F_1 offspring arising from the crossbreeding of a chemotype from South Africa with chemotype II (Israel) and designated Africa ×II, plants of which were grown in an experimental field at the Department of Plant Introduction, Bet-Dagan, Israel by A. Abraham.

Chromatography of the crude methanol extract obtained from the leaves resulted in the separation of six withanolides. The main component (0.44% of the dried leaves) was identified as withanolide D (**1a**), which previously had been shown to be the principal constituent of the parent *W. somnifera* chemotype II [2]. A second major constituent (0.26% of the dried leaves) was 4β,20α-dihydroxy-1-oxo-5β,6β-epoxy-20*R*,22*R*,24*S*,25*R*-witha-2-enolide (**2a**), the 24,25-dihydro derivative of withanolide D, found previously as a constituent of the parent South African chemotype [3]. The third known compound isolated was withaferin A (**3**) which is among the principal constituents of the parent South African chemotype, but was obtained from the offspring in only minor amounts (0.05% of the dried leaves). the three compounds **4a**, **5** and **6** (isolated in 0.22, 0.015 and 0.012% yields, respectively) have not previously been encountered.

Compound **2a** was identified through spectroscopic examination, and comparison with an authentic sample [3]. Irradiation (^{1}H NMR, 270 MHz) of the protons of the C-27 methyl group showed the decoupled 25-H as a doublet ($J_{24,25} = 10$ Hz) thus providing supporting evidence for the *trans* stereochemistry of the methyl substituents of the lactone ring.

The ^{1}H NMR spectrum of **4a**, (Table 1) mp 277–280°, $C_{28}H_{40}O_6$, showed the five methyl groups to have chemical shifts characteristic of the structure of withanolide D **1a** [4], but the spectrum lacked the resonance due to the protons of the 2,3-double bond. The chemical shifts and coupling attributed to 22-H and to 6-H were likewise analogous to those of **1a**, but the proton adjacent to the secondary hydroxyl resonated at δ 3.50 compared with 3.75 in the spectrum of **1a**. This hydroxyl substituent was assigned to the 4-position since on deuterium exchange the resonance ascribed to 4-H was an apparent triplet ($J = 3.5$ Hz) due to coupling with the methylene protons at C-3. Acetylation of the secondary hydroxyl group gave a monoacetate **4b** in which the resonance of 4-H in the ^{1}H NMR spectrum was an apparent triplet at δ 4.59. All that in accordance with 2,3-dihydrowithaferin A and its acetate [5, 6]. The presence of the unsaturated lactone ring in the side chain was confirmed from the fragmentation under electron impact. The intense

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Table 1. NMR signals for relevant protons in certain withanolides*

Compound	2-H	3-H	4-H	6-H	22-H	Methyl groups 19-H	 18-H	 21-H	 27-H	 28-H	Other signals
2a†[3]	6.32*d* (10)	6.94*dd* (10; 6)	3.75*d* (6)	3.23*bs* ($W_{1/2}$ 4)	4.17*dd* (11.5; 3.0)	1.41*s*	0.86*s*	1.24*s*	1.22*d* (6.7)	1.14*d* (6.7)	
2b‡	6.22*d* (10)	7.02*dd* (10; 6)	4.64*d* (6)	3.20 ($W_{1/2}$ 4)	4.13*m*	1.39*s*	0.85*s*	1.24*s*	1.20*d* (6.7)	1.14*d* (6.7)	2.04 (acetate)
4a‡	—	—	3.50*t* (3.2)	3.13*bs* ($W_{1/2}$ 4)	4.20*dd* (12.7; 4.7)	1.30*s*	0.81*s*	1.25*s*	1.97*s*	1.87*s*	
5‡	6.86 2×H *s*		—	3.42*d* (2.3)	4.20*dd*	1.38*s*	0.88*s*	1.28*s*	1.95*s*	1.89*s*	
6‡	6.85 2×H *s*		—	3.42*d* (2.3)	4.15*m*	1.38*s*	0.89*s*	1.26*s*	1.22*d* (6.7)	1.14*d* (6.7)	

* Solvent $CDCl_3$; δ values; coupling constants (Hz) in parentheses.

† Recorded at 270 MHz.

