Cholinesterase Inhibiting Withanolides from *Withania somnifera*

Muhammad Iqbal CHOUDHARY,* Sammer YOUSUF, Sarfraz Ahmad NAWAZ, Shakil AHMED, and ATTA-UR-RAHMAN

H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi; Karachi-75270, Pakistan. Received May 10, 2004; accepted July 12, 2004

A total of two new (1, 2) and four known (3—6) withanolides were isolated from the whole plant of *Withania somnifera*. Their structures were elucidated on the basis of spectroscopic techniques and were characterized as 6α,7α-epoxy-3β,5α,20β-trihydroxy-1-oxowitha-24-enolide (1), 5β,6β-epoxy-4β,17α,27-trihydroxy-1-oxowitha-2,24-dienolide (2), withaferin-A (3), 2,3-dihydrowithaferin-A (4), 6α,7α-epoxy-5α,20β-dihydroxy-1-oxowitha-2,24-dienolide (5), and 5β,6β-epoxy-4β-hydroxy-1-oxowitha-2,14,24-trienolide (6), respectively. Compounds 2, 3, 5, and 6 displayed inhibitory potential against butyrylcholinesterase, but only compounds 3, 4, and 6 were found to be active against acetylcholinesterase.

Key words *Withania somnifera*; Solanaceae; withanolide; withaferin A; acetylcholinesterase inhibition; butyrylcholinesterase inhibition

The withanolides are a group of naturally occurring steroids with a lactone-containing side chain of nine carbons attached at C-17.[1] They have been reported from the plants of Solanaceae, Taccaceae,[2] and Leguminosae[3] and also from some marine organisms.[4] *Withania somnifera* DUNAL. (Aswaghanda or Indian "Ginseng") is widely used in ayurvedic medicines and is consumed as a dietary supplement around the world.[5] The bruised leaves of this plant are used in the treatment of tumors and as an anti-inflammatory agent.[5] The methanolic extracts of different parts of *W. somnifera* exhibit therapeutic potential against various types of cardiovascular problems and are also effective against hyperlipidemia, obesity,[6] aging, and copper-induced pathophysiological conditions.[7] Other investigations indicated that *W. somnifera* also has antistrees,[8] immunomodulatory, cytotoxic, antibacterial, antifungal, and immunosuppressive properties.[1] This plant is reputed to promote vitality during recovery from chronic illnesses[9] and is useful for pain management in arthritic conditions.[10] Previous phytochemical investigations of this plant resulted in the isolation of more than 70 compounds.[10—14]

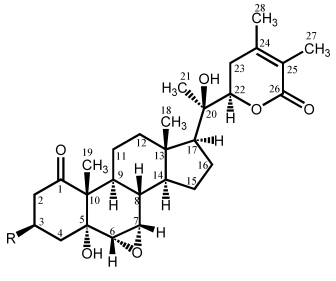
We report here the isolation of two new (**1**, **2**) and four known (**3**—**6**) withanolides from the whole plant of *W. somnifera*, along with their cholinesterase [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] inhibitory activity. AChE is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine.[15] According to the cholinergic hypothesis, memory impairment in patients with senile dementia of the Alzheimers type results from a deficiency in cholinergic function in the brain.[16] Hence the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic supplements. BChE is produced in the liver and enriched in the circulation. In addition, it is also present in adipose tissues, intestine, smooth muscle cells, white matter of the brain, and many other tissues.[17] The exact physiological function of BChE is still elusive. It is generally viewed as a back-up for homologous AChE and as a scavenger for anti-cholinesterase compounds.

The search for new cholinesterase inhibitors seems to be an important strategy to develop new drug candidates to prevent Alzheimer's disease and related dementias.

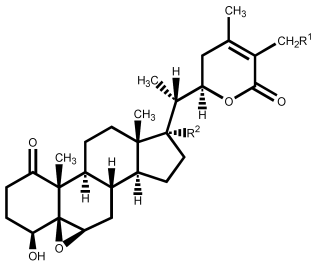
Results and Discussion

The methanolic extract of the whole plant of *W. somnifera* was evaporated to a gum and subjected to repeated column chromatography on silica gel to afford compounds **1**—**6**. Among them, two new compounds were characterized as 6α,7α-epoxy-3β,5α,20β-trihydroxy-1-oxowitha-24-enolide (**1**) and 5β,6β-epoxy-4β,17α,27-trihydroxy-1-oxowitha-2,24-dienolide (**2**).

