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POTENTIAL BIOGENETIC PRECURSORS OF WITHANOLIDES FROM *WITHANIA COAGULANS*

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Key Word Index—*Withania coagulans*; Solanaceae; withanolides; steroidal lactones; ergostene derivative.

Abstract—The structure of two new compounds, a withanolide and an ergostene derivative, are described and identified as 3β,14α,17β,20α$_F$-tetrahydroxy-1-oxo-20*S*,22*R*-witha-5,24-dienolide and ergosta-5,25-diene-3β,24ξ-diol. These are considered interesting intermediates in the biogenetic sequence leading to the formation of withanolides, the ergostene diol being a very early precursor.

INTRODUCTION

Within the framework of a collaborative effort oriented towards the study of the constituents of the fruits of *Withania coagulans* Dunal., a number of compounds were isolated, three of which were identified as sitosterol, 3β-hydroxy-2,3-dihydro-withanolide F (**1a**), and ergosta-5,25-diene-3β,24ξ-diol (**3**). The latter two, which are new compounds, can be considered as intermediate products in the biosynthetic sequence which produces the withanolides.

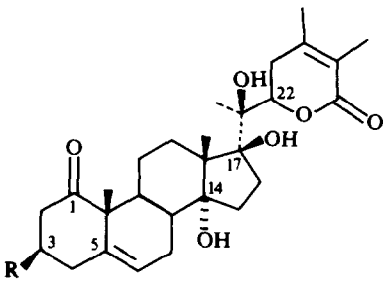
RESULTS

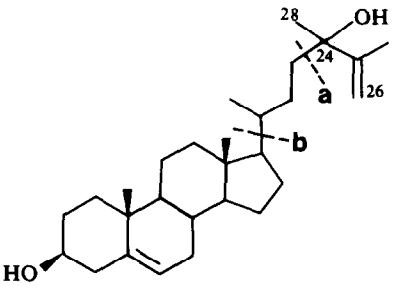
Compound **1a**, $C_{28}H_{40}O_7$, showed in the UV spectrum an absorption at λ_{max} 224 nm (ε 10,800). This value and its intensity are characteristic for a single α,β-unsaturated carbonyl chromophore. The IR spectrum indicated a six-membered ring ketone, an unsaturated δ-lactone and a double bond (1700, 1670 and 1650 cm^{-1}). The ^{1}H NMR spectrum (Table 1) was characteristic in a general way for the steroidal structure of the withanolide class [1] and more specifically for the withanolide E group, i.e. having an α-oriented side chain [2, 3]. Indeed, the 21-Me signal is a singlet at δ 1.39, the 18-Me is at 1.09 and the 22-H shows a double-doublet at 4.88, all values which are close to withanolide E and certain related compounds. Interestingly, in the low field region of the spectrum, no signals could be observed for the usual 2-en-1-one system of

Table 1. ^{1}H NMR spectral signals of relevant protons in compounds **1–3**

Compound	2-H	3-H	4-H	6-H	22-H	26-H	Methyl groups 18	19	21	27	28
1a	—	3.92 (*br octet*)	—	5.63 (*m**7)	4.88 (*dd* 9, 7)	—	1.09 (*s*)	1.26 (*s*)	1.39 (*s*)	1.86 (*s*)	1.94 (*s*)
1b	—	5.03 (*br octet*)	—	5.67 (*m**7)	4.89 (*dd* 9, 7)	—	1.12 (*s*)	1.24 (s)	1.43 (s)	1.88 (s)	1.94 (s)
2a	5.85 (*dd* 10, 2)	6.76 (*ddd* 10, 5, 2)	2.82 (*dd* 19, 6) 3.27 (*br d* 19)	5.60 (*m**7)	4.93 (*dd*, 9, 7)	—	1.13 (*s*)	1.23 (*s*)	1.42 (*s*)	1.88 (*s*)	1.94 (*s*)
2b	—	5.68 (*br d* 10)	6.05 (*br d* 10)	5.70 (*m**10)	4.91 (*dd* 9, 7)	—	1.13 (*s*)	1.37 (*s*)	1.43 (*s*)	1.87 (*s*)	1.93 (*s*)
3	—	3.29	—	5.34 (*d* 5)	—	4.81 (*s**5) 4.95 (*s**4)	0.67 (*s*)	1.00 (*s*)	0.92 (*d* 6.6)	1.73 (*s*)	1.30 (*s*)
	—	[3.85]	—	[5.43]	—	[5.44] [5.75]	[0.67]	[1.06]	[1.02]	[1.94]	[1.56]

