Ashwagandhanolide, a Bioactive Dimeric Thiowithanolide Isolated from the Roots of *Withania somnifera*[⊥]

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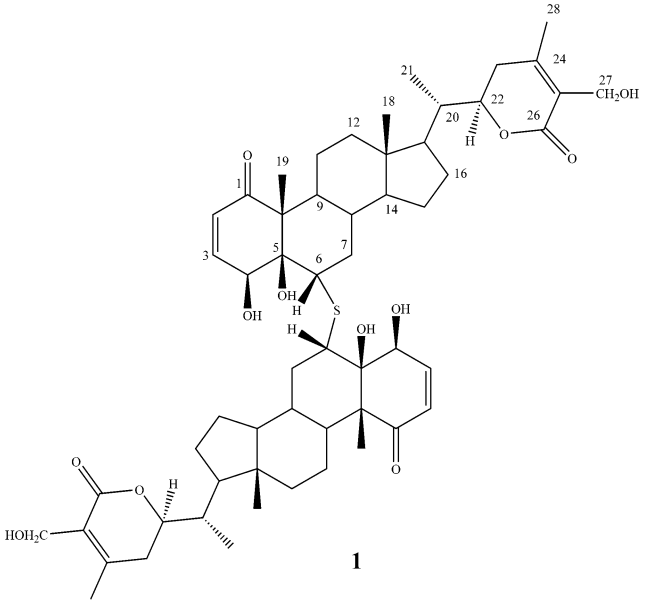
Received April 1, 2006

A new dimeric withanolide, ashwagandhanolide (**1**), was isolated from the roots of an Ayurvedic medicinal herb, *Withania somnifera*. A detailed spectroscopic evaluation revealed its identity as a dimer with an unusual thioether linkage. Compound **1** displayed growth inhibition against human gastric (AGS), breast (MCF-7), central nervous system (SF-268), colon (HCT-116), and lung (NCI H460) cancer cell lines, with IC_{50} values in the range 0.43−1.48 μg/mL. In addition, it inhibited lipid peroxidation and the activity of the enzyme cyclooxygenase-2 in vitro.

Solanaceae is one of the largest families in the plant kingdom and comprises about 85 genera and more than 3000 species.[1] The fruits from several of its species are edible, and some are used in traditional medicine.[2] The plant *Withania somnifera* (L.) Dunal, commonly known as "Ashwagandha", is well known for its therapeutic use in the Ayurveda system of traditional medicine.[3,4] The roots of this plant have been used as an adaptogen and to treat arthritis, asthma, dyspepsia, hypertension, rheumatism, and syphilis.[5] Earlier pharmacological investigations of *W. somnifera* have revealed its antiinflammatory, antioxidant, immunomodulatory, and tumor cell proliferation inhibitory activities.[6] The therapeutic potential of *W. somnifera* has been attributed to the presence of withanolides.[7] Previous studies have shown that the withanolides inhibit tumor cell proliferation and angiogenesis and also induce phase-II enzymes.[8,9] The broad spectrum of biological activities exhibited by *W. somnifera* roots prompted an investigation for further minor active constituents. Examination of the roots of *W. somnifera* resulted in the isolation of a new, dimeric thiowithanolide, named ashwagandhanolide (**1**), along with several known compounds, withaferin A,[10] withanolide A,[11] 12-deoxywithastramonolide,[12] and 20-deoxywithanolide A.[13]

The ethyl acetate-soluble fraction of the methanol extract of the roots of *W. somnifera* was subjected to column chromatography and preparative HPLC to yield compound **1**. Compound **1** was obtained as a colorless solid from MeOH, mp 180−182 °C, $[\alpha]_D$ +161 (*c* 0.09, MeOH), and its molecular formula was determined as $C_{56}H_{78}O_{12}S$ on the basis of the molecular ion observed at *m*/*z* 975.5285 (HRFABMS, $[M + H]^+$). The UV spectrum of **1** showed a strong absorption peak at 220 nm, characteristic of α,β-unsaturated carbonyl and α,β-unsaturated δ-lactone moieties.[14] Its IR spectrum ($CHCl_3$) displayed bands at 3436 and 1681 cm^{-1} and indicated the presence of hydroxyl and α,β-unsaturated carbonyl functions, respectively.[15] The ^1H NMR spectrum of **1** showed signals for three tertiary methyl groups at δ 0.65, 1.12, and 1.99 and a secondary methyl at δ 0.88 (J = 6.4 Hz), as well as two olefinic protons appearing at δ 6.42 and 5.76, similar to those observed for withaferin A,[10] a major withanolide of the extract. Further spectroscopic analysis revealed that the C-26 hydroxymethyl signal appeared as a multiplet between δ 4.10 and 4.20 (2H, m). In addition, the ^1H NMR spectrum showed two resonances at δ 2.66 (J = 15.0 Hz) and 4.29 (dd, J = 13.0 Hz). The latter signal was attributable to the proton at C-22. The ^{13}C NMR spectrum of **1** displayed 28 carbon signals including a carbonyl carbon (δ 200.9), two olefinic carbons (δ 125.6 and 146.7), and an α,β-unsaturated δ-lactone moiety (δ 153.6, 125.5, and 165.3). Further, the ^{13}C NMR and DEPT spectra revealed the presence of an oxygenated methine carbon signal at δ 64.8 and a quaternary carbon at δ 78.8. These signals were assigned to C-4 and C-5, respectively, on the basis of HMBC correlations between H-2/C-4 and H-3/C-5. The DEPT spectrum of compound **1** showed the presence of four methyls, seven methylenes, and 10 methine groups. A broad doublet at δ 2.66 (1H, J = 15 Hz) correlated to a methine carbon at δ 51.7 in the HMQC spectrum and was assigned to C-6 on the basis of HMBC correlations between H-6/C-10 and H-6/C-4. Analysis of all of this evidence suggested that compound **1** is a withaferin A-type withanolide and closely related to (20*S*,22*R*)-4β,5β,6α,27-tetrahydroxy-1-oxowitha-2,24-dienolide[16] with modifications at C-6. The configurations at C-4 and C-5 were ascertained as β by comparison of ^{13}C NMR values with those of similarly substituted compounds.[16] The configuration at C-6 was ascertained as α using $^1H-^1H$ COSY data. The molecular formula of $C_{56}H_{78}O_{12}S$ and a total of 28 carbon signals observed in the ^{13}C NMR spectrum clearly indicated that compound **1** is a symmetrical dimer. The presence of two withanolide (471 amu) and S (32 amu) moieties in the molecule accounted for the observed molecular weight of 503 in