Compound **1** was obtained as a white amorphous powder; which showed UV absorption at 200 nm, characteristic of a saturated hexanone moiety.[18] The overall spectral data of compound **1** closely resembled those of the known compound **5** (6α,7α-epoxy-5α,20β-dihydroxy-1-oxowitha-2,24-dienolide),[19] with the difference that the NMR spectra of



1 R= OH
5 R=H, $\Delta^{2,3}$



2 $R^1 = R^2 = OH$, $\Delta^{2,3}$
3 $R^1 = OH$, $R^2 = H$, $\Delta^{2,3}$
4 $R^1 = OH$, $R^2 = H$
6 $R^1 = R^2 = H$, $\Delta^{2,3}$, $\Delta^{14,15}$

* To whom correspondence should be addressed. e-mail: hej@cyber.net.pk

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) Spectral Data of Compounds **1** and **2** (δ in ppm)

Carbon	**1**[a] (J=Hz)		**2**[b] (J=Hz)	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1	210.8		202.2	
2	45.5	2.77 (dd, 14.1, 7.1) 3.74 (dd, 13.9, 9.8)	141.9	6.15 (d, 10.0)
3	73.8	4.91 (m, $W_{1/2}$=18)	132.1	6.92 (dd, 9.9, 5.7)
4	41.2	2.24 (dd, 13.3, 6.5)	69.8	3.74 (dd, 5.8, 2.3)
5	73.4		63.7	
6	57.4	3.03 (d, 3.2)	62.6	3.21 (br s, $W_{1/2}$=5.2)
7	57.1	3.25 (dd, 3.2, 2.1)	31.2	2.2 (m)
8	36.6	1.90 (m)	42.5	1.88 (m)
9	36.2	1.81 (m)	50.2	1.80 (m)
10	54.3		47.5	
11	22.9	1.74 (m)	22.0	1.62 (m)
12	39.3	1.80 (m)	23.0	1.78 (m)
13	44.8		47.8	
14	55.6	0.88 (m)	43.7	0.81 (m)
15	24.1	1.44 (m)	31.8	1.45 (m)
16	25.5	1.03 (m)	36.3	1.01 (m)
17	53.0	1.01 (m)	84.8	
18	14.4	0.99 (s)	14.6	0.78 (s)
19	16.6	1.21 (s)	9.4	1.44 (s)
20	76.2	1.91 (m)	30.1	1.85 (m)
21	20.4	1.32 (s)	17.4	1.00 (d, 6.6)
22	82.9	4.22 (dd, 13.2, 3.2)	79.1	4.63 (ddd, 11.3, 5.7, 2.8)
23	32.7	2.01 (m)	32.9	2.32 (m)
24	152.2		153.9	
25	122.5		125.2	
26	168.9		166.9	
27	123.5	1.80 (s)	57.4	4.34 (d, 12.5) 4.38 (d, 12.5)
28	20.8	1.99 (s)	19.9	2.00 (s)

a) In CD_3OD. *b*) In $CDCl_3$.

compound **1** lacked the signals for the C-2/C-3 double bond and contained an additional signal resonating at δ 4.91 ($W_{1/2}$=18 Hz) assigned to the C-3 proton, geminal to the secondary hydroxyl group, with a *pseudo* equatorial orientation.[20] The ^{13}C-NMR data (Table 1) of compound **1** showed 28 carbon signals representing five methyl, seven methylene, eight methine, and eight quaternary carbons. A downfield methine carbon signal resonating at δ 73.8 was assigned to the C-3 methine carbon, as inferred by HMBC interactions of H-2 (δ 2.77, 2.99) with C-1 (δ 210.8) and C-3 (δ 73.8), and interaction of H-4 (δ 2.24) with C-3 (δ 73.8). The stereochemistry at C-5, C-6, and C-7 was assigned by comparing the ^{13}C-NMR chemical shifts of compound **1** with those of the known compound nicandrin B, which indicated α orientations of the C-5 hydroxyl, and C-6/C-7 epoxy functionalities.[21] The assignment of stereochemistry was also supported by the NOESY spectrum, which showed interactions of C-6H with C-7H and C-8βH. The spectroscopic evidence thus led to the deduction of the structure **1** for this new withanolide.

