NEW STEROIDAL LACTONES FROM *WITHANIA COAGULANCE*

Atta-ur-Rahman,[a]* Muhammad Shabbir,[a] Dur-e-Shahwar,[a] M. Iqbal Choudhary,[a]* Wolfgang Voelter,[b] and Dirk Hohnholz[b]

[a]International Centre for Chemical Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan

[b]Leiter der Abteilung Für Physikalische Biochemic Physiologisch-Chemisches Institüt, Der Universitate Tübingen Hoppe-Seyler, Strasse 4, D-7400, Tübingen I, Germany

Abstract- Four new withanolides, coagulin B (**1**), coagulin C (**2**), coagulin D (**3**) and coagulin E (**4**) were isolated from the aerial parts of *Withania coagulance*. The structures (**1-4**) have been determined through spectroscopic studies.

Genus *Withania* (Solanaceae) has six species distributed in North Africa, West Asia, South Europe, China and South Asia.[1] *Withania coagulance* Dunal is widely found throughout the South Asian subcontinent.[1] The fruits of the plant are employed as emetic, stomachic, blood purifier, diuretic, febrifuge, bitter tonic in dyspepsia and as a growth promoter in infants.[2-4] The plant is also used in the treatment of asthma, biliousness, strangles and colic infections.[5]

The UV spectrum of coagulin B (**1**) (*m/z* 452.2536; $C_{28}H_{36}O_5$) showed absorption at 223 nm characteristic of α,β-unsaturated ketone and an unsaturated lactone system.[6] The ^{1}H-NMR spectrum of **1** showed two downfield doublet of double doublets at δ 6.77 and 5.88, and a broad doublet at δ 5.60, assigned to the olefinic C-3, C-2 and C-6 protons respectively. These signals are characteristic of a 2,5-dien-1-one system[7] in rings A and B of withanolides. A downfield double doublet at δ 4.38 (J =12.0 Hz, 4.6 Hz), assigned to the C-22 methine protons of the lactone moiety, is a diagnostic signal in all withanolides. In addition to these signals two AB doublets resonating at δ 4.25 and 4.29 (2H, $J_{27a,27b}$ = 13.0 Hz) were assigned to C-27 hydroxymethylene protons. A 3H singlet at δ 1.07 was due to the C-19 methyl protons while other three tertiary methyls (CH_3-21, CH_3-18, CH_3-28) resonated as singlets at δ 1.25, 1.30 and 2.04.

The presence of an ether linkage in **1** was supported by the HREIMS (*m/z* 452.2536, $C_{28}H_{36}O_5$) which indicated eleven degrees of unsaturation. Four of these were accounted for by the tetracyclic steroidal skeleton, three by double bonds, two by the lactone and one by the C-1 ketonic carbonyl. The presence of an additional double bond in the skeleton was ruled out on the basis of ^{1}H- and ^{13}C-NMR spectra which indicated the presence of only three -C=C- (double bonds) in the molecule. The mass fragments at *m/z*

Dedicated to Dr. Koji Nakanishi on the occasion of his 75th birthday.

169.0904 ($C_9H_{13}O_3$) and 311.1944 ($C_{21}H_{27}O_2$) indicated the presence of only three oxygen functionalities in the lactone side chain while two oxygen were present in the cyclopentanophenanthrene part (A,B,C, and D ring). Excluding the ketonic oxygen of ring A, oxygen of primary hydroxyl group and two lactonic oxygens, only one oxygen remained to be incorporated in the skeleton, whereas the ^{13}C-NMR spectra exhibited signals for two oxygen bearing carbons in the ring D and in the C-17 side chain. These results indicated the presence of an ether linkage either between C-14/C-17 or C-14/C-20. The ether linkage is only possible between C-14/C-20 which is less strained as compared to the four-membered ether ring through C-17/C-20 (joining of C-14 with C-17 would afford a 4-membered cyclic ether within a 5-membered ring which would be too strained to exist).[8]

The homonuclear COSY-45° spectrum of **1** showed two important spin- systems in the molecule. Olefinic H-3 (δ 6.77) showed coupling with H-4α (δ 2.63) and H-4β (δ 3.00) as well as with the olefinic H-2 (δ 5.88). The methine H-22 (δ 4.38) showed cross-peaks with methylenic H_2-23 (δ 2.30, 2.00). The spin systems deduced on the basis of the COSY-45° spectrum are shown in Figure **1**. The stereochemistry at C-22 was assumed to be *R* on biogenetic grounds as found in related withanolides of the series.[9] The stereochemistry at C-17 was inferred to be β (*R*) based on the fact the alternative arrangement (*S*) is not possible when α-oriented ether bridge exist between C-17/C-20.[8]