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[⊥] Laila Research Center communication number 59.

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10.1021/np060147p CCC: $33.50
Published on Web 11/22/2006

Table 1. NMR Data for Compound **1** (in $CDCl_3$+DMSO-d_6)

position	δ_H[a] (mult., *J*)	δ_C[a]	DEPT	HMBC correlations
1		200.9	C	
2	5.76 (1H, d, *J* = 10.0 Hz)	125.6	CH	C-4, C-10
3	6.42 (1H, d, *J* = 9.7 Hz)	146.7	CH	C-1, C-5
4	4.71 (1H, d, *J* = 7.1 Hz)	64.8	CH	C-2, C-3
OH-4	5.35 (1H, d, *J* = 7.1 Hz)			C-3, C-4, C-5
5		78.8	C	C-4, C-5, C-6, C-10
OH-5	3.73 (1H, s)			
6	2.66 (IH, d, *J* = 15 Hz)	51.7	CH	C-4, C-5, C-6
7	1.38 (2H, m)	36.6	CH_2	C-6
8	1.60 (1H, m)	34.9	CH	
9	1.10 (1H, m)	44.9	CH	
10		56.3	C	
11		22.5	CH_2	
12		38.5	CH_2	
13		42.6	C	
14		54.6	CH	
15		23.4	CH_2	
16		26.6	CH_2	
17		51.0	CH	
18	0.65 (3H, s)	11.6	CH_3	C-12, C-13, C-14, C-17
19	1.12 (3H, s)	9.3	CH_3	C-1, C-5, C-9, C-10
20		38.3	CH	
21	0.88 (3H, d, *J* = 6.4 Hz)	12.8	CH_3	C-17, C-21
22	4.29 (1H, d, *J* = 13.0 Hz)	77.4	CH	
23	2.36 (1H, t, *J* = 12.0 Hz)	29.2	CH_2	
24		153.6	C	
25		125.5	C	
26		165.3	C	
27	4.13 (1H, d, *J* = 5.1 Hz)	54.7	CH_2	C-24, C-25, C-26
OH-27	4.18 (1H, d, *J* = 5.1 Hz) 4.47 (IH, t)			C-25, C-27
28	1.99 (3H, s)	19.8	CH_3	C-23, C-24, C-25

[a] Supported by DEPT, $^1H-^1H$ COSY, and HMQC data.

its mass spectrum. The upfield shifts of the C-6 proton and carbon signals suggested that the withanolide units are linked via the S atom between the C-6 and C-6′ (6-C−S−C-6′) positions. Also, a significant correlation in its HMBC spectrum was observed for H-6 (δ_H 2.66, br d, *J* = 15.0 Hz) to C-6 (δ_C 51.7). ^1H and ^{13}C NMR, DEPT, $^1H-^1H$-COSY, HMQC, and HMBC spectroscopic assignments for compound **1** are shown in Table 1 and confirmed the structure of compound **1** as a novel dimeric thiowithanolide, which has been named ashwagandhanolide.

