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ANTIFUNGAL STEROIDAL LACTONES FROM *WITHANIA COAGULANCE*

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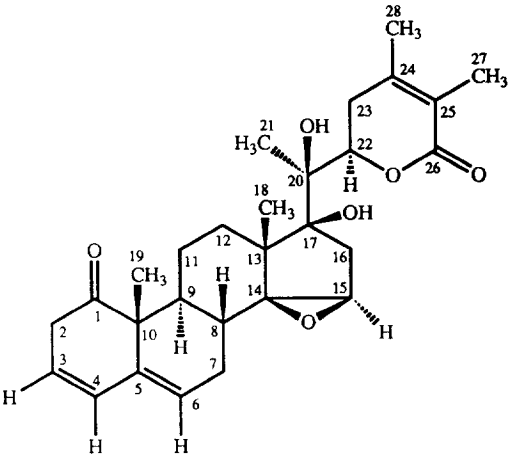
Key Word Index—*Withania coagulance*; Solanaceae; withanolides; 14,15β-expoxywithanolide I; 17β-hydroxywithanolide K; antifungal activity.

Abstract—Two new withanolides, 14,15β-epoxywithanolide I [(20*S*,22*R*) 17β,20β-dihydroxy-14β,15β-epoxy-1-oxo-witha-3,5,24-trienolide] and 17β-hydroxywithanolide K (20*S*,22*R*) 14α,17β,20β-trihydroxy-1-oxo-witha-2,5,24-trienolide] have been isolated from the whole plant of *Withania coagulance* and their structures were elucidated by spectroscopic techniques. The latter compound was found to be active against a number of potentially pathogenic fungi.

INTRODUCTION

Withanolides are C_{28} steroidal lactones, which have been isolated from different plants of the family Solanaceae such as *Withania somnifera*, *W. coagulance*, *Acnistus australis* and *Datura metel*. Plants of genus *Withania* are known to exhibit a variety of pharmacological activities mainly due to the presence of withanolides [1–3].

Our work on the withanoidal constituents of *W. coagulance* has already resulted in the isolation of a number of new withanolides [4]. We now describe the isolation and structure elucidation of two new withanolides, 14,15β-epoxywithanolide I (**1**) and 17β-hydroxywithanolide K (**2**), from this plant. The structures of these new compounds have been elucidated by spectroscopic studies. A crude extract of the plant and compound **2** were found to be active against a number of pathogenic fungi.



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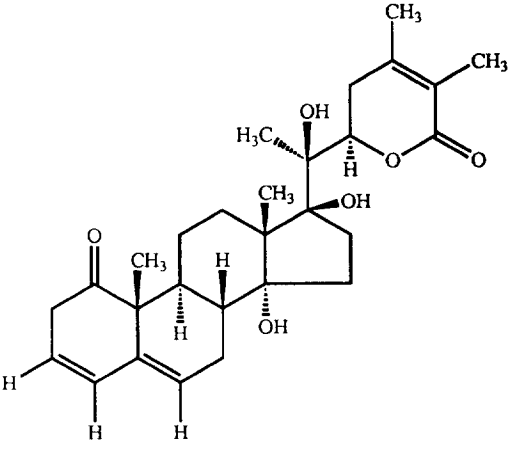
RESULTS AND DISCUSSION

The two new withanolides were isolated from the ethanolic extracts of the whole plant by column and thin-layer chromatographic techniques. The EI mass spectra of **1** showed the $M^+ - 18$ ion at *m/z* 450.2465 analysing for $C_{28}H_{34}O_5$ (0.3 mmu deviation). Hence, compound **1**, $C_{28}H_{36}O_6$, possessed 11 degrees of unsaturation. Seven of these were eventually accounted for by the pentacyclic α,β-unsaturated steroidal lactone skeleton, two were due to double bonds, one for the ketonic carbonyl and one for the epoxide. The FAB mass spectra (positive ion, glycerol) gave a major $[M + H]^+$ ion at *m/z* 469. The ion at *m/z* 125.0666 of composition $C_7H_9O_2$ further confirmed the presence of a six membered lactone



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substituent at the C-20 side chain of the main steroidal skeleton. The ion at *m/z* 169.0902 ($C_9H_{13}O_3$) could arise by the cleavage of the C-17/C-20 bond, while the fragment *m/z* 299.1716 ($C_{19}H_{23}O_3$) represented the remaining half of the molecule. The overall mass fragmentation pattern of **1** was characteristic of withanolides [5].