The broad-band decoupled ^{13}C-NMR spectrum (Table **2**) of **1** indicated the presence of 28 carbon atoms in the molecule. DEPT spectra showed the presence of four methyl, eight methylene and seven methine carbons. The remaining nine quaternary carbon signals appeared in the broad-band ^{13}C-NMR spectrum. Downfield signals at δ 206.3 and 166.3 were due to the ketone and lactone carbonyls, respectively. The three vinylic carbons of rings A and B resonated at δ 127.3 (CH), 146.2 (CH) and 125.9 (CH) were due to C-2, C-3 and C-6, respectively, while the quaternary C-5 appeared at δ 135.70. The C-14 and C-20 linked through an ether linkage resonated at δ 86.2 and 84.5 respectively.

The one-bond ^{1}H/^{13}C chemical shift correlations in **1** were determined by the HMQC experiment ($CDCl_3$ + CD_3OD).[10] The carbon resonating at δ 33.2 (C-4) was coupled with H-4 (δ 2.63, δ 3.00) while the carbon at δ 32.3 (C-23) showed direct ^{1}H/^{13}C interactions with the protons at δ 2.10 and 2.30 (H-23α and H-23β). The downfield methine protons at δ 5.60 (H-6), 6.77 (H-3), 5.88 (H-2) and 4.38 (H-22) showed one-bond heteronuclear interactions with the carbons resonating at δ 125.9 (C-6), 127.3 (C-2), 146.2 (C-3) and 81.7 (C-22).

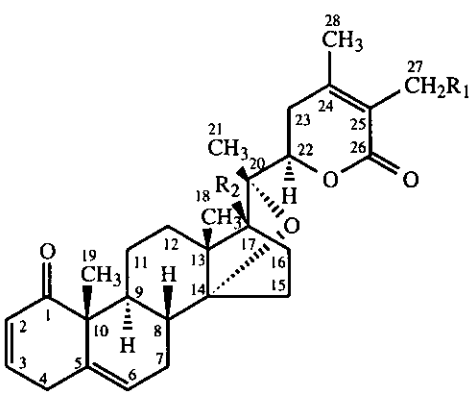
The long-range ^{1}H/^{13}C correlations in **1** were determined by the HMBC experiment[11] ($CDCl_3$ + CD_3OD) which showed that H-4 (δ 33.2) has long-range coupling with C-1. The Me-19 protons (δ 1.07) showed long-range couplings with C-10 (δ 50.7), C-1 (δ 206.3) and C-5 (δ 135.7). The C-18 methyl protons (δ 1.30) also showed long-range correlations with C-13 (δ 47.1), C-12 (δ 25.0) and C-14 (δ 86.2). The C-23 methylene protons resonating at δ 2.30 and 2.10 showed interactions with C-22 (δ 81.7) and C-24 (δ 155.2). The chemical shift assignments were made on the basis of hetero-COSY and HMQC experiments[10] and by comparing the spectral data with those of reported withanolides having a similar skeleton.[12,13]

The HREIMS of **1** showed the M^+ ion at *m/z* 452.2536 corresponding to the molecular formula $C_{28}H_{36}O_5$ (calcd 452.2536). The ion at *m/z* 141.0652 ($C_7H_{10}O_3$) resulted from the cleavage at C-20/C-22 and indicated the presence of C-27 OH and a six-membered lactone moiety,[9] while the fragment *m/z* 311.1944 ($C_{21}H_{27}O_2$) represented the remaining portion of the molecule. On the basis of the above spectroscopic studies structure (**1**) was assigned to coagulin B.

Coagulin C (**2**) *m/z* 452.2588; $C_{28}H_{36}O_5$ showed a UV absorption band at 223 nm. The IR spectrum revealed the presence of a hydroxy group (3400 cm^{-1}), α,β-unsaturated ketone (1660 cm^{-1}) and α,β-unsaturated δ lactone (1700 cm^{-1}) group.

