Published in final edited form as:
Phytochem Lett. 2011 December ; 4(4): 411–414. doi:10.1016/j.phytol.2011.04.016.

Chlorinated Withanolides from *Withania somnifera*

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Abstract

A chlorinated withanolide, 6*α*-chloro-5*β*,17*α*-dihydroxywithaferin A (**1**), and nine known withanolides, 6*α*-chloro-5*β*-hydroxywithaferin A (**2**), (22*R*)-5*β*-formyl-6*β*,27-dihydroxy-1-oxo-4-norwith-24-enolide, withaferin A, 2,3-dihydrowithaferin A, 3-methoxy-2,3-dihydrowithaferin A, 2,3-didehydrosomnifericin, withanone, withanoside IV and withanoside X, were isolated from *Withania somnifera* (Solanaceae). All structures were elucidated on the basis of spectroscopic methods (IR, HRESIMS, 1D/2D NMR). X-ray crystallography confirmed the absolute configuration of **1**.

Keywords

Chlorinated withanolide; *Withania somnifera*; Solanaceae; NMR; Crystal structure

1. Introduction

Withanolides are a group of naturally occurring C_{28}-steroidal lactones built on an intact or rearranged ergostane scaffold in which C-22 and C-26 are appropriately oxidized to form a *δ*-lactone ring on the nine-carbon side chain. These compounds are mainly found in members of the plant family Solanaceae (Mirjalili et al., 2009) and are known to possess various biological activities including anticarcinogenic (Christina et al., 2004), antioxidant (Bhattacharya et al., 1997), adaptogenic (Dhuley, 2000), antiparkinsonism (Nagashayana et al., 2000), antibacterial (Arora et al., 2004), anti-inflammatory (Al-Hindawi et al., 1989), immunomodulatory (Davis and Kuttan, 2000) and antidepressant (Bhattacharya et al., 2000) effects. As part of our ongoing investigation of antiproliferative withanolides (Samadi et al., 2010), we selected *Withania somnifera* L. Dunal as an abundant source of these compounds.

W. somnifera, commonly known as “ashwagandha” and widely cultivated in the drier parts of India, is well known for its use in Ayurvedic medicine. Previous research supports its pharmacological uses, confirming antioxidant, anti-inflammatory (Sumantran et al., 2008), immunomodulatory, anticarcinogenic, antibacterial (Owais et al., 2005), antiparkinsonism (Sankar et al., 2007) and antistress (Geetha and Harish, 2006) properties. Raw plant material and extracts of this species are currently sold as dietary supplements in the USA for

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Supplemental Data: LC-MS of PhycoMyco crude extract of *W. somnifera* using withaferin A and withanone as markers; X-ray crystallography analysis, ^{1}H NMR, and MS spectra as well as HPLC analysis of **1** and ^{1}H NMR of **2**. This material is available free of charge via the internet.

increasing energy and endurance, strengthening immune function, and helping the body overcome imbalance. More than 130 withanolides have been reported in previous investigations from the same species. Herein, we report the isolation and structural elucidation of a new chlorinated withanolide (**1**), named 6*α*-chloro-5*β*,17*α*-dihydroxywithaferin A (Fig. 1), along with other nine known withanolides.

2. Results and discussion

The ethyl acetate-soluble fraction of the crude extract acquired for this study was subjected to repeated column chromatography to yield compound **1**. Compound **1** was obtained as colorless parallelepiped-shaped crystals by recrystallization from MeOH–CH_2Cl_2–EtOAc (5:1:1). The molecular formula $C_{28}H_{39}ClO_7$ was deduced from its HRESIMS (observed *m/z* 523.2440 $[M + H]^+$, calcd for $C_{28}H_{40}ClO_7$ 523.2463) and NMR data. The presence of chlorine was supported by the intensity of the $[M + H + 2]^+$ peak, which was approximately one-third as intense as the pseudomolecular ion peak $[M + H]^+$. IR absorption bands at 3500, 1700, and 1650 cm^{-1} revealed the presence of OH, carbonyl and lactonic groups.

