New Withanolides from Fresh Berries of *Withania somnifera*

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The chloroform extract of the fresh berries of *Withania somnifera* has been investigated to afford stigmasterol, its glucoside, withanone, 27-hydroxy withanolide A along with two new withanolides, namely, iso-withanone (**1**) and $6\alpha,7\alpha$-epoxy-$1\alpha,3\beta,5\alpha$-trihydroxy-witha-24-enolide (**2**). Compound **1** possesses an uncommon 17α-oriented side chain whereas compound **2** has $1\alpha,3\beta$-dihydroxy group which is a molecule of biogenetic significance for withasteroids.

Key words: Solanaceae, *Withania somnifera*, Steroids, Withasteroids, Ashwagandha, Berries

Introduction

Withania somnifera L. Dunal (Solanaceae) is an evergreen perennial herb distributed throughout northern and southern regions of India and have been reported to be invariably useful constituent of various Indian Ayurvedic prescriptions and herbal preparations [1, 2]. In Ayurvedic system of Indian medicine, the fruits of *Withania somnifera* are used as an emetic, sedative, diuretic and for the treatment of asthma, atherosclerosis, *etc.* [3 – 5]. The seeds and fruits of *W. somnifera* have earlier been investigated to isolate 7α-hydroxy withanolide [5], some withanolide glycosides [3] and 17α- and 17β-withanolides [6].

In continuation of our interest on the chemical investigations of Indian medicinal and aromatic plants [7 – 10], we have recently reported the presence of unusually sulfated and oxygenated withanolides from the leaves of *Withania somnifera* [11].

In the present communication, we report the isolation of four withanolides out of which two are new. The structures of known compounds were ascertained by comparing their spectral data with those reported in the literature as well as co-TLC with authentic samples available with us while the structures of the new withanolides were elucidated by spectroscopic methods and chemical transformations which is discussed in this paper.

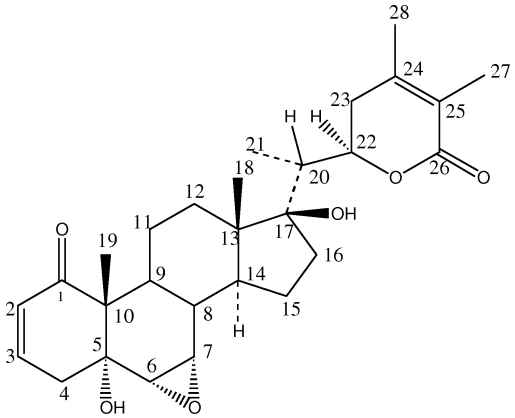
Materials and Methods

Plant material

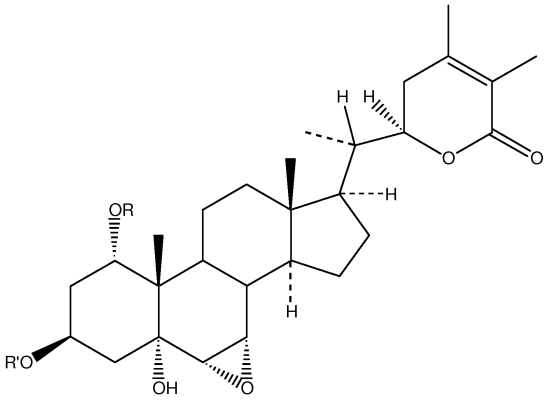
The fresh berries (650 g) of *Withania somnifera* were collected from the experimental farm of our institute in May 2004 where plant material after identification is maintained and propagated by us. The accession is deposited at National Gene Bank (No. RS-NMITLI-IIA) at CIMAP, Lucknow for field conservation and maintenance.