The UV spectrum of **1** showed an absorption maximum at 227 nm, characteristic of an α,β-unsaturated lactone chromophore [6]. The IR spectrum indicated the presence of a hydroxyl group, a six membered cyclic ketone and an α,β-unsaturated lactone [7].

The ^{1}H NMR and ^{1}H ^{1}H COSY spectra were used to obtain further structural information. The ^{1}H NMR spectrum exhibited signals for five tertiary methyl groups at δ1.40, 1.42, 1.70, 1.78 and 1.93. Two mutually coupled olefinic signals resonating at δ5.55 (multiplet) and 6.08 (*dd*, $J_{4,3}$ = 9.7 Hz, $J_{4,2}$ = 2.0 Hz) were assigned to the C-3 and C-4 vinylic protons, respectively. Another downfield proton resonating at δ5.81 (*dd*, $J_{6,7a}$ = 5.1 Hz, $J_{6,7b}$ = 2.4 Hz) was not coupled with any other olefinic proton and was in turn coupled with two protons of a methylene group (δ2.62 and 2.10). These observations indicated a trisubstituted conjugated diene with a quaternary olefinic carbon (C-5) which lies between the olefinic bonds. This arrangement of double bonds is only possible in rings A and B. A one-proton doublet of doublet centred at δ3.10 ($J_{15,16a}$ = 3.2 Hz, $J_{15,16b}$ = 1.6 Hz) was characteristic of a proton geminal to the epoxide function and it was assigned to the C-15 methine proton. A downfield double doublet at δ5.21 ($J_{22,23a}$ = 13.7 Hz, $J_{22,23b}$ = 3.5 Hz) was assigned to the C-22 methine proton of the lactone moiety.

The C-3 vinylic proton (δ5.55) showed vicinal couplings in the COSY-45° spectrum with the C-2α (δ2.70) and β (δ3.32) protons as well as with the C-4 vinylic proton (δ6.08). The C-4 proton also exhibited allylic couplings with the C-2α (δ2.70) and β (δ3.32) protons. The C-15 oxirane proton (δ3.10) exhibited couplings with the C-16 methylene protons (δ1.11 and 1.32). The β-stereochemistry of the C-14/C-15 epoxide was deduced on the basis of ^{1}H NMR chemical shift comparison with other compounds [8, 9] and on the basis of coupling constants ($J_{15,16a}$ = 3.2, $J_{15,16b}$ = 1.6 Hz). The C-22 methine proton showed coupling with the C-23 methylenic protons resonating at δ2.75 and 3.08, respectively. The C-23 methylene protons showed weak homoallylic coupling with the C-27 methyl protons.

The ^{13}C NMR spectra of **1** showed resonances for all 28 carbons. A notable feature was the appearance of a downfield signals for the quaternary and tertiary carbons at δ62.2 and 50.9, respectively, which were assigned to the epoxy bearing C-14 and C-15. The chemical shifts of the various carbons are listed in Table 1. One-bond ^{1}H–^{13}C correlations were determined by the HMQC [10] technique, and the results are presented in Table 1.

The HMBC data was used to connect different structural fragments as well as to confirm the above chemical shift assignments. For instance, the C-4 proton displayed long-range shift correlations with the C-2, C-5 and C-10 carbons, while the C-3 protons showed couplings with the C-6 and C-10 carbons. The C-18 methyl protons exhibited long-range shift correlations with the C-17 and C-14 carbons. Other important long-range couplings were between C-25 and 23-H and 28-H, while the C-28 protons also showed coupling with C-27. Other couplings of C-20 with 18-H and 16-H helped to confirm the proton–carbon assignments. These data led to structure **1** for the compound.