Chemical shifts are as δ values; coupling constants (in Hz) are in parentheses; data measured in C_5D_5N are in square brackets; *for $W_{1/2}$ values.





1a R = OH

1b R = OAc

2a Δ^2 R = H

2b Δ^3 R = H

3

ring A of the withanolides, and only one olefinic proton was present at δ 5.63, which was assigned to 6-H for a Δ^5-bond. A broad octet at δ 3.92 was related to a proton (3α-H) adjacent to the 3β-OH and was characteristic for such a group. All these data together with the location of the 19-Me (δ 1.26) were in close agreement with those of a semi-synthetic derivative, obtained from previous work, possessing a 1-oxo-3β-OH-Δ^5 system [4], and therefore structure **1a** was assigned to this compound. Upon acetylation, a mono-3β-acetate (**1b**) was obtained, in which the proton geminal to the acetoxy group had moved, as expected, to δ 5.03.

The ^{13}C NMR data of compounds **1a** and **1b** are given in Table 2. All signals for the carbon atoms of rings C, D and the side chain have values similar to those of withanolide E and its derivatives [5]. The outstanding values, however, are for the specific substitutions of ring A in **1a** and **1b** respectively; they are related to a C-1 regular carbonyl, to the C-3 to which an alcohol (or an acetate) is attached, and to the C-5 and C-6 of the Δ^5-bond. It is noteworthy that when converting the 3-OH to 3-OAc, the C-2 and C-4 became more shielded and showed upfield shifts of 4 and 2 ppm, respectively.

Further confirmation for the structure was adduced by mass spectrometry. As for withanolide E, the molecular ion was absent, but fragments corresponding to the loss of 1–4 times H_2O were present. The cleavage of the C(20)–C(22) bond was observed through fragments indicating the loss of $C_7H_9O_2$, together with H_2O; the cleavage of the C(17)–C(20) bond resulting in loss of $C_9H_{13}O_3$, and again minus H_2O, was observed as well. All these are characteristic fragmentations of the withanolide class of compounds.

Since compound **1a** was considered an intermediate in the biogenesis of the withanolides, it was further subjected to a biomimetic reaction, namely the elimination of the 3β-OH, in order to produce the characteristic 2-en-1-one system of ring A of the withanolides. To this end, elimination of the 3-acetoxy group in **1b** was carried out with alkali in dioxane, as described earlier with a semi-synthetic model [4]. In the present case, withanolide F (**2a**) was obtained as a major product and was found to be in all respects identical with an authentic sample. This compound was obtained together with its Δ^3-isomer, namely Δ^3-isowithanolide F (**2b**). The ^{1}H NMR and ^{13}C NMR spectra of compound **2b** show the characteristic signals for the protons at C-3, C-4 and C-6, and for C-3 to C-6 reported under different circumstances [5, 6].