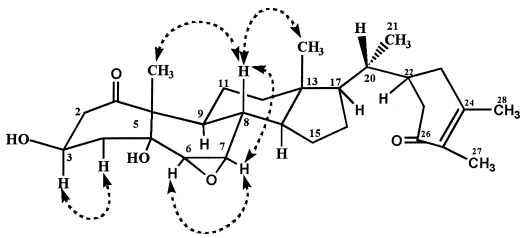


Fig. 1. Important NOESY Correlations of Compound **1**

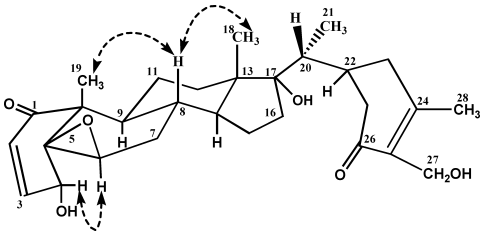


Fig. 2. Important NOESY Correlations of Compound **2**

Compound **2** was obtained as a white amorphous powder that showed spectral data closely resembling those of withaferin-A,[22,23] a well-known constituent of *Withania* plants. The main difference was the presence of a hydroxy group at C-17 in compound **2** as deduced from the downfield signal of C-22H appearing as a doublet of a double doublet at δ 4.63 ($J_{22\alpha,23\alpha}$=11.3 Hz, $J_{22\alpha,23\beta}$=5.7 Hz, $J_{22\alpha,20\beta}$=2.8 Hz).[24] The Newman projection of C-17 and C-20 bonds indicated an interaction between 17-OH and 22H, responsible for a downfield shift of C-22H.[22] The ^{13}C-NMR data of compound **2** showed signals for all 28 carbons (Table 1) including four methyl, seven methylene, nine methine, and eight quaternary carbons. A downfield quaternary carbon signal resonating at δ 84.8 was assigned to the hydroxyl-bearing C-17, as supported by HMBC interactions of H-21 (δ 1.00) and H-18

Table 2. Summary of the *in Vitro* Anticholinesterase Activities of Compounds **1**—**6**

Compound	IC_{50} (μM)±S.E.M.[a]	
	AChE	BChE
1	Inactive	Inactive
2	161.5±1.1	Inactive
3	84.0±1.5	125±3.2
4	Inactive	500±3.2
5	50±2.0	Inactive
6	124.0±1.1	62.5±2.0
Galanthamine[b]	0.50±0.001	8.2±0.01
Eserine[c]	0.04±0.0001	0.85±0.0001

a) Standard mean error of five assays. *b*, *c*) Positive controls used in the assays.

(δ 0.78) with C-17. The stereochemistry at C-17 was deduced to be α by comparing the ^{13}C-NMR data with those reported in the literature.[24]

Comparison of the spectral data of compounds **3**—**6** with the reported values led to their identification as known withanolides, withaferin-A (**3**),[22,23] 2,3-dihydrowithaferin-A (**4**),[23] 6α,7α-epoxy-5α,20-dihydroxy-1-oxowitha-2,24-dienolide (**5**),[19] and 5β,6β-epoxy-4β-hydroxy-1-oxowitha-2,14,24-trienolide (**6**).[22]

Compounds **1**—**6** were screened for their anti-cholinesterase activity in a mechanism-based assay (Table 2). Compounds **2** (IC_{50} 161.5 μM), **3** (IC_{50} 84.0 μM), **5** (IC_{50} 50.5 μM), and **6** (IC_{50} 124.0 μM) were found to be active against AChE. Similarly, compounds **3** (IC_{50} 125.0 μM), **4** (IC_{50} 500.0 μM), and **6** (IC_{50} 62.5 μM) inhibited the activity of BchE significantly.

