Steroidal Constituents from *Withania somnifera* Root

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From the roots of *Withania somnifera* two new sterols, namely 3-epi-β-sitosterol and 20(21)-dehydrocampesterol, have been isolated along with β-sitosterol and campesterol. The structures of the new phytoconstituents have been established as stigmasta-5-en-3α-ol and (24R)-ergost-5,20(21)-dien-3β-ol.

Withania somnifera Dunal (solanaceae) is an erect, evergreen, perennial shrub, which is reported to have adaptogenic, tonic, analgesic, antipyretic, antiinflammatory, and abortifacient properties[1-3]. This plant is a source of various withanolides[4]. The present paper describes the isolation and characterization of two new sterols from the root of the plant.

W. somnifera roots were procured from a local market of Delhi and authenticated in the Botany Department of this University. The roots of *W. somnifera* were dried in an oven at a temperature below 45° for 2-3 days and coarsely powdered. The ground roots (3.9 kg) were extracted exhaustively with ethanol (95%) in a Soxhlet apparatus. The ethanolic extract obtained was concentrated to yield a dark brown viscous mass (355 g). The extract was analyzed chemically for determining the presence of different chemical constituents.

The non-alkaloidal portion was dissolved in ethanol (95%), made neutral, dried and concentrated on a water bath. The viscous dark brown mass was adsorbed on silica gel (60-80 mesh) for preparation of slurry. It was dried and loaded on silica gel column packed in petroleum ether. The column was eluted with petroleum ether, chloroform and methanol in the order of increasing polarity to isolate the following compounds:

Fractions eluted with $CHCl_3$ furnished as colourless pearl white crystals of compound 1, purified by preparative TLC ($CHCl_3$: MeOH, 9.5:0.5), recrystallised from $CHCl_3$: MeOH (1:4), 62 mg (0.00138%); MP 119-120°; R_f 0.409 ($CHCl_3$:MeOH, 9.5:0.5); $[\alpha]_D^{22}$ -28.9° ($CHCl_3$, 0.1); UV λ_{max} (MeOH) 204 nm (log ∈ 3.7); IR ν_{max} (Nujol) 3410, 2945, 2850, 1610, 1465, 1380, 1140, 1055, 960, 840, 725 cm^{-1}; ^1H NMR: Table 1; ^{13}C NMR: Table 1; EIMS *m/z* (rel. int.) 414 $[M]^+$ ($C_{29}H_{50}O$) (43.0), 399 (36.1), 396 (18.5), 273 (25.7), 255 (45.1), 213 (40.0), 159 (34.3), 145 942.8), 133 (39.2), 119 (29.5), 105 (44.7), 95 (63.0), 83 (22.6), 81 (80.2), 69 (63.3), 67 (37.9), 55 (100).

To compound 1 (15 mg) a mixture of acetic anhydride (5 ml) and pyridine (2 ml) was added and heated on a steam bath for three hours. The reaction mixture was poured in ice-cold water (15 ml) and extracted with chloroform (3×10 ml). The organic phase was washed with water (3×10 ml), dried (Na_2SO_4) and evaporated to obtain monoacetyl product, MP 110-111°, n_{max} 1725 cm^{-1}.

Compound 1 (20 mg) was dissolved in Me_2CO (20 ml) and treated with freshly prepared Jones reagent (5 ml). The reaction mixture was stirred at room temperature till the reaction was completed (TLC monitoring). It was diluted with water and extracted with diethyl ether. The ether layer was dried (Na_2SO_4) and evaporated to yield 3-oxo derivative, MP 102-103°, ν_{max} 1705 cm^{-1}.

The 3-oxo compound was dissolved in methanol (5 ml) and 1 mg of $NaBH_4$ was added in portions with stirring. After dilution with water, the mixture was extracted with chloroform. The chloroform layer was washed with water, dried (Na_2SO_4) and evaporated to giveβ-sitosterol (2), MP 138-139° C.

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Further elution of column with $CHCl_3$ furnished colourless amorphous powder of compound 2, purified by preparative TLC ($CHCl_3$: MeOH, 9.5:0.5), recrystallised from $CHCl_3$:MeOH (1:4), 145 mg (0.003%); MP and mixed MP 138-140°; $[\alpha]_D^{22}$ -36.8° ($CHCl_3$, 0.1); IR ν_{max} (Nujol) 3410; ^1H NMR: δ 5.34 (1H, d, *J* = 5.2 Hz, H-6); 3.52 (1H, br m, $w_{1/2}$ 18.6 Hz, H-3α), 1.00, 0.91, 0.85, 0.82, 0.78, 0.67 (methyls); ^{13}C NMR: δ 144.76 (C-5), 121.62 (C-6), 71.75 (C-3); EIMS *m/z* (rel. int.) 414 $[M]^+$ ($C_{29}H_{50}O$) (43.0).