Table 2. ^{13}C NMR spectral data of compounds **1a**, **1b** and **2b**

Carbon	1a	1b	2b	Carbon	1a	1b	2b
1	209.7	211.3	210.5	16	37.1	37.1	37.9
2	47.6	43.6	39.7	17	87.9	88.1	87.9
3	68.6	70.2	127.9	18	20.6	20.6	20.6
4	40.0	38.0	129.5	19	18.4	17.3	20.5
5	135.4	134.9	140.5	20	78.7	79.2	79.1
6	125.9	126.3	121.2	21	19.1	20.0	19.9
7	25.9	26.0	25.9	22	81.5	80.0	79.8
8	36.2	36.1	33.9	23	32.5	32.4	32.4
9	35.9	36.1	36.1	24	152.3	150.4	150.4
10	53.1	53.0	52.3	25	121.4	121.7	121.5
11	22.2	22.0	21.8	26	166.0	165.8	165.9
12	34.6	34.3	34.2	27	12.4	12.4	12.4
13	54.1	54.9	53.8	28	20.7	20.6	20.6
14	82.5	82.0	83.1	$\underline{C}H_3$-CO	—	21.1	—
15	30.4	30.4	30.1	CH_3-$\underline{C}O$	—	170.1	—

Compound **3** was found to be a sterol, $C_{28}H_{46}O_2$, showing in the ^{1}H NMR the characteristic values for a 3β-hydroxy-5-ene system (Table 1). Two low-field, sharp one-proton signals (δ 4.81 and 4.95), together with a signal at 1.73 for a methyl group on a double bond, were assigned to the terminal methylene group shown in structure **3**. By comparing the values of the two methylenic protons, which are 0.14 ppm apart, with those of similar systems [7] in 2-methyl-2-propenol (δ 4.8 and 4.9) and in 3-methyl-3-butenol (δ 4.75 for two protons), it was deduced that in the present case a hydroxy group could be adjacent as in the first example, and therefore located at C-24. Indeed, the 28-Me attached at C-24 (as in ergostane) appeared as a singlet at δ 1.30, forming a side chain as drawn in **3**. This assumption was strongly supported by measuring the ^{1}H NMR spectral shifts in C_5D_5N for this compound. Due to the collision complex formed with the 24-OH, the 27 and 28-Me exhibited a shift of $\Delta^{CDCl_3}_{C_5D_5N}$ 0.21 and 0.26 ppm, respectively, whereas an even stronger shift was measured for the 26-methylenic protons (0.63 and 0.80 ppm). All these data indicate the proximity of the tertiary hydroxy group to the methylene group, which was therefore placed at C-25.

Further support for the side chain as drawn in **3** was adduced through the high-resolution mass spectrum. Two cleavages *a* and *b* could be well visualized. The first one provided C_5H_9O (85) fraction of the molecule which gave the 100% peak. Also present were M − 85 = 329 and M − 18 − 85. The fraction C_4H_8 (56) for cleavage *b* following *a* was also well represented. All peaks for the carbocyclic skeleton minus cleavage *a* and *b* with loss of the elements of water were present, as described in the Experimental.

DISCUSSION

In a series of preceding papers [8–10] dealing with the study of withanolides in different hybrids obtained by selected cross-breeding, in addition to genetic studies at the chemical level, it was possible to account for biogenetic pathways based on the structure of the different components fitting into the proposed sequence [4, 11]. Generally speaking, studies on additional chemotypes or species may contribute and support the ideas presented at different occasions. For example, it has been previously suggested that ring A is produced following a sequence **a**–**d** shown in Scheme 1. Presently, the study of *Withania coagulans* and the isolation of compound **1a** from natural sources having the substitution shown in **c** and its conversion to **d** provide now the complete pattern, and thereby all the combinations shown in **a** to **d** have now been actually isolated from different withanolide-producing plants.

Along the same line, the identification of the sterol **3** is of importance and may provide additional information and the clue to the way whereby the lactonic side chain is formed in the plant. 24-Methylenecholesterol (**a** in Scheme 2) has been shown to be a precursor and at the origin of the δ-lactone side chain of the withanolide [12]. It is now proposed that compound **3** (**b** in Scheme 2) is actually one of the intermediate steps leading to the lactone. In such a case, **b** should undergo 22-hydroxylation to **c**, a well-known reaction in the steroidal pathway in nature [13]. From this point two alternative hypotheses may be considered as shown in the scheme: one going through cyclization (**d**) and oxidation (**e**); and the second through oxidation (**g**) of the allylic isomer (**f**), followed by cyclization to **e**. The latter compound, a lactol, has been found to be present in a number of withanolides [14–17]. Ultimate oxidation will lead to the δ-lactone **h**.