Experimental

General Experimental Procedures The UV spectra were measured on a Hitachi U-3200 spectrophotometer. The IR spectra were recorded on a Jasco A-302 spectrophotometer. Optical rotations were measured on a Schmidt+Haensch Polartronic D polarimeter. The ^{1}H-NMR spectra were recorded on Bruker AM 400 and AMX 500 NMR spectrometers using the UNIX data system at 400 MHz, while the ^{13}C-NMR spectra were recorded at 100 MHz on the same instruments, using $CDCl_3$ and CD_3OD as solvents. The positive FAB-MS ion modes using xenon (8000 eV) and HR-EI-MS [ion source energy (70 eV), ion source temperature 250 °C] were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. Column chromatography (CC) was performed on silica gel (70—230 mesh size).

Plant Material The fresh plants (60 kg) of *W. somnifera* Dun., were collected from Karachi (Pakistan) and identified by the plant taxonomists in the Botany Department, University of Karachi. A voucher specimen (KUH 1550) was deposited in the herbarium of the University of Karachi.

Extraction and Isolation The plant material was dried in the shade. The dried plant material was crushed to powder (15.5 kg) and then soaked in methanol (60 l) for 3 weeks. The methanolic extract was then evaporated to a gum under a vacuum (1.1 kg). This gummy material was later suspended in H_2O (2 l), defatted with pet. ether (10 l), and then extracted with CH_2Cl_2 (15 l) and BuOH (5 l), respectively.

The dichloromethane extract was concentrated to a gum (95.5 g) and loaded on a silica gel column and eluted with the increasing polarities of pet. ether–CH_2Cl_2 and then with CH_2Cl_2–MeOH. The fraction obtained (185 mg) on elution with CH_2Cl_2–MeOH (95 : 5) was again subjected to silica gel CC to obtain subfractions A (35 mg), B (60.2 mg), C (20 mg), D (20.5 mg), and E (25.5 mg). Fractions A and B were again subjected to CC on silica gel, and as a result fraction A afforded compound **1** (7.2 mg) on elution with CH_2Cl_2–MeOH (94 : 6) and fraction B afforded compounds **2**, **3**, and **4** (15.2, 15.4, 12.0 mg, respectively) on elution with CH_2Cl_2–MeOH (98 : 2). Fraction C was subjected to preparative TLC in CH_2Cl_2 : MeOH (97 : 3), affording compound **5** (10.3 mg). Fraction D yielded compound **6** (12.5 mg) on elution with CH_2Cl_2–MeOH (99 : 1).

6α,7α-Epoxy-3β,5α,20β-trihydroxy-1-oxowitha-24-enolide (**1**): White amorphous powder (7.2 mg, 3.5×10^{-3}% yield); $[\alpha]_D^{25}$ −196° (c=0.006, MeOH); UV λ_{max} (MeOH) nm (log ε): 200 (3.46); IR (MeOH) cm^{-1}: 3417, 1706; FAB-MS $[M+H]^+$ m/z: 489; EI-MS m/z (rel. int. %): 263 (18.4), 238 (8.3), 211 (4.6), 169 (13.5), 171 (15.6), 152 (12.5), 125 (100), 97 (30.1), 55 (21); ^{1}H-NMR (CD_3OD, 400 MHz) for δ, see Table 1. ^{13}C-NMR (CD_3OD, 100 MHz) for δ, see Table 1.

5β,6β-Epoxy-4β,17α,27-trihydroxy-1-oxowitha-2,24-dienolide (**2**): White amorphous powder (15 mg, 7.5×10^{-3}% yield); $[\alpha]_D^{25}$ +12° (c=0.11, CH_2Cl_2); UV λ_{max} ($CDCl_3$) nm (log ε) 221 (2.68); IR ($CDCl_3$) cm^{-1}: 3445, 1682; FAB-MS $[M+H]^+$ m/z 487; EI-MS m/z (rel. int. %): 416 (15.8), 309 (2.6), 281 (3.9), 124 (100), 141 (51.5), 171 (15.6); ^{1}H-NMR ($CDCl_3$, 400 MHz) for δ, see Table 1. ^{13}C-NMR ($CDCl_3$, 100 MHz) for δ, see Table 1.