The ^{13}C NMR (DEPT) spectrum of **1** displayed 28 signals which were assigned to four methyl, seven methylene (including one oxygenated at *δ*57.3), nine methine (including two olefins at *δ*127.8 and 142.8), and eight quaternary carbons (including one keto carbonyl at *δ*200.2 and one ester carbonyl at *δ*167.0, two olefins at *δ*154.2 and 125.2, two oxygenated at *δ*84.8 and 78.1) corresponding to $C_{28}H_{35}$. Thus, the remaining four hydrogen atoms were assigned to four OH groups. These data were consistent with the characteristic features of withanolides, C_{28}-steroidal lactones with a *δ*-lactone ring on the nine-carbon side chain.

The ^1H NMR spectrum of **1** also showed typical signals for a withanolide. The four methyl groups resonated at *δ*0.99 (3H, d, *J* = 7.0 Hz), *δ*0.78 (3H, s), *δ*1.27 (3H, s), and *δ*2.03 (3H, s), corresponding to the secondary methyl of C-21 and the quaternary methyls C-18, 19 and 28 of a withanolide.

The NMR data of **1** was very similar to those of another chlorinated withanolide, 6α-chloro-5*β*-hydroxywithaferin A **2** (Nittala et al., 1981), isolated during this study. The obvious difference between **1** and **2** was the presence of a quaternary carbon (*δ*84.8) in **1** and a methine carbon (C-17) in the latter (^1H NMR *δ* 1.07, ^{13}C NMR *δ*51.8), suggesting that **1** was a 17-hydroxy derivative of **2**. This observation was supported by the high-frequency shift of C-13 (*δ*48.3 in **1** and *δ*43.2 in **2**), C-16 (*δ*36.5 in **1** and *δ*27.2 in **2**), and C-20 (*δ*42.6 in **1** and *δ*38.7 in **2**) in the ^{13}C NMR spectra. It was also supported by the ^1H-^1H COSY and HSQC spectra (a fragment CH_3-CH-CH(O)-CH_2- of the side chain linked to the oxygenated quaternary C-17 in **1** and a fragment CH_3-CH-CH(O)-CH_2- of the side chain linked to methine C-17 in **2**), and the HMBC correlations (Fig. 2) between H_3-21 (*δ*0.99) and C-17 (*δ*84.8), between H_3-18 (*δ*0.78) and C-17 (*δ*84.8) in **1**. The full assignments of NMR data (Table 1) were obtained by 2D NMR including ^1H-^1H COSY, HSQC and HMBC spectra.

Finally, the structure of **1** was confirmed through single-crystal X-ray diffraction experiments (Fig. 3). The absolute configuration of **1** was confirmed on the basis of anomalous scattering of the chlorine atoms in the crystal. Thus, the structure of the new withanolide **1** was established as 6*α*-chloro-5*β*,17*α*-dihydroxywithaferin A.

The known compounds were identified, by comparison of their NMR and MS data with those reported in the literature, as 6*α*-chloro-5*β*-hydroxywithaferin A (**2**) (Nittala et al., 1981); (22*R*)-5*β*-formyl-6*β*,27-dihydroxyl-1-oxo-4-norwith-24-enolide (Nittala and Lavie, 1982); withaferin A, 2,3-dihydrowithaferin A and 3-methoxy-2,3-dihydrowithaferin A (Pelletier et al., 1979); 2,3-didehydrosomnifericin (Choudhary et al., 1996); withanone (Gottlieb and Kirson, 1981); withanoside IV (Matsuda et al., 2001) and withanoside X

(Zhao et al., 2002). Full assignments of NMR data for the known chlorinated withanolide **2** were listed in Table 1 because those values reported in the literature (Nittala et al., 1981) and, especially the assignments of its ^{13}C NMR data (C-4, 6, 10, 14, 17), need to be revised.

Chlorinated compound**s** such as **1** and **2** are not commonly encountered in higher plants. To date, only five other chlorinated withanolides have been reported from *W. somnifera*, including withanolide C (Bessalle and Lavie, 1992), withanolide Z (Pramanick et al., 2008), 6*α*-chloro-5*β*-hydrowithanolide D, 4-deoxyphysalolactone (Nittala et al., 1981) and 27-acetoxy-5*β*-chloro-6*α*-hydroxywithaferin A (Choudhary et al., 2010). With the exception of 27-acetoxy-5*β*-chloro-6*α*-hydroxywithaferin A and withanolide Z, all the reported chlorinated withanolides contain a 6*α*-chloro-5*β*-hydroxy system. This suggests that the epoxide rings that are usually encountered at C-5 and C-6 have undergone a diaxial opening (Nittala et al., 1981).