General experimental procedures

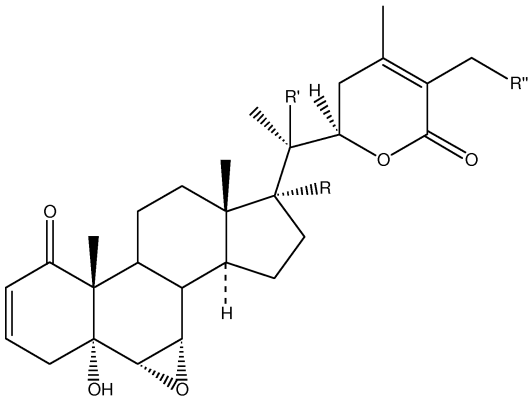
Instrumentation has been followed as described in our earlier publication [11]. The fresh plant material (650 g) after grinding in the presence of liquid nitrogen was extracted three times with 25% methanol (3×450 ml) by keeping at r. t. overnight. The extracts were pooled together and concentrated on rotavapor at 40 °C. The concentrated extract was defatted with *n*-hexane (3×500 ml) then was extracted with chloroform (3×500 ml). The chloroform extract was dried (1.25 g) and adsorbed on silica gel. The column chromatography over Si gel was done in *n*-hexane-EtOAc as mobile phase with increasing order of polarity by adding EtOAc stepwise, affording 90 fractions (150 ml each). Frs. 1 to 11 (*n*-hexane-EtOAc, 3 : 1 v/v) were rich in fatty acids and therefore, not processed further. Frs. 12 to 27 (*n*-hexane-EtOAc, 3 : 2 v/v) on crystallization yielded stigmasterol (19 mg). Frs. 28 to 30 (*n*-hexane-EtOAc, 1 : 1 v/v) on re-crystallization yielded **1** (TLC, $CHCl_3$-EtOAc-MeOH-C_6H_6 70 : 2 : 4 : 24 v/v, R_f 0.62, 11 mg) while frs. 31 to 34 (*n*-hexane-EtOAc, 1 : 1 v/v) also on re-crystallization afforded withanone (**3**,



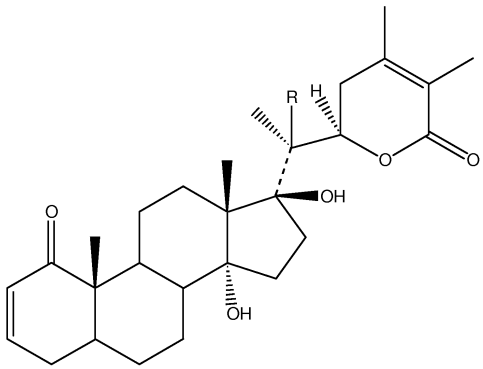
1



2, R= R'= H
2a, R= R'= Ac



3, R= OH, R'= R"=H
4, R'= R"= OH, R= H



5, R= OH, 5,6b-epoxy
6, R= H, 5,6-didehydro

27 mg). Frs. 35 to 39 (*n*-hexane-EtOAc, 1 : 1 v/v) on recrystallization afforded 27 hydroxy withanolide A (**4**, 21 mg) while frs. 40 to 45 were discarded as they were complex mixture. Similarly, frs. 46 to 50 (*n*-hexane-EtOAc, 2 : 3 v/v) on crystallization (EtOAc) afforded **2** (TLC, $CHCl_3$-EtOAc-MeOH-C_6H_6, 70 : 2 : 8 : 20 v/v, R_f 0.52, 52 mg) whereas frs. 51 to 61 (*n*-hexane-EtOAc, 3 : 7 v/v) on further purification yielded stigmasterol glucoside (16 mg). Frs. 62 to 90 (EtOAc) were discarded as these were an inseparable complex mixture.