The isolation and characterization of ashwagandhanolide (**1**) showed it to be the first example of a dimeric withanolide from *W. somnifera* with a thioether linkage. Thiowithanolides are very rare, although withaperuvin H, isolated from *Physalis peruviana*, contains a thioether functionality at C-6.[17]

Ashwagandhanolide (**1**) was evaluated for its ability to inhibit cell proliferation against human lung (NCI H-460), breast (MCF-7), colon (HCT-116), CNS (SF-268), and gastric (AGS) tumor cell lines using the MTT cell viability assay, as described earlier.[18] Compound **1** exhibited 50% growth inhibitory concentrations (IG_{50}) at 0.43, 1.45, 0.84, 1.25, and 1.48 μg/mL against AGS, breast, CNS, colon, and lung tumor cells, respectively. Among the tested cell lines, gastric cancer cells were the most susceptible to **1**. Ashwagandhanolide (**1**) was also tested against cyclooxygenase enzymes (COX-1 and -2) and for lipid peroxidation inhibitory activities at a concentration of 100 μg/mL. It inhibited lipid peroxidation by 65% and the COX-2 enzyme by 60%, but did not show any activity against the COX-1 enzyme. On the basis of our bioassay results, the consumption of *W. somnifera* root extract may be beneficial in preventing the progression of certain tumors. However, further in vivo studies are needed to establish the efficacy and mechanism of action of **1**.

Experimental Section

General Experimental Procedures. The melting point was determined with a Bristolscope Bristoline melting point apparatus and is uncorrected. The optical rotation was obtained on a Perkin-Elmer 341 polarimeter and the circular dichroism (CD) spectrum on a JASCO model J-720 spectropolarimeter in MeOH at 25 °C. UV and IR spectra were obtained on a Shimadzu UV-240 and a Perkin-Elmer BX FT-IR spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-400 FT NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents. The EIMS data were obtained on a JEOL D-300 spectrometer. HRMS data were recorded on a Finnigan MAX 95XL mass spectrometer. LC-MS data were carried out using an Agilent 1100 LC/MSD instrument. Analytical HPLC experiments were performed with an Alltima C_{18}, 5 μm 4.6 × 250 mm column equipped with a variable-wavelength UV detector operating at 215 and 227 nm, using 0.1% phosphoric acid in H_2O-CH_3CN (1:1, isocratic) as mobile phase, at a flow rate of 1 mL/min. Separations were carried out with a Phenomenex Luna 10 C_{18}, 250 × 21.2 mm, 10 μm column (UV detector at λ_{max} 215 nm) with H_2O-CH_3CN (1:1) as eluant, at a flow rate of 20 mL/min. Column chromatography was conducted on silica gel (200−400 mesh size) and thin-layer chromatography (TLC) on precoated silica gel (5 × 10 cm, 0.2 mm thick, E. Merck) using $CHCl_3$−acetone−H_2O (6:3.9:0.1). HPTLC on RP-18 plates was developed with methanol−water (70:30) as solvent system. Spots or bands on TLC were visualized by spraying with 5% sulfuric acid in methanol followed by heating at 70 °C.

Plant Material. Authenticated roots of *Withania somnifera* were collected from Kalyanadurg, Andhra Pradesh, India, in July 2003. The plant material was identified by Dr. K. Hemadri, and a voucher specimen is on deposit at the herbarium of the Laila Research Centre, Vijayawada, India (voucher no. LIH-6852).

Extraction and Isolation. The dried roots of *W. somnifera* (1.5 kg) were extracted with MeOH (5 L × 3) at room temperature, and the resulting extract was concentrated under vacuum to yield a dark gummy residue (180 g). The residue was then defatted with hexane (1 L × 2), and the resulting residue was stirred with ethyl acetate (1 L × 3). The ethyl acetate extract was concentrated (38 g) and subjected to column chromatography (silica gel, 200−400 mesh column size), eluted with hexane and EtOAc mixtures. Similar fractions were combined after TLC examination to provide three fractions: I (5.9 g), II (6.2 g), and III (18.5 g). Successive column chromatographic and preparative HPLC purification of fraction III using a Phenomenex Luna 10 C_{18}, 250 × 21.2 mm, 10 μm column (UV detector at λ_{max} 215 nm) with H_2O-CH_3CN (1:1) as eluant, at a flow rate of 20 mL/min, afforded ashwagandholide (**1**, t_R 8.40 min, 35 mg).

Ashwagandhanolide (1): colorless solid; mp 180−182 °C; $[\alpha]_D$ +161 (*c* 0.09, MeOH); +240.1 (*c* 0.385, DMSO); CD (nm) $\Delta\epsilon$ +28.2 (239, *c* 0.0005, MeOH); UV λ_{max} (log ϵ) 220 nm (4.45); IR ν_{max} 3436, 1681, 1023, 801 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRMS *m/z* 975.5285 [M + H^+] (calcd for $C_{56}H_{79}O_{12}S$, 976.2347).

Bioassays. Tumor cell proliferation,[18] lipid peroxidation,[19] and cyclooxygenase[18] enzyme inhibitory assays were performed according to previously published procedures.

Acknowledgment. The authors thank the Sophisticated Instrumentation Facility at the Indian Institute of Science, Bangalore, for providing spectroscopic data and Mr. G. Ganga Raju at Laila Impex, India, for providing the plant material.

Supporting Information Available: NMR spectra for compound **1**. The information is available free of charge via the Internet at http://pubs.acs.com.

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NP060147P