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BIOACTIVITIES AND STRUCTURAL STUDIES OF WITHANOLIDES FROM *Withania somnifera*

S. Arshad Jamal, Samina Qureshi, Samina Noor Ali, M. Iqbal Choudhary, and Atta-Ur-Rahman

Four bioactive withanolides withalactone (1), withaoxylactone (2), quresimine-A (4β-hydroxy, 3β-methoxy-5β,6β-epoxy-(22R)-witha-24-enolide) (3) and quresimine-B (4β, 27-dihydroxy-3β-methoxy-5β,6β-epoxy-(22R)-witha-24-enolide (4) have been isolated from the herbs of Withania somnifera, *Dunal* (Solanaceae). *The elucidation of their structures is based on extensive spectroscopic studies, such as ^{1}H-NMR, COSY-45°, HMBC, HMQC, HOHAHA, E.I., FAB (+ ve), and HR MS, etc.*

INTRODUCTION

The withanolides are steroidal lactones, one of which, withaferin-A, was isolated for the first time from the leaves of *Withania somnifera* in 1956. Since then a large number of withanolides have been isolated from different species of *Withania*, *Physalis*, and *Datura*. Some of these were found to have antitumor, cytotoxic and antimicrobial activities [1-5]. Different parts of *Withania somnifera* have shown a significant importance since long time, as a medicinal remedies for many diseases [6-13] (see Table 1). Our studies have revealed that *Withania somnifera* is a rich source of interesting new withanolides with potentially useful biological activities (see Tables 2-6).

RESULTS AND DISCUSSION

The extraction procedure for methanolic extract of the whole plant of *Withania somnifera* afforded chloroform extract and pure compounds from it. Minimum concentrations of these compounds exhibited inhibitory, bactericidal, fungicidal, antifungal and antibacterial activities were determined (see Tables 2-6).

The compound **1**, withalactone was isolated from the defatted methanolic extract of the whole plant of *W. somnifera*. The high-resolution electron-impact mass spectrum afforded the molecular ion at *m/z* 454.2767 establishing the molecular formula as $C_{28}H_{38}O_5$ (calcd. 454.0754) indicating eight degrees of unsaturation in the molecule. The M^+ was further confirmed by positive FAB MS. The UV spectrum showed absorptions at 225 nm indicating the presence of an α,β-unsaturated lactone [14]. The IR spectrum displayed bands at 1685, 1700, and 3500 cm from which the presence of an α,β-unsaturated lactone, a ketone, and hydroxylic groups in the molecule could be inferred [15].

The ^{1}H NMR spectrum of **1** showed four 3H singlets at δ 0.83, 1.22, 1.85, and 1.91 for the C-18, C-19, C-28, and C-27 tertiary methyl protons, respectively, while a 3H doublet at δ 1.01 ($J_{21,20\beta}$ = 7.0 Hz) was due to the C-21 secondary methyl protons. A doublet of doublets at δ 5.82 ($J_{2,3}$ = 10.2 Hz, $J_{2,4\beta}$ = 2.3 Hz) was due to vinylic C-2H. Another doublet of double doublets at δ 6.57 ($J_{3,2}$ = 102 Hz, $J_{3,4\alpha}$ = 5.0 Hz, $J_{3,4\beta}$ = 2.1 Hz) was assigned to the C-3 olefinic proton conjugated with a carbonyl. A doublet of double doublets appeared at δ 2.68 ($J_{4\beta,4\alpha}$ = 20.1 Hz, $J_{4\beta,3}$ = 4.9 Hz, $J_{4\beta,2}$ = 0.3 Hz) and a doublet of doublets at δ 2.51 ($J_{4\alpha,4\beta}$ = 20.1 Hz, $J_{4\alpha,3}$ = 5.0 Hz) were assigned to the C-4 allylic methylene protons. A disubstituted oxirane ring was inferred from the signals of the two mutually coupled adjacent protons at δ 3.03 and 3.30, the former (C-6H) being a doublet ($J_{6,7}$ = 3.8 Hz) while the second (C-7H) was a double doublet ($J_{7,6}$ = 3.8 Hz, $J_{7,8}$ = 2.1 Hz). This indicates that one of the oxirane protons has an additional neighboring proton, while the other has a quaternary car-