*5α,17β-dihydroxy-6α,7α-epoxy-1-oxo-witha-2,24-dienolide (**1**)*

Crystals (EtOAc) M. p. 198 °C. – $[\alpha]_D^{30} + 80.6°$ ($CHCl_3$; 0.25). – IR (KBr) $\nu = 3465$, 2990, 2950, 2900, 1710, 1680, 1130 cm^{-1}. – MS (HR, 70 eV): *m/z* (%) = 470.6028 $[M]^+$ calcd. for $[M(C_{28}H_{38}O_6)] = 470.6022$. – MS (FAB, 70 eV): *m/z* (%) = 471 (5) $[M+1]^+$, 452 (4) $[M- H_2O]^+$, 434 (2) $[M-2H_2O]^+$, 348 (5), 273 (10), 262 (50), 185 (30), 125 (100), 77 (20). – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.87$ (1H, d, $J =$ 10.0 Hz, 2-H), 6.60 (1H, m, 3-H), 2.85, 2.45 (1H each, dd, $J = 10.0$, 4.5 Hz, 4-H), 3.13 (1H, br s, OH at 5-C), 3.07 (1H, d, $J = 3.0$ Hz, 6-H), 3.32 (1H, dd, $J = 3.0$, 2.0 Hz, 7-H), 0.98 (3H, *s*, 18-H), 1.21 (3H, s, 19-H), 1.13 (3H, d, $J = 7.0$ Hz, 21-H), 4.73 (1H, dt, $J = 9.0$, 5.5, 3.0 Hz, 22-H), 2.58 (2H, m, 23-H), 1.88 (3H, s, 27-H), 1.93 (3H, s, 28-H). – ^{13}C NMR: see Table 1.

*6α,7α-epoxy-1α,3β,5α-trihydroxy-witha-24-enolide (**2**)*

Shining crystals (EtOAc) M. p. 217 – 219 °C. – $[\alpha]_D^{30} +$ 86.00° ($CHCl_3$; 0.25). – IR (KBr) $\nu = 3490$, 2995, 2940, 2880, 1710, 1110 cm^{-1}. – MS (HR, 70 eV): *m/z* (%) = 474.6342 $[M]^+$ calcd. for $[M(C_{28}H_{42}O_6)] = 474.6338$. – MS (FAB, 70 eV): *m/z* (%) = 475(2.5) $[M]^+$, 456 (5) [M-

Table 1. ^{13}C NMR data (δ) of Compounds **1** and **2** in $CDCl_3$ (75 MHz).

Carbon	**1**	**2**	Carbon	**1**	**2**
1	203.2 s	73.2 d	2	129.1 d	39.9 t
3	139.2 d	63.9 d	4	36.7 t	41.7 t
5	73.1 s	74.2 s	6	56.7 d	58.3 d
7	56.3 d	58.0 d	8	35.2 d	35.4 d
9	36.7 d	36.2 d	10	50.9 s	40.1 s
11	21.6 t	20.6 t	12	32.6 t	39.2 t
13	48.6 s	44.3 s	14	46.2 d	51.7 d
15	22.9 t	27.7 t	16	36.7 t	23.8 t
17	85.2 s	52.1 d	18	12.3 q	12.3 q
19	15.0 q	16.5 q	20	40.9 d	39.3 d
21	12.3 q	12.7 q	22	78.5 d	78.5 d
23	32.6 t	30.1 t	24	150.5 s	149.3 s
25	121.3 s	122.3 s	26	167.2 s	167.2 s
27	14.7 q	13.6 q	28	20.3 q	20.8 q

Assignments based on the reported values for similar Compounds [21].

$H_2O]^+$, 438 (5) [M- $2H_2O]^+$, 420 (5) [M- $3H_2O]^+$, 349 (65), 326 (25), 292 (5), 125 (100), 123 (35). – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.63$ (1H, t, $J = 2.0$ Hz, 1-H), 4.45 (1H, m, 3-H), 3.57 (1H, s br, OH at 5-C), 2.96 (1H, d, $J = 3.0$ Hz, 6-H), 3.28 (1H, dd, $J = 3.0$, 2.0 Hz, 7-H), 0.74 (3H, *s*, 18-H), 0.82 (3H, s, 19-H), 1.01 (3H, d, $J = 7.0$ Hz, 21-H), 4.42 (1H, m, 22-H), 2.42 (2H, m, 23-H), 1.86 (3H, s, 27-H), 1.99 (3H, s, 28-H). – ^{13}C NMR : see Table 1.