‡ Recorded at 90 MHz.

peaks at *m/e* 125 (lactone) and at 169 were attributed to cleavage of the bonds C-20–C-22 or C-17–C-20, respectively. The principal peaks at higher mass number were also due to loss of these two fragments together with molecules of water, *m/e* 347 (M − 125), 329 (M − 125 − H_2O) and 311 (M − 125 − 2 × H_2O) [3].

Since the withanolides are generally colourless, the yellow colour of compound **5**, mp 273–274°, $C_{28}H_{36}O_5$, provided immediate evidence of a new chromophoric system. The ^1H NMR spectrum showed differences in the substitution pattern of rings A and B whereas the other signals complied well with those of withanolide D. In the low field region, a two-proton singlet at δ 6.85 is characteristic for a Δ^2-1,4-diketone system in ring A. The UV showed a λ_{max} at 224 nm (ε 20 300) which is a combination of the side chain unsaturated δ-lactone chromophore which is located at 225 nm (ε 9600), and the cissoid Δ^2-1,4-diketone system absorbing at 224 nm (ε 13 000) as shown in compound **6**. Oxidation of withanolide D (**1a**) with MnO_2 afforded a product identical with the natural product (**5**). A similar reaction had previously been done with withaferin A [5].

Compound **6**, mp 260–263°, $C_{28}H_{38}O_6$, contained two more hydrogen atoms than compound **5**. It was yellow and the ^1H NMR showed a two-proton singlet at δ 6.85 due to the vinylic protons of the enedione system. Indeed, oxidation of **2a** with MnO_2 yielded a diketone which was identical with the natural compound **6**. The presence of the side chain as a saturated lactone was supported by the fragmentation under electron impact. The peaks at *m/e* 127 and 128 and at 171 were two mass units higher than those arising from the unsaturated lactone in the compounds above, and they were likewise attributed to cleavage of the C-20–C-22 and C-17–C-20 bonds. At higher mass number the principal peaks could be attributed to loss of these fragments along with additional molecules of water, as previously described [3]. The stereochemistry of the saturated lactone in **6** is consequently the same as in **2a**, and this had previously been established through studies of circular dichroism to have the configuration 22*R*, 24*S*, 25*R* [7].

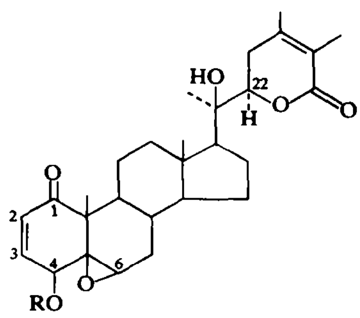
When the molecular rotation (M_D) differences were calculated (see Table 2) for the natural products **4a**, **5** and **6** and compared to withanolide D (**1a**), 24,25-dihydro-withanolide D (**2a**), withaferin A (**3**), and its 4-dehydro derivative **7**, excellent values were obtained. These values confirm the identity of the various natural and synthetic derivatives due to oxidation of the allylic alcohol at C-4 or reduction of the Δ^2 and Δ^{24}. They also confirm that no conformational changes took place in the molecules during these transformations in the hybrid offspring.

DISCUSSION

The constituents of *Withania somnifera* chemotype II include withanolide D (**1a**), 27-hydroxy-, 14α-

Table 2. Molecular rotation differences (ΔM_D) between certain basic withanolides and their natural oxidation or reduction products

Compound and M_D values		ΔM_D
Withaferin A **3** M_D 535.8	4-Dehydro-withaferin A **7** [5] M_D 687.9	−152.1
Withanolide D **1a** M_D 376	4-Dehydro-withanolide D **5** M_D 531.2	−155.2
24,25-Dihydro-withanolide D **2a** M_D 80.2	24,25-Dihydro-4-dehydro-withanolide D **6** M_D 231.2	−151.0
Withaferin A **3** M_D 535.8	2,3-Dihydro-withaferin A **8** M_D 37.8	498.0
Withanolide D **1a** M_D 376	2,3-Dihydro-withanolide D **4a** M_D − 132.2	508.2