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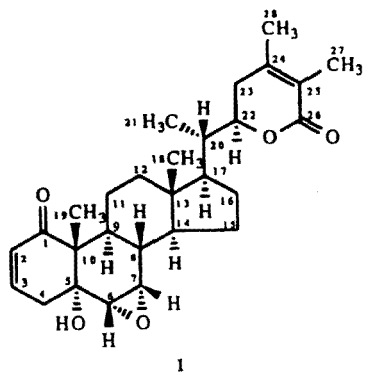

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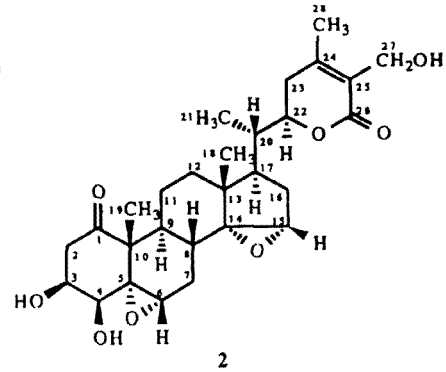


2

bon in its vicinity. The α-stereochemistry of the C-6/C-7 epoxide was assigned on the basis of chemical shift comparisons with known withanolides. A downfield doublet of double doublets at δ 4.59 ($J_{22,20}$ = 11.3 Hz, $J_{22,23\beta}$ = 5.3 Hz, $J_{22,23\alpha}$ = 2.7 Hz) was due to the C-22 methine proton geminal to the ester functionality. These observations convincingly support a tetracyclic steroidal skeleton with a lactone substituent, as found in other common withanolides. The compound contains an α,β-unsaturated ketone in ring A and an epoxide in ring B.

The COSY-45° spectrum of **1** revealed the presence of three different spin systems in the molecule. The C-3H showed COSY-45° connectivity with the C-2H, C-4β, and α protons (δ 2.68 and 2.51) respectively. The C-7H showed strong crosspeaks with the C-6H and C-8H (δ 1.75). The couplings of the C-22H (δ 4.59) with the C-23 methylenic protons (δ 2.45 and 1.84) and with the C-20H (δ 2.28) were also apparent in the COSY-45° spectrum of compound **1**.

The ^{13}C NMR spectra (DEPT and BB) of withalactone **1** exhibited resonances for all twenty-eight carbons. The ^{13}C NMR signals in the broad-band decoupled ^{13}C NMR spectrum at δ 150.4, 121.5, and 167.1 were assigned to the C-24 and C-25 vinylic and C-26 carbonyl carbons, respectively. The C-6 and C-7 epoxide carbons resonated at δ 56.3 and 57.2, respectively. The downfield signal of a quaternary carbon at δ 73.3 was assigned to C-5 bearing an α-oriented hydroxyl group.

In the HMQC [16, 17] spectrum of **1**, the carbon resonating at δ 36.8 (C-4) was found to be coupled with the protons at δ 2.68 (H-4β) and 2.51 (H-4α). The carbon at δ 32.9 (C-23) showed one-bond interactions with signals at δ 2.45 and 1.84 (H-23β and α). The downfield methine protons at δ 5.82 (H-2), 6.57 (H-3), 3.03 (H-6), 3.30 (H-7), and 4.59 (H-22) showed one-bond heteronuclear interactions with the carbons at δ 129.0 (C-2), 139.7 (C-3), 56.3 (C-6), 57.2 (C-7), and 78.8 (C-22), respectively [18]. The HMQC data are presented in Table 7.