Acetylation of **2**

Compound **2** (5 mg) was taken in a pear shape flask and 2 ml Ac_2O was added to it in the presence of 2 drops of pyridine and left over night, after usual work up it afforded **2a** (5 mg). R_f 0.45 ($CHCl_3$-EtOAc-MeOH-C_6H_6, 70 : 2 : 4 : 24). – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.81$ (1H, t, $J = 2.0$ Hz, 1-H), 5.40 (1H, m, 3-H), 3.25 (1H, s br, OH at 5-C), 2.96 (1H, d, $J = 3.0$ Hz, 6-H), 3.12 (1H, dd, $J = 3.0$, 2.0 Hz, 7-H), 0.74 (3H, *s*, 18-H), 0.92 (3H, s, 19-H), 1.0 (3H, d, $J = 7.0$ Hz, 21-H), 4.35 (1H, m, 22-H), 2.42 (2H, m, 23-H), 1.88 (3H, s, H-27), 1.94 (3H, s, 28-H), 2.02 (6H, s, OCOCH_3 at 1–C and 3-C).

Results and Discussion

The IR spectrum of **1** showed bands at 3465, 1710, 1680 and 1130 cm^{-1} for hydroxyl, lactone, ketone and epoxide functionalities, respectively, along with other typical bands for withanolides. The HRMS spectrum showed $[M]^+$ at *m/z* 470.6028 corresponding to the molecular formula $C_{28}H_{38}O_6$. The ^{1}H NMR spectrum showed the typical pattern of five methyl signals of withanolides at $\delta = 0.98$ (s), 1.21 (s), 1.13 (d, $J = 7.0$ Hz), 1.88 (s), 1.93 (s) for H-18, H-19, H-21, H-27 and H-28, respectively. The typical downfield methine double triplet at δ = 4.73 ($J = 9.0$, 5.5, 3.0 Hz) was assigned to H-22. The downfield signal at $\delta = 5.87$ (1H, d, $J = 10.0$ Hz) and 6.60 (1H, m) were assigned to H-2, H-3 conjugating to the ketone in A ring. The multiplicity of H-3 as a multiplet and its upfield chemical shift clearly suggested that H-4 was not oxygenated. Further, the presence of a typical set of signals, *i. e.* a doublet at $\delta = 3.07$ ($J = 3.0$ Hz) for H-6 and a double doublet at $\delta = 3.32$ (1H, $J = 3.0$, 2.0 Hz) for H-7 along with a broad singlet at $\delta = 3.13$ for OH suggested the presence of 2-en-1-one system with 5α-hydroxy and 6, 7α epoxy functional groups in A and B rings. This type of functionalities are quite comparable to the withanone (**3**) type of carbon skeleton.