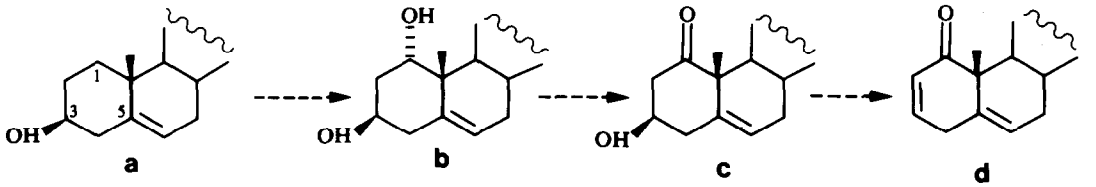
EXPERIMENTAL

Mps were measured on a Fischer–Johns apparatus and are uncorr. Optical rotations were determined in $CHCl_3$ solns. IR spectra were recorded with KBr pellets; UV spectra were recorded for EtOH solns; ^{1}H NMR and ^{13}C NMR spectra were determined on a Bruker WH 270 and WH 90 (22.63 MHz) instruments respectively, for solns in $CDCl_3$, containing TMS as internal standard. Liquid column chromatography was done over Si gel H or G 60 and over Al_2O_3. Analytical TLC was carried out using chromatoplates (50 × 75 mm, Si gel F_{254}), and prep. TLC using chromatoplates, 200 × 200 × 2 mm, Si gel 60 F_{254}. Mass spectra were determined under the direction of Dr. Z. Zaretskii, with a Varian MAT 731 for HR, and an improved Atlas CH4 for LR.

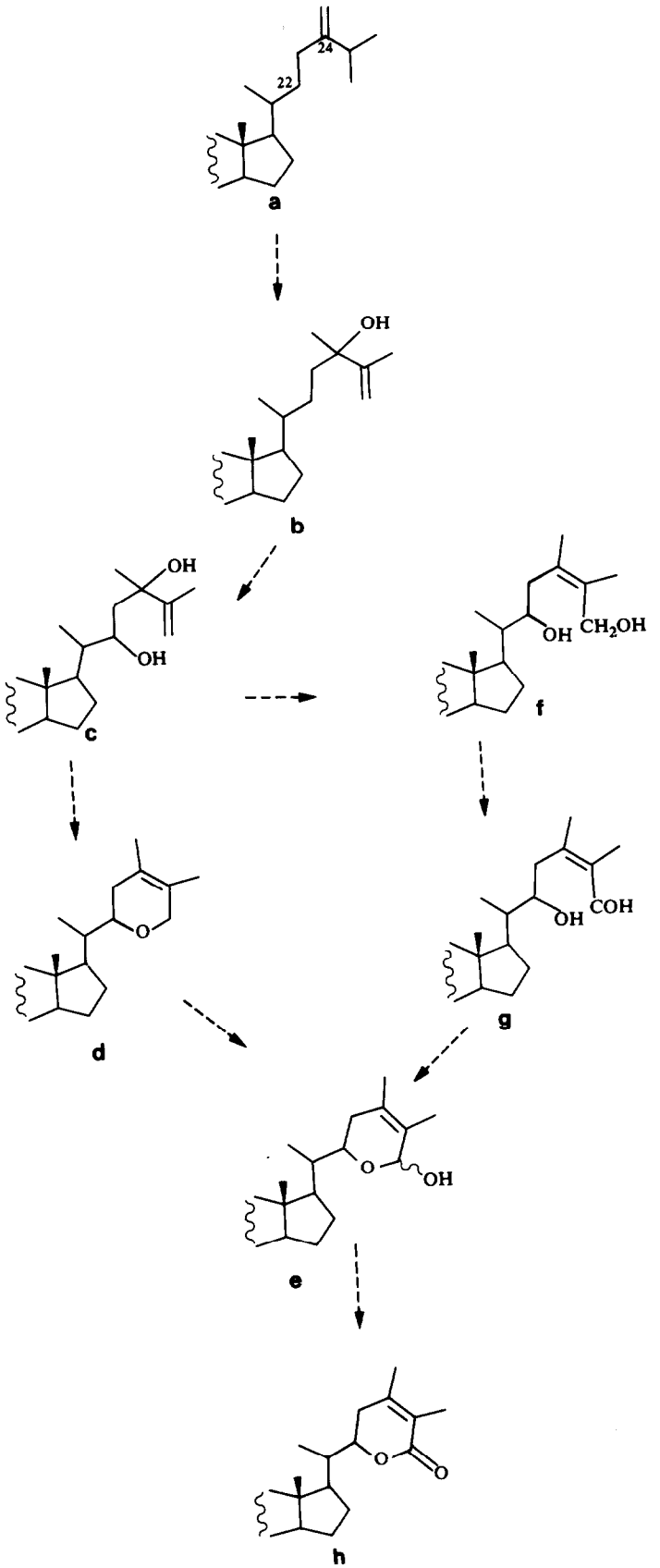
Isolation of compounds **1a** *and* **3**. Air-dried powdered fruits of *Withania coagulans* Dunal. (authenticated by the Director, Regional Research Laboratory, Jammu Tawi) were extracted with H_2O by repeated maceration; this fraction was extracted with $CHCl_3$, washed with H_2O, dried (Na_2SO_4), and evaporated at red. pres. The residue was chromatographed over Si gel H, using successively petrol (bp 60–80°), C_6H_6, $CHCl_3$, EtOAc and MeOH as eluants. The first three yielded fatty material, whereas from EtOAc a crystalline product was obtained, which was purified by repeated crystallizations from MeOH–Me_2CO to yield compound **1a**.