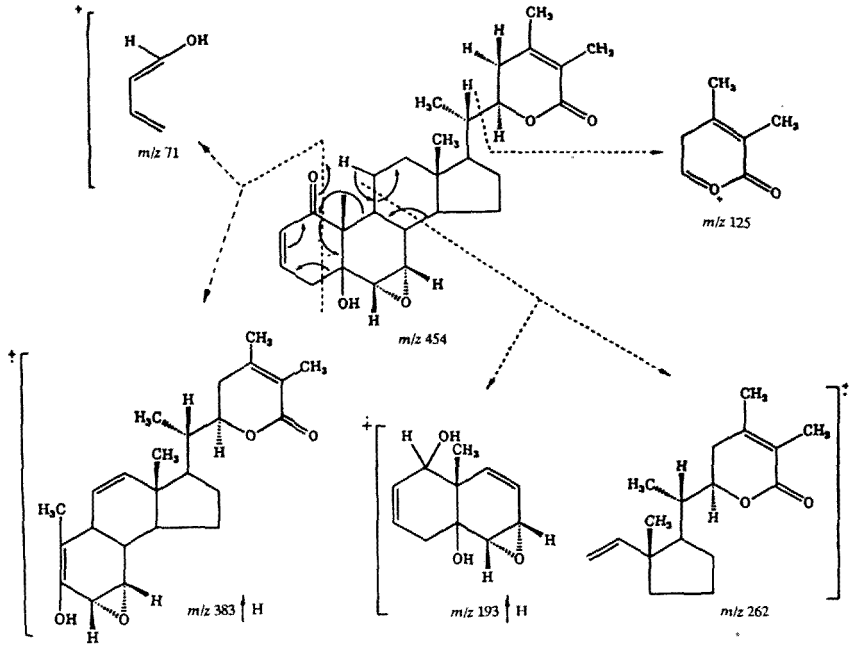
The HMBC spectrum of **1** showed that the C-4 α and β protons (δ 2.68 and 2.51) have long-range correlation with the carbons at δ 203.1 (C-1), 129.0 (C-2), 139.7 (C-3), 73.3 (C-5), and 15.1 (C-19). Similarly C-6H showed cross-peaks with C-5, C-7, C-8, C-9, C-10, and C-19 in the HMBC spectrum. These connectivities confirmed various carbon-proton assignments of rings A and B. Long-range heteronuclear correlations between the C-22H and C-20, C-21, C-23, C-24, C-25, and C-26 were also observed in the HMBC spectrum.

The HREI MS of **1** showed the M+ at *m/z* 454.2767 corresponding to the molecular formula $C_{28}H_{38}O_5$ (calcd 454.2719), with eight double bond equivalents in the molecule. The ion at *m/z* 125.0754 ($C_7H_9O_2$) could result from the cleavage of the C-20/C-22 bond. The peak at *m/z* 153 ($C_9H_{13}O_2$) may arise by cleavage of the C-17/C-20 bond and indicated the presence of a six-membered lactone substituent of the C-20 side chain. The fragment at *m/z* 301 ($C_{19}H_{25}O_3$) in turn represented the remaining part of the molecule. The mass fragment at *m/z* 193 ($C_9H_{13}O_3$), arising by the cleavage of ring C, indicated the presence of three oxygen functions and one double bond in rings A and B of the molecule. The ion at *m/z* 71.0429 (C_4H_7O) may arise by cleavage of ring A. The above mentioned spectroscopic studies led to the structure **1** (α-hydroxy-6α, 7α-epoxy-1-oxo (22R)-witha-2,24-dienolide) for this withanolide.

The second compound, named withaoxylactone **2**, $C_{28}H_{38}O_5$, showed UV absorption at 215 nm, characteristic of an α,β-unsaturated lactone chromophore [14]. The IR spectrum showed peaks at 1701 (α,β-unsaturated lactone) and 3500 (OH) cm^{-1}.

The ^1H NMR spectrum showed three 3H singlets at δ 0.63, 1.22, and 2.03 for the three tertiary methyl groups which could be assigned to the C-17, C-18, and C-28 methyl protons respectively, while a 3H doublet at δ 0.95 ($J_{21,20\beta}$ = 6.6 Hz) was due to the C-21 secondary methyl protons. A doublet of double doublets centered at δ 3.63 ($J_{3\alpha,2\beta}$ = 7.4 Hz, $J_{3\alpha,2\alpha}$ =

Scheme 1



Mass fragmentation pattern of withalactone **1**

7.4 Hz, $J_{3\alpha,4\alpha}$ = 3.5 Hz) was assigned to the 3α methine proton geminal to the oxygen function. Another methine proton (4Hα) resonating as a doublet at δ 3.40 ($J_{4\alpha,3\alpha}$ = 3.0 Hz) is also geminal to the oxygen function. A downfield doublet of double doublets, centered at δ 4.38 ($J_{22,20}$ = 13.3 Hz, $J_{22,23\alpha}$ = 3.5 Hz) was assigned to the C-22 methine proton geminal to the lactone oxygen [19]. Two AB doublets at δ 4.32 and 4.25 ($J_{27\alpha,\beta}$ = 12.3 Hz) were ascribed to the hydroxy methylene protons [20]. These observations once again supported a withanolide skeleton with two epoxides, one primary hydroxyl and two secondary hydroxyl substituents. Two broad singlets at δ 3.14 and 3.31 were characteristic of the protons on the epoxide bearing carbons. The presence of four epoxide-bearing carbon signals [δ 64.3 (−C−), 55.7 (CH), 63.5 (−C−), 58.4 (CH)] but only two mutually uncoupled methine protons bearing epoxide oxygen suggested that both epoxides were such that one end was a quaternary carbon and the other end was a tertiary carbon. The α-stereochemistry of both epoxides was again assigned on the basis of chemical shift comparisons with known withanolides [21-24].