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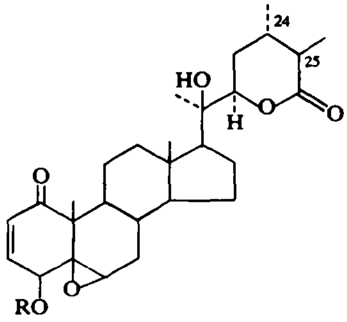
1b R = Ac



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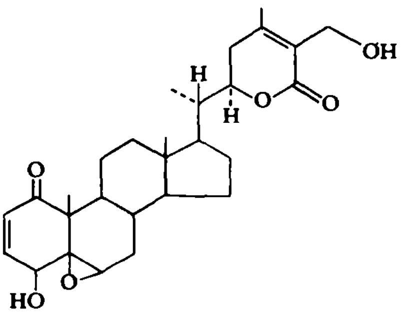

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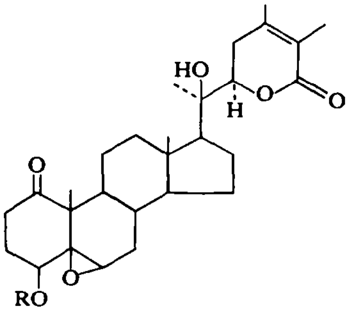

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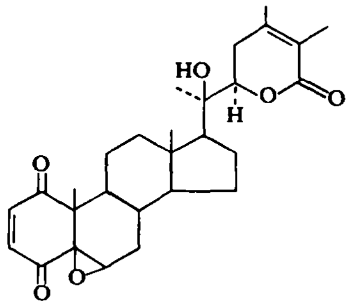
4b R = Ac



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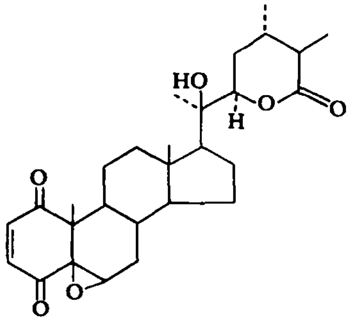

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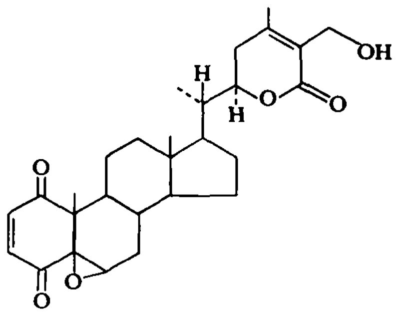

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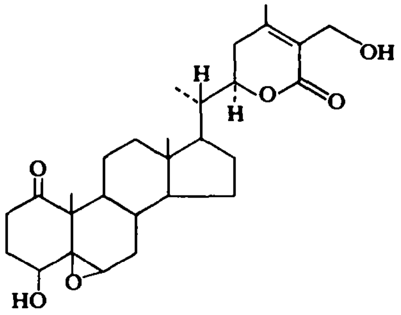

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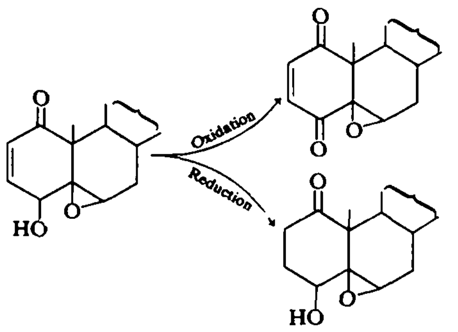
hydroxy-, and 17α-hydroxy-withanolide D, together with withanolide G [2]. The dominant feature is that all the compounds carry a hydroxy group at C-20, and the side chain in each consists of an unsaturated lactone. The chemotype of South Africa provides, however, two principal variations of substitutions, and it is the only one containing both type of compounds: those having a hydroxy substituent at C-20, and others in which C-20 is not hydroxylated. Furthermore, it contains compounds which are unsaturated lactones, and others in which the lactone ring is saturated. The constituents included withanolide D (**1a**) and 24,25-dihydrowithanolide D (**2a**) in the first category, and withaferin A (**3**), 27-deoxywithaferin A and 24,25-dihydro-27-deoxywithaferin A in the second [3].