The ^{13}C NMR and DEPT spectra of compound **1** also supported the presence of 28 carbon resonances including five methyl, six methylene, nine methine and eight quaternary carbons. The downfield signals at $\delta =$ 203.2, s for C-1; 129.1, d for C-2; 139.2, d for C-3 were indicative of the α,β-unsaturated ketone. The signals for E ring (α,β-unsaturated δ lactone at $\delta = 167.2$ for C-26; $\delta = 78.5$ for C-22; $\delta = 150.5$ for C-24 and $\delta = 121.3$ for C-25) suggested that C-26 and C-22 have oxidized to form the usual E ring of a withasteroid. The singlet at $\delta = 73.1$ for C-5 along with the doublets at $\delta = 56.7$ for C-6 and $\delta = 56.3$ for C-7 typically corresponded to the hydroxyl and epoxide groups, respectively, matching with the withanone (**3**) [11, 12]. Since the compound remained unchanged on acetylation with Ac_2O in the presence of pyridine, it clearly supported the absence of primary and secondary hydroxyls. The singlet at $\delta = 85.2$ in its ^{13}C NMR was indicative of the presence of another hydroxyl at the quaternary carbon. There are two possible positions for this hydroxyl, one at C-14 and the other at C-17. The placement of OH at C-14 is ruled out as it should have restricted the chemical shift of H-22 from $\delta = 4.40$ down fielding to $\delta = 4.73$. Also, the 14α- as well as 14β-hydroxyls influence the chemical shift of H-18 down fielding by 0.11 ppm and 0.31 ppm, respectively, along with the down field chemical shift of H- 7β by 0.45 ppm [12]. The second option at C-17 compares with the structure of **3** having a hydroxyl at C-17. However, the down field chemical shifts of H-22, H-21 and H-18 at $\delta = 4.73$, 1.13 and 0.98, respectively, in the ^{1}H NMR spectrum of **1** were not comparable to that of withanone (**3**) which shows these signals at $\delta = 4.64$, 1.03 and 0.86, respectively. Such kind of down field

Table 2. Pyridine induced shifts of H-18, H-21 and H-22 in compounds **1** and **2** (300 MHz, in δ).

Compound	H	$CDCl_3$	C_5D_5N	Δ ($CDCl_3$- C_5D_5N)
1 (17S)	H-22	4.73	4.81	−0.08 (negligible)
	H-21	1.13	1.40	−0.27 (sizeable)
	H-18	0.98	1.22	−0.24 (sizeable)
2 (17R)	H-22	4.42	4.92	−0.50 (sizeable)
	H-21	1.01	1.36	−0.35 (sizeable)
	H-18	0.74	0.80	−0.06 (moderate)

shifting is typical for the compounds possessing 17α-side chain, like in the ^{1}H NMR spectra of withanolide E (**5**), withanolide P (**6**) [13 – 16]. Therefore, compound **1** has got no other possibility than having the structure of withanone but with 17α- side chain.

The change of stereochemistry at C-17 as (β-OH) was further ascertained following the standard method of ^{1}H NMR spectral experimentation by recording in $CDCl_3$ and C_5D_5N and observing the differences in the chemical shifts of H-22 along with H-21 and H-18 [16, 17]. According to this method, the 17R configuration shows sizeable differences (δ $CDCl_3$- C_5D_5N) at H-22, H-21 and moderate at H-18 while in the case of 17S, the pyridine induced shifts are sizeable for H-21 and H-18 only (Table 2) [15, 18]. Therefore, it is evident that **1** possesses 17S configuration.

However, the configuration at C-22 in all the isolated steroids as been assigned as R, since in their ^{1}H NMR spectra, the H-22 has appeared as doublet appearing like a double triplet by having couplings with the protons of C-23 whereas in the case of S configuration, it shows negligible coupling with H-23 [11, 19] which is a normal configuration for the steroids isolated from *W. somnifera*, so far [13] and has got the biogenetic support also [20]. The stereochemistry at other carbons of similar withasteroids have already been established by X-ray [18]. The 2D NMR experiments were also in agreement with the proposed structure (Table 3). Therefore, these data clearly supported that the structure of **1** is (17S,22R)-5α,17β-dihydroxy-6α,7α-epoxy-1-oxo-witha-2,24-dienolide which has been, tentatively, named as iso-withanone.