The petrol extract of the fruits was saponified and the unsaponifiable fraction extracted with Et_2O. The residue following evaporation was chromatographed over Al_2O_3 and eluted successively with petrol, C_6H_6, $CHCl_3$ and MeOH in various combinations. The fraction eluted with C_6H_6–$CHCl_3$ (1:1) gave compound **3**, which crystallized from $CHCl_3$. Further elution with the same solvent mixture gave a compound which crystallized from Me_2CO, mp 137–138°, identified as sitosterol by comparison with an authentic sample, similar ^{1}H NMR and mmp.

20S,22R-3β,14α,17β,20α_F-*Tetrahydroxy-1-oxo-witha-5,24-dienolide* (**1a**). Mp 260–261° (from MeOH–Me_2CO); $[\alpha]_D$ + 61.4° (*c* 0.2); UV λ_{max} nm: 224 (ε 10800); IR ν_{max} cm^{-1}: 3400, 1700,



Scheme 1.



Scheme 2.

1670 and 1650; MS (LR) *m/z* (rel. int.): 452 (3)[M − 2 × H_2O]$^+$, 434 [M − 3 × H_2O]$^+$ (4), 416 [M − 4 × H_2O]$^+$ (1), 345 [M − 125 − H_2O]$^+$ (9), 327 [M − 125 − H_2O]$^+$ (9), 309 [M − 125 − 3 × H_2O]$^+$ (6), 283 [M − 169 − 2 × H_2O]$^+$ (17), 125 [cleavage C20–C22]$^+$ (62), 169 [cleavage C17–C20]$^+$ (32). MS (HR) *m/z* (rel. int.): 434.2446 [$C_{28}H_{34}O_4$]$^+$ (10), 345.2065 [$C_{21}H_{29}O_4$]$^+$ (14), 327.1931 [$C_{21}H_{27}O_3$]$^+$ (16), 309.1847 [$C_{21}H_{25}O_2$]$^+$ (11), 301.1788 [$C_{19}H_{25}O_3$, M − 169 − H_2O]$^+$ (36), 283.1696 [$C_{19}H_{23}O_2$]$^+$ (23), 255.1774 [$C_{18}H_{23}O$, M − 169 − 2 × H_2O − CO]$^+$ (13), 241.1600 [$C_{17}H_{21}O$, M − 125 − 2 × H_2O—cleavage of ring A]$^+$ (14), 197.1318 [$C_{15}H_{17}$, M − 169 − 2 × H_2O—cleavage of ring A]$^+$ (21), 125.0595 [$C_7H_9O_2$]$^+$ (89), 169.0874 [$C_9H_{13}O_3$]$^+$ (42). (Found: C, 68.70; H, 8.16. $C_{28}H_{40}O_7$ requires: C, 68.85; H, 8.20%, MW 488.6.)