3. Experimental

3.1. General

Optical rotations were obtained on a Rudolph Research Analytical Autopol IV Automatic polarimeter. Melting points were recorded using a MPA100 OptiMelt Automated Melting Point System (Stanford Research Systems, Sunnyvale, CA). UV data were acquired using an Agilent Technologies 1200 HPLC (Diode Array Detector). HPLC was conducted on Agilent Technologies 1200 series system. IR data were obtained using a Thermo Nicolet Avatar 360 FT-IR instrument. NMR spectra were recorded on either a Bruker DRX-400 with a qnp probe or on a Bruder AV-500 with a cryoprobe. ^{1}H and ^{13}C spectra were recorded using the residual protonated signal in the $CDCl_3$ solvent (δ_H 7.24) or the central peak of the $CDCl_3$ triplet (δ_C 77.00) as the internal standard. High resolution mass spectrometry data were collected on a LCT Premier time of flight mass spectrometer (Waters Corp., Milford, MA). Normal phase TLC was performed on Sorbent Technologies Silica G TLC plates (200 μm, w/UV 254) using the solvent system CH_2Cl_2–EtOAc–MeOH (1:8:1), and reverse phase TLC was performed on Sorbent Technologies C18 TLC plates (150 μm, w/UV 254) using H_2O-MeOH (1:1). Spots were visualized using UV light (254 nm) and spraying with vanillin-sulfuric acid reagent.

3.2. Plant Material

The plant crude extract was acquired from PhytoMyco Research Corporation, Greenville, NC in March 2009. The extract was prepared according to published procedures by sequential extraction of the dried and ground leaves of *W. somnifera* with a mixture of CH_2Cl_2 and MeOH (1:1, v/v) followed by MeOH and H_2O to obtain three fractions (Jayaprakasam and Nair, 2003). All fractions were then combined, filtered and dried under reduced pressure. The original extract has been stored at -20 °C in our laboratory to serve as a reference material.

3.3. Extraction and Isolation

The powdered, dried plant extract (189 g) of *W. somnifera* was dissolved in 200 mL of water to form a suspension. The mixture was defatted with hexane (2 L × 3), and the aqueous phase was extracted with EtOAc (2 L × 6) and BuOH (2 L × 3) to obtain 32.9 g of EtOAc extract and 80.0 g of BuOH extract. The EtOAc extract was then subjected to passage over a Si gel column (600 g, 32 ~ 63 μm, Sorbent Technologies), eluted sequentially with CH_2Cl_2-CH_3COCH_3 solvent gradient (0–100%) followed by MeOH, to give eight major fractions (Fraction A-H). Fraction F was chromatographed on a Si gel (200 g, 12 ~ 26 μm, Sorbent Technologies) MPLC column, eluted with CH_2Cl_2–EtOAc–MeOH (40:10:1), to afford four further fractions (F1–F4). Fraction F2 was applied onto a C-18 reverse phase column (100 g,

Sorbent Technologies), eluted with MeOH–H_2O (3:7) to remove the green color. The colorless fraction F2 was then separated on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH to obtain the major components, and then purified by separation over a Si gel column (50 g, 12 ~ 26 μm, Sorbent Technologies), eluted with CH_2Cl_2–MeOH–EtOAc (18:1:1), to yield **1** (5 mg). Fraction F1 was purified on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH, to afford 2,3-didehydrosomnifericin (21 mg). Compound withanone (800 mg) was obtained as fine needles by recrystallization of fraction D from $CHCl_3$–CH_3COCH_3 (1:1). Fraction E was chromatographed on a Si gel column, eluted with CH_2Cl_2–CH_3COCH_3 (10:1), to collect twelve fractions (E1–El2), and E6 was identified as withaferin A (34 mg). The major withanolide in fraction E7 was obtained as a white powder yielding 3-methoxy-2,3-dihydrowithaferin A (22 mg) by recystallization from $CHCl_3$–CH_3COCH_3 (1:1). E12 was separated over a C-18 reverse phase SPE column (5 g, 20 mL, Phenoment strata C-18), eluted with MeOH-H_2O (1:1), to give a mixture of two compounds. Further purification of the mixture by HPLC using isocratic elution with CH_3CN–H_2O (45:55) provided (22*R*)-5*β*-formyl-6*β*,27-dihydroxy-1-oxo-4-norwith-24-en (1.5 mg) and 2,3-dihydrowithaferin A (1.2 mg). Compound **2** (7.3 mg) was purified from fraction E11 by a C-18 reverse phase SPE column (5 g, 20 mL, Phenoment strata C-18) using MeOH–H_2O (1:1).