Fractions eluted with $CHCl_3$:MeOH (99:1) furnished colourless as pearl white crystals of compound 3, purified by preparative TLC ($CHCl_3$:MeOH, 95.5:0.5), recrystallised from $CHCl_3$:MeOH (1:1), 62 mg (0.00138%); MP 119-120°; R_f 0.25 ($CHCl_3$: MeOH, 9.5:0.7); $[a]_D^{22}$ -35.6° ($CHCl_3$, 0.1); IR n_{max} (Nujol) 3435, 2955, 2850, 1620, 1455, 1380, 1160, 1055, 960, 840, 725 cm^{-1}; ^1H NMR: d 5.35 (1H, d, *J* = 5.08 Hz, H-6); 5.12 (1H, d, *J* = 8.64 Hz, H-21a), 5.00 (1H, d, *J* = 5.46 Hz, H-21b), 3.52 (1H, br m, $w_{1/2}$ 15.60 Hz, H-3b), 1.01 (3H, br s, Me-19), 0.90 (3H, d, *J* = 4.26 Hz, Me-28), 0.84 (3H, d, *J* = 6.78 Hz, Me-26), 0.82 (3H, d, *J* = 6.70 Hz, Me-27), 0.67 (3H, br s, Me-18); ^{13}C NMR: Table 1; EIMS *m/z* (rel. int.) 398 $[M]^+$ ($C_{28}H_{46}O$) (58.4), 383 (64.9), 380 (10.4), 368 (9.9), 297 (10.2), 255 (19.1), 236 (29.0), 213 (29.0), 177 (10.1), 159 (15.3), 147 (24.5), 120 (10.1), 109 (27.2), 106 (27.8), 95 (51.3), 83 (59.2), 69 (71.1), 57 (87.6), 43 (100).

Fractions eluted with $CHCl_3$:MeOH (98:2) furnished colorless amorphous of compound 4, purified by preparative TLC ($CHCl_3$:MeOH, 95.5:0.5), recrystallized from $CHCl_3$:MeOH (1:3), 36 mg (0.0009%); MP 152-158°; $[a]_D^{22}$-35.6° ($CHCl_3$, 0.1); IR n_{max} (Nujol) 3435, 2955, 2850, 1620, 1455, 1380, 1160, 1055, 960, 840, 725 cm^{-1}; ^1H NMR: d 5.34 (1H, d, *J* = 5.05 Hz, H-6), 3.52 (1H, br m, $w_{1/2}$ 15.60 Hz, H-3a), 1.00 (3H, br s, Me-19), 0.91 (3H, d, *J* = 6.50 Hz, Me-21), 0.90 (3H, d, *J* = 6.00 Hz, Me-28), 0.84 (3H, d, *J* = 6.20 Hz, Me-26), 0.82 (3H, d, *J* = 6.52 Hz, Me-27), 0.67 (3H, br s, Me-18), ^{13}C NMR: Table 1; EIMS *m/z* (rel. int.) 400 $[M]^+$ ($C_{28}H_{48}O$) (14.63), 314 (18.9), 271 (16.1), 255 (14.0), 159 (26.9), 145 (36.7), 122 (27.7), 107 (40.9), 95 (50.0), 81 (58.8), 69 (51.0).

Compound 1, 3-epi-β-sitosterol, was obtained as a colourless amorphous powder from chloroform eluents. The compound responded positively to Libermann-Burchard test for steroids. Its IR spectrum showed absorption bands for hydroxyl group (3410 cm^{-1}) and unsaturation (1610 cm^{-1}). Its Mass spectrum had a molecular ion peak at *m/z* 414 corresponding to a steroidal formula, $C_{29}H_{50}O$. It indicated five double bond equivalents, four of them were adjusted in the steroidal carbon skeleton and one in olefinic linkage. The important other diagnostic peaks were at *m/z* 399 $[M\text{-}Me]^+$, 396 $[M\text{-}H_2O]^+$, 273 [M-side chain]$^+$, 255 $[273\text{-}H_2O]^+$ and 213 [255-ring D fission]$^+$. These fragments suggested that it was a C_{29} sterol possessing one double bond and a C_{10} saturated side chain. The fragments at *m/z* 55 $[C_{1,10}\text{-}C_{4,5}$ fission-$H_2O]^+$, 69 $[C_{2,3}\text{-}C_{5,10}\text{-}C_{6,7}$ fission]$^+$ and 83 $[C_{2,3}\text{-}C_{5,10}\text{-}C_{7,8}$ fission]$^+$ indicated that the hydroxyl group was located in ring A which was placed at C-3 on biogenetic grounds. The mass spectrum indicated the presence of an ethyl group in the side chain, which was placed at C-24 on the basis of biological analogy, as well as similarities in chemical shifts of the protons and carbons of the side chain with related compounds[5,10]. Therefore, the compound 1 has identical basic carbon framework as sitosterol. However, the compound as well as their derivatives differed widely in melting points, suggesting that they were isomers.