In the F_1 offspring Africa × II, the principal constituent is withanolide D (**1a**), and with the exception of some withaferin A (**3**), which is present only as a minor component, all the remaining compounds isolated from this hybrid carry a 20-hydroxyl substituent, and the 20-hydroxylation is clearly a dominant genetic factor. Such a behaviour could have been anticipated and has been described for previous similar experiments [1, 8].

In the Africa × II hybrid, one major (**2a**), and one minor (**6**) component have a saturated lactone so that this is definitely a genetic factor arising from the African chemotype. The outstanding feature is the presence of a new oxidizing system which apparently oxidizes ring A to a diketone in the presence of either the saturated (**6**) or unsaturated lactone (**5**).

A further notable finding is the isolation of a compound having C-2 and C-3 saturated, a feature which has previously only been noted in a minor constituent of chemotype I, and found only on one previous occasion [6]. Only subsequently was it found that the plant processed at the time [6] belonged to chemotype I [2]. Further attempts to isolate this compound were unsuccessful. These findings suggest that enzyme systems are present which can both oxidize and reduce the basic 4-hydroxy-1-oxo-2-ene system present in almost all withanolides (Scheme 1).

It is interesting to note that 2,3-dihydro-withaferin A has been isolated from *Withania aristata* Pauq [9].



EXPERIMENTAL

Mps were measured on a Fischer-Johns apparatus and are uncorr. Optical rotations refer to $CHCl_3$ solns. IR spectra refer to KBr pellets; UV spectra were recorded for EtOH solns; ^1H NMR spectra were determined on a Brucker WH 90 instrument for 5–10% solns in $CDCl_3$, containing TMS as int. standard. Analytical TLC was carried out using chromatoplates of Si gel F (Riedel-De Haën), and prep. TLC using chromatoplates (Merck, 200 × 200 × 2 mm, Kieselgel 60 F_{254}). Mass spectra were determined with an Atlas CH4 instrument under the direction of Dr. Z. Zaretskii. (MWs are given for the principal isotopic species.) Analyses were performed in the microanalytical laboratory of our Institute, under the direction of Mr. R. Heller.

Plant material and isolation procedure. Crossbreedings between *Withania somnifera* chemotype South Africa and chemotype II (Israel) were performed by pollination and isolation of the flowers (by A. A.). Crushed air-dried leaves (1 kg) were extracted in the usual way to yield a direct ethereal extract (30.4 g). The crude extract was adsorbed on Si gel (50 g) and introduced at the top of a chromatographic column made up of Si gel (Woelm 63–100, 550 g) and elution was performed with CH_2Cl_2 followed by CH_2Cl_2–EtOAc, fractions of 60 ml each being collected (Table 3).

Compound **1a**, mp 254° (from $CHCl_3$–EtOAc) was identified with an authentic sample of withanolide D by TLC, mmp and ^1H NMR and IR spectra. The acetate (**1b**) crystallized from EtOH or from EtOAc–hexane, mp 258° (lit. [4] 247° from EtOH) ν_{max} cm^{-1}: 3550, 1725, 1705, 1680 and 1245.

Compound **2a** crystallized from $CHCl_3$–EtOAc to yield colourless crystals of 24,25-dihydro-withanolide D, mp 273° (lit. [3] 275°), $[\alpha]_D + 17°$ (*c* 0.22) (lit. [3] $[\alpha]_D + 14°$). ν_{max} cm^{-1}: 1730 and 1681; λ_{max} nm: 214 (ε 7500). The acetyl derivative **2b** crystallized from EtOAc or Me_2CO–hexane, mp 231° (lit. [3] 237–238°) $[\alpha]_D - 37°$ (*c* 0.18) (lit. [3] $[\alpha]_D - 38°$).

Compound **3**, mp 241–243° (EtOAc), was isolated from the combined fractions 222–275 by prep. TLC (EtOAc). It was identified with an authentic sample of withaferin A by mmp and ^1H NMR and IR spectra.