The *n*-BuOH extract was subjected to passage over a Si gel column (2 kg, 12 ~ 26 μm, Sorbent Technologies), eluted sequentially with MeOH–CH_2Cl_2 solvent gradient (30%–100%), to give 24 fractions. Fraction 4–5 was purified on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH–H_2O (8:2), to afford a withanoside-rich fraction. This faction was further purified by PTLC eluting with CH_2Cl_2–MeOH–H_2O (40:10:1) and semi-prep HPLC eluting with CH_3CN–H_2O (25:75) to afford withanoside IV (5.6 mg) and withanoside X (6.3 mg).

3.4 6*α*-chloro-5*β*,17*α*-dihydroxywithaferin A (1)

Colorless crystal (MeOH); mp 238-240 °C; $[\alpha]^{25}_{546}$ +53.1 (c 0.001, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.93) nm; IR (film) ν_{max} 3500, 2980, 1700, 1650, 1630 and 1050 $cm^{-1;}$ ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; ESIMS *m/z* 523 $[M + H]^+$; HRESIMS *m/z* 523.2440 (calc. for $C_{28}H_{40}ClO_7$, 523.2463).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge financial assistance from the Center for Cancer Experimental Therapeutics NIH Grant 5P20 RR015563-10 (to B.N.T.) and the University of Kansas fund KU 2506014-910/099 (to B.N.T.). The authors thank Dr. Victor Day, Director of the Molecular Structures Group, University of Kansas, for the X–ray analysis.

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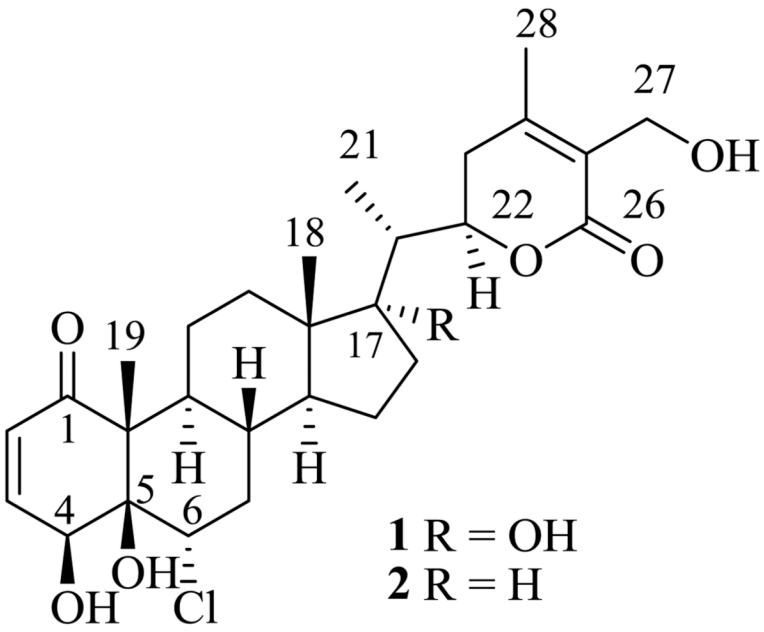

Fig. 1.
Chlorinated withanolides from *Withania somnifera*.