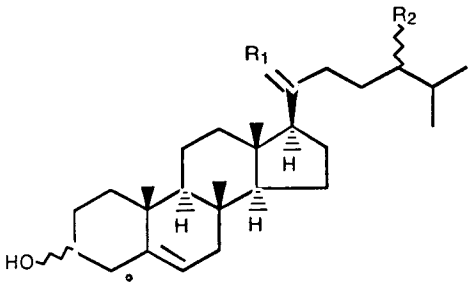
The ^1H NMR spectrum of compound 1 exhibited a one-proton doublet at δ 5.34 (*J* = 5.0 Hz) assigned to H-6 proton. A multiplet at δ 3.51 with $w_{1/2}$ 13.35 Hz showed the presence of 3β-methine protons (equatorial) interacting with C-2 equatorial, C-2 axial, C-4 axial, C-4 equatorial protons. Four doublets, integrating three protons each, at δ 0.92 (*J* = 6.32 Hz), 0.80 (*J* = 6.60 Hz), 0.82 (*J* = 6.00 Hz) and 0.84 (*J* = 7.32 Hz) were due to C-21, C-26, C-27 secondary and C-29 primary methyls, respectively. The remaining two tertiary C-18 and C-19 methyl protons resonated as singlets at d 0.69 and 1.00. A broad multiplet δ at 1.46 was assigned to 17α proton. The appearance of all the methyls in the region of d 0.69 and 1.00 suggested that these functionalities were attached to saturated carbons. The remaining methylene and methine groups appeared in the region δ 2.28-1.08 Table 1.

Further evidence for the structure of compound 1 was provided by its ^{13}C NMR spectral data, which showed the evidence of 29 carbon atoms in the molecule. Signals at δ 140.75, 121.71 and 71.80 were assigned to C-5, C-6 unsaturated carbons and C-3 carbinol carbon, respectively. The β-configuration of the ethyl group was confirmed by comparison of chemical shifts of carbons and protons of the side chain in ^1H and ^{13}C NMR spectra of compound 1 with a series of sterols having similar configuration at C-24, particularly stigmast-5-en-3-one, stigmas-5-en-6β-ol-3-one[5] and lawsaritol[6]. The H_3-29

TABLE 1: ^{1}H NMR SPECTRAL DATA OF 1 AND ^{13}C NMR CHEMICAL SHIFTS OF COMPOUNDS 1,3 AND 4 ($CDCl_3$)

POSITION	^{1}H NMR (300 MHz)		^{13}C NMR (100 MHz)		
	α	β	1.	3.	4.
1.	1.25 dddd (16.10, 9.76, 5.34, 5.06)	2.28 dddd (12.16, 5.84, 5.06, 11.34)	37.25	37.25	37.06
2.	1.865 m	1.83 m	30.27	31.69	31.52
3.	3.51 oct (J= 4.58, 6.28, 4.76, 4.95, 6.12, 4.58, 11.07, 0) ($w_{1/2}$ 13. 35)	-	71.80	71.82	71.76
4.	2.26 d (5.2)	1.99 (10.32)	40.49	39.81	39.10
5.	-	-	140.75	140.77	140.60
6.	5.34 d (5.0)	-	121.71	121.74	121.78
7.	1.64 m	1.64 m	31.89	31.95	31.91
8.	-	1.58 m	31.65	29.74	29.22
9.	1.49 m	-	50.12	50.17	50.44
10.	-	-	36.50	36.55	36.08
11.	2.02 m	1.47 m	36.14	33.76	33.71
12.	1.10 m	1.85 m	21.07	21.12	21.11
13.	-	-	42.29	42.34	42.22
14.	1.16 m	-	56.76	56.81	56.71
15.	1.08 m	1.47 m	24.30	24.34	24.20
16.	1.57 m	1.51 m	28.24	29.74	28.36
17.	1.46 m	-	56.05	56.01	56.14
18.	0.69 s	-	11.97	11.90	11.06
19.	1.00 s	-	19.39	19.43	19.41
20.	-	2.00 m	35.87	128.31	35.83
21.	0.92 d (6.32)	-	18.25	98.99	16.35
22.	1.54 m	1.08 m	33.69	33.95	33.71
23.	1.25 m	1.83 m	26.06	26.22	26.14
24.	1.16 m	1.57 m	45.82	42.34	42.22
25.	1.56 m	-	29.13	29.06	30.68
26.	0.80 d* (6.60)	-	20.19*	19.43	21.08
27.	0.82 d* (6.00)	-	19.01*	19.06	18.96
28.	1.16 m	1.64 m	23.06	20.16	21.20
29.	0.84 d (7.32)	-	11.85	-	-

*The chemical shifts of the methyl protons and carbon signals in a vertical column may be interchanged with the same superscripts. Coupling constants in Hertz are given in parenthesis.