The EI mass spectra of **2** afforded the M^+ at *m/z* 470.2677 corresponding to the formula $C_{28}H_{38}O_6$, and indicated 10 degrees of unsaturation. The presence of hydroxyl, ketonic carbonyl and α,β-unsaturated lactone was indicated by the IR absorptions at 3420, 1690 and 1675 cm^{-1}. The presence of an α,β-unsaturated lactone was also indicated by the UV absorption at 223 nm.

The ^{1}H NMR spectrum of **2** showed distinct resemblance to that of **1**. The only notable difference was the lack of a doublet of doublet centred at δ3.10, indicating that **2** did not contain a C14–C15 epoxide. Similarly, the ^{13}C NMR spectrum showed an additional downfield signal at δ82.0, which may be assigned to an oxygen-bearing carbon. Based on spectral observations, such as one less degree of unsaturation, the presence of a downfield ^{13}C resonance and the absence of characteristic epoxide signals in the ^{1}H and ^{13}C NMR spectra of the compound, as compared to **1**, structure **2** was deduced for the compound, which contains a hydroxyl group at C-14 instead of an expoxide functionality.

Compound **2** was obtained earlier by partial derivatization of withanolide F by Vande Velde *et al.* [11], but has not been isolated from natural sources. Compound **2** exhibited antifungal activity against human pathogens *Nigrospora oryzae*, *Aspergillus niger*, *Curvularia lunata*, *Stachybotrys atra*, *Allescheria boydii*, *Drechslera rostrata*, *Microsporum canis* and *Epidermophyton floccosum* and plant pathogen *Pleurotus ostreatus* (MIC 300 μg/mL) [12]. It also showed activity against gram positive *Staphylococcus aureus* [13].

EXPERIMENTAL

General experimental procedure. Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a JASCO 302-A spectrophotometer. UV spectra were recorded on a Hitachi U 3200 spectrophotometer. EI, FAB and HREI MS were recorded on JMS HX110 with data system and on JMS-DA 500 mass spectrometers. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker spectrometers operating at 500, 300 and 125 MHz. The chemical shifts values are reported in ppm (δ) units and the coupling constants (J) are in Hz. Standard pulse sequences were used for COSY, DEPT, HMQC and HMBC experiments.

Chromatographic conditions. TLC: precoated silica G-25, UV_{254} plates. *CC*: silica gel, 230–400 mesh. Visualization of the TLC plates was achieved at 250 and 336 nm and Dragendorff's spray reagent was used for detection.

Table 1. ^{13}C NMR data and ^{1}H ^{13}C connectivities in **1** and **2**

	Compound **1**			Compound **2**		
Carbon	Chemical shift (δ)	Multiplicity	^{1}H ^{13}C† Connectivity (J = Hz)	Chemical shift (δ)	Multiplicity	$^{1}H/^{13}C$† connectivity (J = Hz)
C-1	210.0	C		197.0	C	
C-2	39.7	CH_2	3.32 *m* 2.70 *m*	39.2	CN_2	3.32 *m* 2.71 *m*
C-3	127.8	CH	5.55 *m*	127.1	CH	5.57 *m*
C-4	129.5	CH	6.08 *dd* ($J_{4,3}$ = 9.7, $J_{4,2}$ = 2.0)	129.0	CH	6.01 *dd* ($J_{4,3}$ = 9.6, $J_{4,2}$ = 2.3)
C-5	140.4	C		140.1	C	—
C-6	121.7	CH	5.81 *dd* ($J_{6,7a}$ = 5.1, $J_{6,7b}$ = 2.4)	125.2	CH_2	5.71 *dd* ($J_{6,7b}$ = 4.9, $J_{6,7a}$ = 2.1)
C-7	32.7	CH_2	2.62 *m* 2.10 *m*	25.2	CH_2	2.10 *m* 2.01 *m*
C-8	35.2	CH	1.80 *m*	35.4	CH	1.75 *m*
C-9	32.4	CH	1.68 *m*	35.1	CH	1.87 *m*
C-10	52.3	C		52.3	C	—
C-11	21.6	CH_2	1.55 *m*	21.5	CH_2	1.50 *m*
C-12	25.9	CH_2	1.58 *m*	34.0	CH_2	1.35 *m*
C-13	52.0	C		54.0	C	—
C-14	62.2	C		82.0	C	—
C-15	50.9	CH	3.10 *dd* ($J_{15,16a}$ = 3.2, $J_{15,16b}$ = 1.6)	29.8	CH_2	2.01 *m* 1.85 *m*
C-16	37.9	CH_2	1.32 *m* 1.11 *m*	36.1	CH_2	1.34 *m* 1.02 *m*
C-17	87.9	C		87.0	C	
C-18	20.1	CH_3	1.42 *s*	19.8	CH_3	1.02 *s*
C-19	20.3	CH_3	1.70 *s*	18.2	CH_3	1.19 *s*
C-20	79.9	C		78.2	C	
C-21	17.7	CH_3	1.40 *s*	18.3	CH_3	1.32 *s*
C-22	80.4	CH	5.21 *dd* ($J_{22,23a}$ = 13.7, $J_{22,23b}$ = 3.5)	81.0	CH	4.84 *dd* ($J_{22,23a}$ = 12.2, $J_{22,23b}$ = 4.2)
C-23	30.1	CH_2	3.08 *m* 2.75 *m*	31.7	CH_2	2.55 *m* 2.45 *m*
C-24	151.5	C		151.2	C	
C-25	121.5	C		120.5	C	
C-26	164.0	C		167.5	C	
C-27	12.0	CH_3	1.78 *s*	11.2	CH_3	1.78 *s*
C-28	20.6	CH_3	1.93 *s*	19.8	CH_3	1.86 *s*