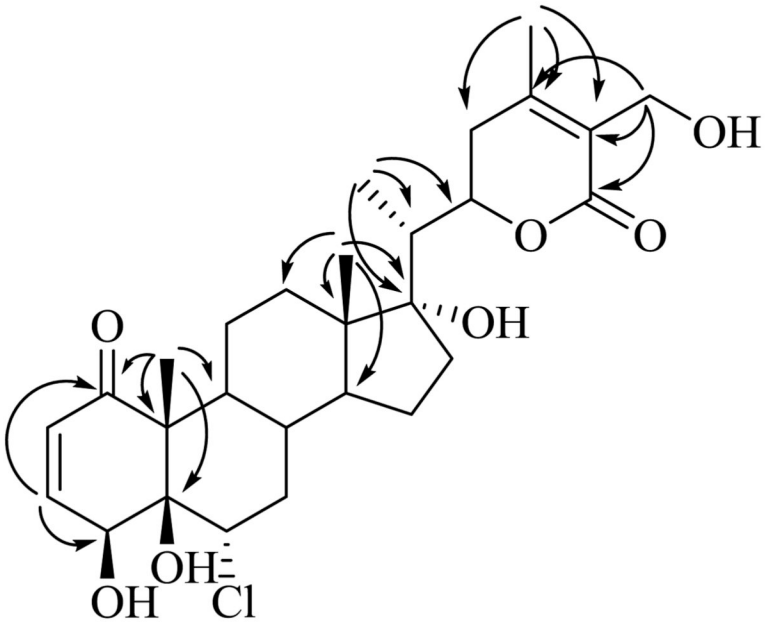

Fig. 2.
Key HMBC correlations of compound **1**.

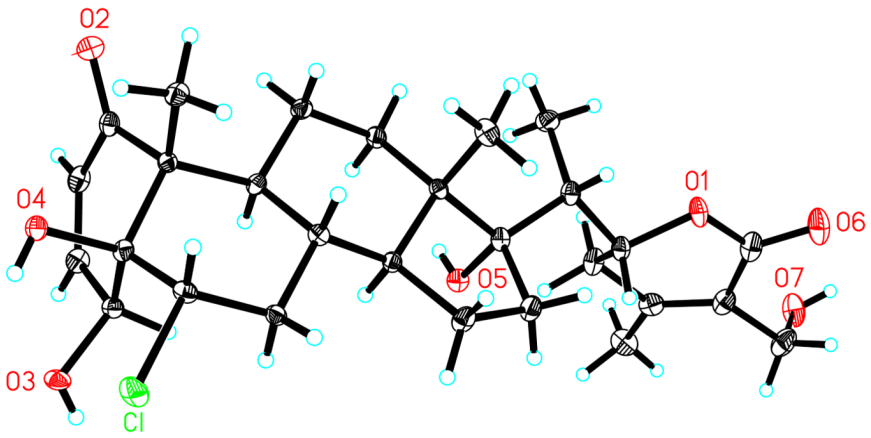

Fig. 3.
ORTEP view of chlorinated withanolide **1**.

Table 1

^{1}H and ^{13}C NMR data of chlorinated withanolides **1** and **2** in $CDCl_3$.

No.	1		2	
	$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{a}$	$^{13}C^{b}$
1		200.2		200.1
2	6.02 dd (10.4, 2.2)	127.8	6.00 dd (10.4, 2.2)	127.8
3	6.50 dd (10.4, 2.5)	142.8	6.47 dd (10.4, 2.5)	142.8
4	5.06 br s	66.1	5.01 br s	66.2
5		78.1		78.1
6	4.45 dd (12.7,4.6)	66.5	4.42 dd (12.5,4.9)	66.7
7	2.30; 1.71	39.3	2.30; 1.63	39.4
8	1.63	35.2	1.62	35.1
9	1.27	45.3	1.27	45.8
10		57.1		57.2
11	1.33; 0.97	22.4	1.33; 0.90	22.7
12	1.58; 1.31	31.6	1.89; 1.04	39.0
13		48.3		43.2
14	1.64	49.6	1.05	55.3
15	1.71; 1.21	23.3	1.63; 1.18	24.0
16	1.97; 1.68	36.5	1.67; 1.37	27.2
17		84.8	1.07	51.8
18	0.78 s	14.8	0.65 s	11.8
19	1.27 s	9.9	1.24 s	9.9
20	2.26	42.6	1.87	38.7
21	0.99 d (7.0)	9.4	0.95 d (7.0)	13.3
22	4.65 m	78.9	4.38 m	78.6
23	2.47, 1.91	32.9	2.45, 1.93	29.8
24		154.2		152.7
25		125.2		125.7
26		167.0		166.9
27	4.39 d (12.6); 4.35 d (12.6)	57.3	4.37 d (12.6); 4.33 d (12.6)	57.4
28	2.03 s	20.0	2.00 s	20.0

[a] δ(ppm) 500 MHz; multiplicities; *J* values (Hz) in parentheses.

[b] δ(ppm) 125 MHz.