The IR spectrum of **2** showed absorption bands at 3490, 1710 and 1120 cm^{-1} for hydroxyl, lactone and epoxide functionalities, respectively, along with other typical bands as in case of **1**. The HRMS showed $[M]^+$ at *m/z* 474.6342 corresponding to $C_{28}H_{42}O_6$. The ^{1}H NMR spectrum showed the typical pattern of five methyl signals of a withanolide at $\delta = 0.74$ (s), 0.82 (s), 1.01 (d, $J = 7.0$ Hz), 1.86 (s), 1.99 (s) for H-18, H-19, H-21, H-27 and H-28, respectively. The characteristic down field methine multiplet at $\delta = 4.42$ was assigned to H-22. The doublet at $\delta = 2.96$ (1H, $J = 3.0$ Hz) and double doublet at $\delta = 3.28$ (1H, $J = 3.0, 2.0$ Hz) along with a broad singlet at $\delta = 3.57$ were also characteristically assigned to 5α-hydroxy-6,7α-epoxy system in A/B ring. The absence of typical signals of conjugated vinylic protons indicated that A ring is saturated and the multiplet at $\delta = 4.45$ and broad triplet at $\delta = 3.63$ ($J = 2.0$ Hz) were assigned to the H-3 and H-1, respectively. Since in its ^{1}H NMR spectrum, the signals for H-3 and H-22 overlapped with each other, it was acetylated to ascertain the stereochemistry at C-3 and C-1. On acetylation **2** yielded **2a** showing the clear signals for H-3 and H-1 at $\delta = 5.40$ and 4.81, respectively, in addition to a broad singlet (6H) at $\delta = 2.02$ for the acetates. The position of hydroxyls at C-1 and C-3 was supported by ^{1}H^{1}H COSY spectrum of its acetate (**2a**) wherein H-1 showed correlation with H-2 at $\delta = 2.00$ and 2.10. Similarly, H-3 showed additional correlation with H-4 at $\delta = 1.85$ and 2.15 (Table 3). The H-1 in **2a** appeared as a sharp triplet ($J = 2.0$ Hz) indicating that the hydroxyl is α-oriented whereas the H-3 appeared as a multiplet which is typical for β-substitution [4, 11, 21].

Table 3. 2D NMR correlations in compounds **1** and **2** (300 MHz, $CDCl_3$).

Compound	H	^{1}H^{1}H COSY	H	HMBC
1	H-22	H-23, H-20	H-3	C-1, C-2, C-4, C-5
	H-21	H-20	H-20	C-17, C-21, C-22, C-23
	H-20	H-21	H-22	C-23, C-24, C-20, C-21, C-17
	H-6	H-7		
	H-7	H-6, H-8[$]		
	H-3	H-2, H-4		
	H-2	H-3		
2	H-22	H-23, H-20	H-3	C-1, C-2, C-4, C-5
	H-21	H-20	H-22	C-23, C-24, C-20, C-21, C-17
	H-20	*		
	H-17	*		
	H-3	H-2, H-4		
	H-1	H-3		

[$] Weak correlation; * signals obscured.

The ^{13}C NMR and DEPT spectra of compound **2** supported the presence of 28 carbon resonances including five methyl, seven methylene, ten methine and six quaternary carbons. The down field signals at $\delta = 167.2$ for C-26; $\delta = 78.5$ for C-22; $\delta = 149.3$ for C-24 and $\delta = 122.3$ for C-25 along with the signals $\delta = 73.2$ for C-1; $\delta = 63.9$ for C-3 and $\delta = 74.2$ for C-5. The stereochemistry at C-17 and C-22 was established as 17R,22R following the reasons of

experimental data as explained in the case of compound **1** (Table 2). These data suggested that the structure of **2** is (17R,22R)-6α,7α-epoxy-1α,3β,5α-trihydroxy-witha-24-enolide.

The biogenesis of withanolides having 2-en-1-one system has earlier been suggested to route through the oxidation and dehydration of 1α,3β-dihydroxy compounds [22]. Therefore, the isolation of compound **2** from *W. somnifera* has got biogenetic significance also. Further, the presence of withanolides in good concentration suggested that the berries of Ashwagandha (*W. somnifera*) can also be exploited for biologically active withasteroids.

Acknowledgements

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