1. R_1 = α-Me, H; R_2 = (24 R)-Et; 3α-OH
2. R_1 = α-Me, H; R_2 = Et; 3β-OH
3. R_1 = CH_2; R_2 = (24 R)-Me; 3β-OH
4. R_1 = α-Me, H; R_2 = (24 R)-Me; 3β-OH

resonance of 24-R configuration (d 0.84) is more upshielded as compared to 24 S resonance (d 0.86)[7].

Conclusive evidence for the structure of compound 1 was derived from the results of chemical reactions. Acetylation of compound 1 with acetic anhydride-pyridine yielded a monoacetyl derivative (γ max 1725 cm^{-1}). Oxidation of compound 1 with Jones reagent formed 3-oxo product (γ max 1705 cm^{-1}), which responded positive to Zimmermann test[11] confirming the location of 3-hydroxyl group at C-3 in compound 1. The sodium borohydride reduction of compound 1 regenerated β-sitosterol (2), indicating the equatorial orientation of the hydroxyl group in compound 1. Based on these evidences the structure of compound 1 was formulated as (24R) stigmasta-5-en-3α-ol. This novel compound is a new natural product and constitutes the first report of occurrence of stigmasta-5-ene type compound in *Withania somnifera*. Usually β-sitosterol is of common occurrence in these plants.

Compound 2, β-sitosterol, obtained from chloroform eluents, was identified as stigmasta-5-en-3β-ol on the basis of melting and mixed melting points, spectral data analysis and chemical reactions.

Compound 3, 20(21)-dehydrocampesterol, an amorphous powder from chloroform-methanol (99:1) eluents, showed positive Lieberman-Burchard test, and IR absorption bands for hydroxyl group (3435 cm^{-1}) and unsaturation (1620 cm^{-1}). Its mass spectrum displayed a molecular ion peak at m/z 398 ($C_{28}H_{46}O$) and other important ion peak at m/z 383 $[M\text{-}Me]^+$, 380 $[M\text{-}H_2O]^+$, 255 $[380\text{-}C_9H_{17}$, side chain$]^+$, 213 [255-ring D fission]$^+$, 83 $[C_{2,3}\text{-}C_{5,10}\text{-}C_{7,8}$ fission$]^+$, 69 [83- CH_2]$^+$, 106 $[C_{9,10}\text{-}C_{6,7}$ fission-$H_2O]^+$ and 120 $[C_{9,10}\text{-}C_{7,8}$ fission-$H_2O]^+$ indicating the presence of hydroxyl group in ring A which was placed at C-3 on biogenetic consideration and a C_9-unsaturated side chain. The ^1H NMR spectrum of 3 showed three one-proton deshielded doublets at δ 5.35 (J = 5.08 Hz), 5.12 (J = 8.64 Hz) and 5.00 (J = 8.46 Hz) assigned to H-6, H-21a and H-21b, respectively, a one proton broad multiplet at δ 3.52 ($w_{1/2}$ = 15.6 Hz) ascribed to C-3a-carbinol proton, two tertiary methyl signals at d 1.01 (Me- 19) and 0.67 (Me- 18) and three secondary methyl doublets at d 0.90 (J = 4.26 Hz, Me- 28), 0.84 (J = 6.78 Hz, Me- 26) and 0.86 (J = 6.70 Hz, Me- 27), all attached to saturated carbons. The absence of signal near δ 0.96 in the ^1H NMR spectrum and near d 18.2 in the ^{13}C NMR spectrum for C-21 methyl group supported the existence of olefinic linkage at C-20(21). The ^{13}C NMR signals at δ 140.60, 121.78, 128.31 and 98.99 were accounted for unsaturated C-5, C-6, C-20 and C-21 carbons, respectively. Based on these evidences, the structure of 3 was formulated as (24R)-ergost-5,20(21)-dien-3β-ol. This is a new member of steroidal compound.

Compound 4, campesterol, obtained from chloroform-methanol eluents (98:2), was identified as (24R)-ergost-5-en-3β-ol.

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