Compound **4a** was purified by fractional crystallization from Me_2CO to yield 4β,20α-dihydroxy-1-oxo-5β,6β-epoxy-20*R*,22*R*-witha-24-enolide as colourless crystals, mp 277–280° $[\alpha]_D - 28°$ (*c* 0.20); ν_{max} cm^{-1}: 1700; λ_{max} nm: 227 (ε 8400). (Found: C, 71.0; H, 8.6; M^+ 472. Calc. for $C_{28}H_{40}O_6$: C, 71.2; H, 8.5%; MW 472.60).

Compound **5**, 20α-hydroxy-1,4-dioxo-5β,6β-epoxy-20*R*,22*R*-witha-2,24-dieholide, was purified by prep. TLC, mp 273–274° (EtOAc); $[\alpha]_D + 113.5°$ (*c* 0.22); ν_{max} cm^{-1}: 1710, 1685 and 1620; λ_{max} nm: 224 (ε 20 300). (Found: M^+ 468. Calc. for $C_{28}H_{36}O_6$: MW 468.57).

Compound **6**, 20α-hydroxy-2,4-dioxo-5β,6β-epoxy-20*R*,22*R*-witha-2-enolide, mp 260–263° (EtOAc), $[\alpha]_D + 49.2°$ (*c* 0.25); ν_{max} cm^{-1}: 1740, 1685 and 1615; λ_{max} nm: 224 (ε 13 000). (Found: C, 71.4; H, 8.1; M^+ 470; calc. for $C_{28}H_{38}O_6$: C, 71.5; H, 8.1% MW 470.58).

Acetylation of **4a**. Compound **4a** (50 mg) was acetylated with Ac_2O/Py at room temp. for 24 hr. The crude acetyl derivative (**4b**) (49 mg) crystallized from $CHCl_3$–EtOAc, mp 278–280°; $[\alpha]_D - 37°$ (*c* 0.22) (lit. [3] − 38°); ν_{max} cm^{-1}: 1728, 1710 and 1250; λ_{max}: 226 nm (ε 8200) (Found: C, 69.8; H, 8.1. Calc. for $C_{30}H_{42}O_7$: C, 70.0; H, 8.2%; MW 514.64).

Hydrogenation of withanolide D acetate **1b**. Compound **1b** (mp 258°, 200 mg) was hydrogenated over 10% Pd/C in absolute EtOH to yield a mixture of products. Separation of the dihydro compound by prep. TLC (EtOAc) and crystallization from EtOAc gave the 2,3-dihydro compound (**4b**), mp 250° (lit. [4] 241°); ν_{max} cm^{-1}: 1738 sh, 1710, 1650w and 1250. The ^1H NMR spectrum was identical with that recorded for **4b** except that it showed the presence of a trace of **1b** which could not be removed by recrystallization.

Oxidation of withanolide D **1a** *with* MnO_2. MnO_2 (200 mg) was added to a solution of **1a** (50 mg) in Me_2CO (50 ml) and the mixture was stirred at room temp. for 48 hr. The product crystallized from EtOAc in pale-yellow crystals, mp 275°; $[\alpha]_D + 114°$ (*c* 0.22) (lit. [10] + 115°); ; (Found: C, 71.7; H, 7.7; M^+ 468; calc. for $C_{28}H_{36}O_6$: C, 71.8; H, 7.7%; MW 468.57). The compound was shown to be identical with the natural product (compound **5**) by mmp and by comparison of the ^1H NMR and the IR spectra.

Oxidation of compound **2a** *with* MnO_2. Compound **2a** (50 mg) was oxidized with MnO_2 in Me_2CO as above. The product crystallized from EtOAc in pale-yellow crystals, mp 257–260°; $[\alpha]_D + 48°$; (*c* 0.21). (Found: M^+ 470; calc. for $C_{28}H_{40}O_6$; MW 470.60). The compound was shown to be identical with the natural product (compound **6**) by mmp and by comparison of the IR and ^1H NMR spectra.

Table 3. Chromatographic separation of withanolides on silica gel

Withanolide	Fraction No.	Eluted with CH_2Cl_2–EtOAc	Amount isolated (mg)	R_f (EtOAc)
6	82–93	9:1	125	0.61
5	94–105	8:2	150	0.56
2a	136–142	7:3	2600	0.42
1a	146–174	7:3, 6:4	4400	0.36
4a	198–221	6:4	2200	0.29
3	222–275	6:4, 5:5	500	0.19

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