The ^1H NMR spectrum of **2** showed three olifinic signals at δ 6.75 (1H, ddd, *J*= 10.0 Hz, *J* = 4.0 Hz, *J* = 2.4 Hz H-2), 5.84 (1H, ddd, *J* = 10.0 Hz, *J* = 3.0 Hz, *J* = 1.0 Hz) and 5.58 (1H, br d, *J* =5.7 Hz) The proton attached to the C-22 carbon appeared as a double doublet at δ 4.93 (J_1=12.0 Hz, J_2 = 4.5 Hz) which was found to be further downfield than **1** indicating the presence of oxygen functionalities at C-20 and C-17 carbons. The molecular formula of **2** was established as $C_{28}H_{36}O_5$ from the HREIMS. The ion at *m/z* 125 of composition $C_7H_9O_2$ resulted by the cleavage of the C-20/C-22 bond, confirming the presence of a six-membered lactone substituted at C-20 of the steroidal skeleton, while the fragment ion at *m/z* 327.1900 ($C_{21}H_{27}O_3$) represented the remaining portion of the molecule. The main difference between the ^1H-NMR spectra of **2** and **1** was the absence of downfield signals at δ 4.25 and 4.29 due to the C-27 methylene protons in **2**.

The broad-band decoupled ^{13}C-NMR (Table **2**) and DEPT spectra of **2** indicated the presence of twenty eight carbons in the molecule. The downfield signal for C-27 present in **1** was absent in the ^{13}C-NMR spectrum. However C-27 resonated as a methyl at δ 12.5 An additional downfield hydroxyl-bearing quaternary carbon signals at δ 79.2 was also observed which was ascribed to C-17. The C-17 hydroxyl group was proposed to be β-oriented on the basis of ^{13}C-NMR chemical shift as present in several known withanolides.[15]



1 R_1 = OH, R_2 = H
2 R_1 = H, R_2 = OH
3 R_1 = R_2 = H

The HREIMS of couglin D (**3**) showed the molecular ion at *m/z* 436.2614 consistent with the molecular formula $C_{28}H_{36}O_4$ (calcd 436.2614). In the EIMS, two significant peaks were at *m/z* 125 and 311 corresponding to the ions $C_7H_9O_2$ and $C_{21}H_{27}O_2$ respectively. The ^{1}H-NMR spectrum closely resembled that of couglin C (**2**). The major difference was the absence of a hydroxy group at the C-17 carbon. The absence of a hydroxyl group was further confirmed by the broad-band decoupled ^{13}C-NMR (Table **2**) and DEPT spectra. The NMR assignments for coagulin D (**3**) were made with the help of DEPT and COSY-45° spectra.

The EIMS spectrum of coagulin E (**4**) $C_{28}H_{36}O_4$ (*m/z* 436.2613) showed characteristic fragments at *m/z* 125 formed by the loss of a lactone ring and at *m/z* 311 for the steroidal skeleton.

The ^{1}H-NMR spectrum of coagulin E (**4**) resembled that of coagulin D (**3**), the only difference being the presence of a downfield proton signal as a double doublet at δ 5.56. This indicated that the double bond in coagulin E (**4**) was located between C-3 and C-4. The COSY-45° spectrum of **4** exhibited three isolated spin systems. The first spin system involved the protons at C-2, C-3 and C-4 (δ 3.24, H-2β; δ 2.71, H-2α; δ 5.56, H-3 and δ 5.66, H-4). The second spin system includes protons at C-6 and C-7 (δ 5.62, H-6; δ 1.98, H-7β; δ 2.30, H-7α). Third spin system occurred between the protons at C-22 (δ 4.90) and C-23 (δ 2.48, δ 1.88). The structure of coagulin E (**4**) was established on the basis of HMBC and HMQC experiments. The ^{13}C-NMR chemical shifts were assigned in Table **2**.

Table-1 ^{1}H-NMR shifts of withanolides (**1-4**).