* Multiplicities determined by DEPT experiments.

† One-bond heteronuclear connectivities determined by HMQC experiment.

Plant material. The whole plant of *W. coagulance* Dun. was collected from the suburban area of Karachi in April 1990. The plant material was identified by Mr Tahir Ali, Plant Taxonomist, Department of Botany, University of Karachi. A voucher specimen was deposited in the herbarium (KUH-46528) of Karachi University.

Extraction and isolation. The dried plant (50 kg) was extracted with EtOH at room temp. for 1–2 weeks and the resulting extract was concd to a gum. This gum (976 g) was partitioned between *n*-hexane and MeOH. The defatted MeOH extract was evapd and dissolved in H_2O. The aq. extract was extracted with $CHCl_3$ at different pH values (pH 9–10 and pH 2–3), the pH being adjusted by the addition of AcOH and NH_4OH solns. The fr. containing the bases (pH 9–10) was subjected to CC on silica gel. Elution with *n*-hexane and then with *n*-hexane–$CHCl_3$ yielded several frs. A fr. obtained by

elution with *n*-hexane–$CHCl_3$ (1:4) was further sepd on the basis of solvent–solvent sepn (Et_2O and $CHCl_3$) and fine crystals of **1** were obtained. The fr. containing the acids (pH 2–3) was subjected to CC on silica gel. Elution with *n*-hexane and then with *n*-hexane–$CHCl_3$ yielded several fractions. Fractions obtained by elution with *n*-hexane–$CHCl_3$ (1:9) were combined and again subjected to CC on silica gel. The column was eluted with *n*-hexane–$CHCl_3$ (1:1). First few fractions of the column were found to contain white precipitate of **2**.

14,15β-Epoxywithanolide I (**1**): Crystals, mp 203–204°, $[\alpha]_D$ 113.8° ($CHCl_3$). UV λ_{max} (MeOH): 227: IR ν_{max} ($CHCl_3$) cm^{-1}: 3375 (O–H), 1705, 1695 (lactone carbonyl and ketone carbonyl). ^1H NMR (C_5D_5N, 300 MHz) δ: see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: see Table 1.

17β-Hydroxywithanolide K (**2**): Greenish amorphous solid, mp 190–191° $[\alpha]_D$ + 42.3° ($CHCl_3$). UV λ_{max} (MeOH): 223 nm. IR ν_{max} ($CHCl_3$): 3420, 1690, 1675. ^1H NMR (CD_3OD + $CDCl_3$, 500 MHz) δ: see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: see Table 1.

Antimicrobial assays. Antifungal assay was performed by agar tube diffusion method using griseofulvin as positive control. Antibacterial activity was determined by agar well diffusion method using ampicillin as positive control.

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