20S,22R-3β-*Acetoxy*-14α, 17β-20α$_F$-*trihydroxy*-1-*oxo*-*witha*-5,24-*dienolide* (**1b**). Acetylation was carried out with Ac_2O–pyridine at room temp. for 24 hr. Cryst. from EtOAc, mp 154–155°; $[\alpha]_D$ + 33° (*c* 0.15); UV λ_{max} nm: 224 (ε 16 000); IR ν_{max} cm^{-1}: 3400, 1735, 1705, 1670, 1650 and 1230; MS (LR) *m/z* (rel. int.): 512 [M − 18]$^+$ (1), 452 [M − 60 − 18]$^+$ (5), 434 [M − 60 − 2 × 18]$^+$ (2), 387 [M − 125 − 18]$^+$ (3), 369 [M − 125 − 2 × 18]$^+$ (2), 327 [M − 60 − 18 − 125]$^+$ (7), 309 [M − 60 − 125 − 2 × 18]$^+$ (12), 169 [cleavage of C17–C20, $C_9H_{13}O_3$]$^+$ (60), 125 [cleavage C20–C22, $C_7H_9O_2$]$^+$ (38). (Found: C, 67.98; H, 7.99. $C_{30}H_{42}O_8$ requires: C, 67.51; H, 7.82%. MW 530.6.)

20S,22R-14α,17β-20α$_F$-*Trihydroxy*-1-*oxo*-*witha*-2,5,24-*trienolide* (*withanolide F*) (**2a**). The acetate **1b** (35 mg) was treated for 15 min in 1 M KOH in dioxane and the reaction worked up as usual. A mixture of **2a** and **2b** was obtained which was resolved on chromatoplates. The lower spot (21 mg) was identified as withanolide F (**2a**), mp 191–192° (from EtOAc); UV λ_{max} nm: 226 (ε 17 000); IR ν_{max} cm^{-1}: 1680; undepressed mmp with an authentic sample. The second compound could not be induced to crystallize and was identified only through its ^{1}H NMR spectrum as **2b** (isowithanolide F).

3β,24ξ-*Dihydroxy*-*ergosta*-5,25-*dienolide* (**3**). Mp 208–209° (from $CHCl_3$); $[\alpha]_D$ + 68.9° (*c* 0.14); UV λ_{max} nm: 210 sh (ε 7600); IR ν_{max} cm^{-1}: 3300, 3080 ($R_2C{=}CH_2$ stretching), 1675, 1625 (C=C $\Delta^{5,25}$), and 895 (C–H out of plane deformation). MS (LR) *m/z* (rel. int.): 414 [M]$^+$ (2), 396 [M − 18]$^+$ (12), 329 (2), 311 (1), 255 (10), 85 (91), 56 [cleavage *a* and *b* C_4H_8]$^+$ (15), 57 [C_4H_8 + H]$^+$ (90). MS (HR) *m/z* (rel. int.): 414.3536 [M]$^+$ (1), 329.2848 [cleavage *a* $C_{23}H_{37}O$]$^+$ (3), 311.2749 [M − 18 − 85 $C_{23}H_{35}$]$^+$ (1), 255.2101 [M − 18 − 141 $C_{19}H_{27}$]$^+$ (12.5), 85.0671 [cleavage *a* C_5H_9O]$^+$ (100), 141.1245 [cleavage *b* $C_9H_{17}O$]$^+$ (1), $C_{28}H_{46}O_2$, MW 414.65.

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