Compd.	2H	3-H	4-H	6-H	22-H	18-H$_3$	19-H	21-H	27-H$_3$	28-H$_3$
*1	5.88 ddd (10,3,1)	6.77 ddd (10,5,2.5)	-	5.60 br d (5.1)	4.38 dd (12,4.6)	1.30s	1.07s	1.25s	2.04s	-
2	5.84 ddd (10,3,1)	6.75 ddd (10,4,2.4)	-	5.58 br d (5.7)	4.93 dd (12,4.5)	1.21s	1.11s	1.40s	1.86s	1.92s
3	5.84 ddd (10,3,1)	6.76 ddd (10,5,2.5)	-	5.61 br d (5.3)	4.35 dd (11.6,5)	1.30s	1.22s	1.42s	1.87s	1.95s
4	-	5.56 ddd (10,3,1)	5.66 dt (9.5,2)	5.62 dd (5,2.8)	4.90 dd (11.8, 5)	1.28s	1.18s	1.43s	1.86s	1.96s

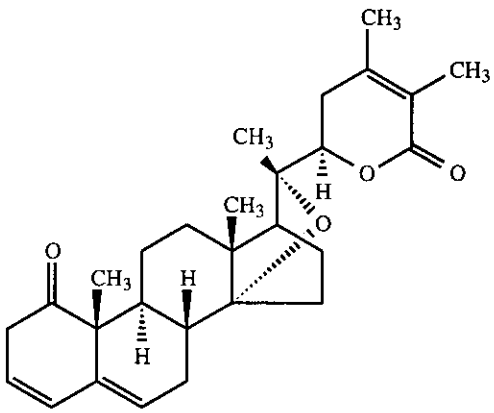
Spectra were recorded at 300 MHz in $CDCl_3$ solution, chemical shifts are in δ units. Coupling constants (in Hz) are in parentheses.

*Spectrum was recorded at 300 MHz in $CDCl_3$ + CD_3OD solution.

Table-2: ^{13}C-NMR Chemical Shifts (δ) of **1-4** in $CDCl_3$ at 125 MHz.

	Coagulin B(**1**)	Coagulin C (**2**)	Coagulin D (**3**)	Coagulin E (**4**)
Carbon	Chemical Shift (multiplicities)*	Chemical Shift (multiplicities)*	Chemical Shift (multiplicities)*	Chemical Shift (multiplicities)*
1	206.3 (C)	205.7 (C)	204.6 (C)	210.0 (C)
2	127.3 (CH)	128.1 (CH)	127.8 (CH)	39.7 (CH_2)
3	146.2 (CH)	146.6 (CH)	145.7 (CH)	127.7 (CH)
4	33.2 (CH_2)	34.0 (CH_2)	33.3 (CH_2)	129.5 (CH)
5	135.7 (C)	135.6 (C)	134.4 (C)	140.4 (C)
6	125.9 (CH)	126.0 (CH)	125.6 (CH)	121.2 (CH)
7	32.0 (CH_2)	32.7 (CH_2)	31.8 (CH_2)	37.9 (CH_2)
8	35.1 (CH)	36.4 (CH)	38.5 (CH)	36.2 (CH)
9	35.9 (CH)	37.8 (CH)	37.1 (CH)	34.0 (CH)
10	50.7 (C)	51.0 (C)	50.9 (C)	54.8 (C)
11	22.0 (CH_2)	23.7 (CH_2)	23.3 (CH_2)	21.9 (CH_2)
12	25.0 (CH_2)	26.1 (CH_2)	26.4 (CH_2)	26.0 (CH_2)
13	47.1 (C)	48.2 (C)	49.1 (C)	52.4 (C)
14	86.2 (C)	88.1 (C)	86.4 (C)	88.0 (C)
15	29.3 (CH_2)	35.0 (CH_2)	29.7 (CH_2)	29.7 (CH_2)
16	31.6 (CH_2)	37.0 (CH_2)	32.3 (CH_2)	30.2 (CH_2)
17	49.1 (CH)	79.2 (C)	55.3 (CH)	54.9 (CH)
18	18.5 (CH_3)	18.2 (CH_3)	18.1 (CH_3)	20.0 (CH_3)
19	17.1 (CH_3)	17.8 (CH_3)	17.5 (CH_3)	17.8 (CH_3)
20	84.5 (C)	83.3 (C)	82.2 (C)	81.9 (C)
21	19.6 (CH_3)	20.3 (CH_3)	20.7 (CH_3)	20.3 (CH_3)
22	81.7 (CH)	82.1 (CH)	81.2 (CH)	79.9 (CH)
23	32.3 (CH_2)	32.4 (CH_2)	31.6 (CH_2)	31.1 (CH_2)
24	155.2 (C)	152.8 (C)	148.8 (C)	150.3 (C)
25	125.0 (C)	121.5 (C)	122.6 (C)	121.6 (C)
26	166.3 (C)	168.6 (C)	166.0 (C)	166.0 (C)
27	55.6 (CH_2)	12.5 (CH_3)	12.5 (CH_3)	12.3 (CH_3)
28	20.3 (CH_3)	20.6 (CH_3)	20.6 (CH_3)	20.6 (CH_3)

*Multiplicities determined from DEPT spectra.