***In Vitro* Cholinesterase Inhibition Assay** Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), eserine, and galanthamine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grades. AChE and BChE inhibition was measured using the spectrophotometric method developed by Ellman *et al.*[25] Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay AChE and BChE activities, respectively. The reaction mixture contained (100 mM) 150 μl of sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 10 μl of test compound solution and 20 μl of AChE or BChE solution, which were mixed and incubated for 15 min (25 °C). The reaction was then initiated with the addition of 10 μl acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of a yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). Test compounds and the positive control (eserine and galanthamine) were dissolved in EtOH. All the reactions were performed in triplicate (3 wells) in 96-well microplates in SpectraMax 340 (Molecular Devices, U.S.A.). The percentage (%) inhibition was calculated as $(E-S)/E\times100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Determination of IC_{50} Values The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) (Table 2) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, MA, U.S.A.).

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References and Notes

1) Atta-ur-Rahman, Yousaf M., Gul W., Qureshi S., Choudhary M. I., Voelter W., Hoff A., Jens F., Naz A., *Heterocycles*, **48**, 1801—1811 (1998).
2) Chen L. Z., Wang B. D., Chen M. Q., *Tetrahedron Lett.*, **28**, 1673—1676 (1987).
3) Srivastava C., Siddiqui I. R., Singh J., Tiwari H. P., *J. Ind. Chem. Soc.*, **69**, 111 (1992).
4) Ksebati M. B., Schmitz F. J., *J. Org. Chem.*, **53**, 3926—3929 (1988).
5) Jayaprakasam B., Zhang Y., Seeram N. P., Nair M. G., *Life Sci.*, **74**, 125—132 (2003).
6) Mary N. K., Babu B. H., Padikkala J., *Phytomedicine*, **10**, 474—482 (2003).
7) Gupta S. K., Dua A., Vohra B. P., *Drug Metab. Drug Interact.*, **19**, 211—222 (2003).
8) Bhattacharya S. K., Muruganandam A. V., *Pharmacol. Biochem. Behav.*, **75**, 547—555 (2003).
9) Singh A., Naidu P. S., Kulkarni S. K., *J. Med. Food*, **5**, 211—220 (2002).
10) Bandyopadhyay M., Jha S., *J. Trop. Med. Plants*, **4**, 273—284 (2003).
11) Atta-ur-Rahman, Jamal A. S., Choudhary M. I., *Heterocycles*, **34**, 689—698 (1992).
12) Atta-ur-Rahman, Jamal A. S., Choudhary M. I., Asif I., *Phytochemistry*, **30**, 3824—3825 (1991).
13) Choudhary M. I., Abbas S., Jamal A. S., Atta-ur-Rahman, *Heterocycles*, **42**, 555—563 (1996).

14) Atta-ur-Rahman, Abbas S., Dur-e-Shawar, Jamal A. S., Choudhary M. I., *J. Nat. Prod.*, **56**, 1000—1006 (1993).
15) Tougu V., *Curr. Med. Chem.*, **1**, 155—170 (2001).
16) Perry E. K., *Br. Med. Bull.*, **42**, 63—69 (1986).
17) Schwarz M., Glick D., Loewensten Y., Soreq H., *Pharmacol. Ther.*, **67**, 283—289 (1995).
18) Scott A. I., "Interpretation of UV Spectra of Natural Products," Pergamon Press, Oxford, 1964, p. 30.
19) Anjaneyulu A. S. R., Rao D. S., *Ind. J. Chem.*, **36B**, 424—433 (1997).
20) Ramaiah P. A., Lavie D., Budhiraja R. D., Sudhir S., Garg K. N., *Phytochemistry*, **23**, 143—149 (1984).
21) Bagchi A., Neogi P., Sahai M., Ray A. B., Oshima Y., Hikino H., *Phytochemistry*, **23**, 853—855 (1984).
22) Kirson I., Glotter E., Lavie D., *J. Chem. Soc. C*, **11**, 2032—2044 (1971).
23) Kupchan S. M., Anderson W. K., Bolinger P., Doskotch R. W., Smith R. M., Renauld J. A. S., Schones H. K., Burlingeame A. L., Smith D. H., *J. Org. Chem.*, **34**, 3858—3866 (1969).
24) Nittala S. S., Lavie D., *Phytochemistry*, **29**, 2741—2748 (1981).
25) Ellman G. L., Courtney D., Andres K. D. V., Featherstone R. M., *Biochem. Pharmacol.*, **7**, 88—95 (1961).