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EXPERIMENTAL

General Details:- MS spectra were recorded on a Varian MAT 312 double focusing mass spectrometer connected to DEC PDP 11/34 computer system. The ^{1}H-NMR spectra were recorded in $CDCl_3$ and $CDCl_3$ + CD_3OD on Bruker AM-300 spectrometer, while the ^{13}C-NMR spectra were recorded at 125 MHz with TMS as internal standard. The IR spectra were recorded on a JASCO IRA-1 spectrophotometer. The UV spectra were recorded on a Shimadzu UV 240 instrument. The purities of the samples were checked by TLC on silica gel G-254 precoated plates

Material:- *Withania coagulance* Dun. (whole plant) 250 kg were collected from the suburban area of the Karachi (Pakistan) in 1991. The plant was identified by Mr. Tahir Ali, Plant Taxonomist and a voucher specimen (KUH # 46258) was deposited in the herbarium of the Department of Botany, University of Karachi.

Extraction and Isolation:- The EtOH extract of the whole plant of *W. coagulance* (250 kg) was concentrated to gum (1.8 kg). This gum was dissolved in MeOH[14] (4000 mL) and defatted with pet. ether (7000 mL). The defatted MeOH extract (1.4 kg) was again evaporated and dissolved in water (1000 mL).[15] The aqueous layer was extracted with ethyl acetate and chloroform (5000 mL). The ethyl acetate fraction (5 gm) was loaded on silica gel (20-240 mesh) column and eluted first with $CHCl_3$ and then with increasing polarities of $CHCl_3$:MeOH and finally with pure MeOH. A fraction (100 mg) obtained at 0.3% MeOH:$CHCl_3$ was found to contain four major compound (**1-4**) were purified using $CHCl_3$-MeOH-NH_4OH (99.7:0.3 + 1 drop of NH_3) as solvent system on TLC (silica gel).

Coagulin B (**1**) was isolated as yellowish white solid yield 1.3 10^{-3}% $R_f = 0.33$;UV λ_{max} (MeOH) 224 nm (ε 18000); IR ν_{max} ($CHCl_3$) 3400, 1690, 1660 cm^{-1}; FABMS *m/z* 453, EIMS *m/z* (rel. int. %) [M$^+$] 452 (17), 311 (16),169 (26), 171 (71), 142 (25), 141 (23), 124 (100). ^{1}H NMR and ^{13}C NMR ($CDCl_3$ + CD_3OD) δ see Tables **1** and **2**.

Couglin C (**2**) was isolated as white powder, yield 1.1 10^{-3}% , R_f=0.34; UV λ_{max} (MeOH) 223 nm

(ε 18000) IR ($CHCl_3$) 3400, 1700, 1660, cm^{-1}; FABMS *m/z* 453, EIMS *m/z* (rel. int. %), 452 (7), 327 (32), 283 (91), 153 (94), 125 (100). ^1H- NMR and ^{13}C-NMR ($CDCl_3$) δ see Tables **1** and **2**.

Couglin D (**3**) white crystalline, yield 1.2 10^{-3}%, R_f =0.57; UV λ_{max} (MeOH) 224 nm, IR ($CHCl_3$) 1705 and 1690 cm^{-1}. EIMS *m/z* (rel. int. %) [M^+], 436 (8), 311 (45), 283 (83), 153 (35) 125 (100). ^1H and ^{13}C-NMR ($CDCl_3$) δ see Tables **1** and **2**.

Couglin E (**4**) white amorphous, yield 1.5 10^{-3}% R_f = 0.94, UV λ_{max} (MeOH) 228 nm, IR ν_{max} ($CHCl_3$) 1705 and 1690 cm^{-1}. EIMS *m/z* (rel. int. %) [M^+] 436(8), 311 (29), 283 (83) ,153 (18) 125 (100). ^1H and ^{13}C-NMR ($CDCl_3$) δ see Tables **1** and **2**.

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