The C-3 proton showed COSY-45° connectivities with the C-4 proton (δ 3.40) and with the C-2β and α protons (δ 2.84 and 2.58). The C-6H of epoxide showed strong cross-peaks with the C-7β and α protons (δ 2.13 and 1.35) in the COSY spectrum. Similarly the C-15 epoxide proton exhibited vicinal couplings with the C-16β and α protons respectively. Couplings of the C-22H with the C-23β and α protons and with the C-20H were also apparent in the spectrum.

The ^{13}C NMR spectrum of withaoxylactone **2** exhibited twenty-eight carbon resonances. The signals at δ 154.9, 124.9, and 167.1 were assigned to the vinylic C-24 and C-25 and carboxylic C-26 respectively. The oxygen-bearing C-3 and C-4 resonated at δ 77.4 and 73.9, whereas the epoxide carbons, i.e., C-5, C-6, C-14, and C-15, resonated at δ 64.3, 55.7, 63.5, and 58.4, respectively. The chemical shifts are presented in Table 7.

The HMQC spectrum of **2** established one-bond $^{1}H/^{13}C$ coupling. The carbon at δ 39.8 (C-2) was found to be coupled with the protons at δ 2.84 and 2.58 (C-2β and α H). The C-23 carbon at δ 29.5 showed one-bond coupling interactions with the protons at δ 2.46 and 1.90 (C-23β and αH) respectively. The downfield methine protons at δ 3.63 (H-3), 3.40 (H-4), 3.14 (H-6β), 3.31 (H-15β), and 4.38 (H-22) showed one-bond interactions with the carbons resonating at β 77.4 (C-3), 73.9 (C-4), 55.7 (C-6), 58.4 (C-15), and 78.6 (C-22) respectively. The HMQC data of **2** is presented in Table 7.

TABLE 1. Biological Importance of *Withania somnifera*

Nos.	Part of the Plant	Biological Activity Reported
1	Leaves	Anti-inflammatory, anti-helminthic, antibiotic, anti-pyretic, protective against hepatotoxicity and syphilitic sores [6]
2	Fruit	Diuretic, anti-tumor, anti-inflammatory and used for the treatment of carbuncle, tubercular glands and ulcers [7]
3	Seeds	Hypnotic, coagulant, diuretic and masticatory [8]
4	Tubers	Anti-bronchitic, anti-psoriatic, anti-ulcer, anti-inflammatory, anti-scabietic and anti-helminthic [9, 10]
5	Roots	Anti-rheumatic [11], nutritive, health restorative protective against cold, chill [12], loss of memory, dyspepsia, nervous exhaustion and hypertension [13]

TABLE 2. Antibacterial Activity of Chloroform Extract of *Withania somnifera* and Its Pure Compound 2,3-Dihydrowithaferine-A

Name of the organism	Concentration used (μg/100μl) with the zone of inhibition (mm)						Control (DMSO) (100μl)
	Chloroform extract			2,3-Dihydrowithaferine-A			
	50	100	200	50	100	200	
B. anthracis	+	25	36	+	30	42	+
B. subtilis	18	23	39	25	35	44	+
C. diphtheriae	+	26	35	+	30	40	+
C. pseudodiphthericum	+	25	35	+	30	40	+
Ps. aeruginosa	+	18	28	+	30	42	+
S. aureus	+	15	25	+	30	40	+
S. fecalis	+	36	45	+	40	48	+
S. agalactiae	+	+	+	+	15	20	+

(+) = Bacterical growth.

TABLE 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Chloroform Extract of *Withania comnifera* and Its Pure Compund 2,3-Dihydrowithaferine-A

Name of the organism	Chloroform extract		2,3-Dihydrowithaferine-A	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
C.diphtheriae	250	Bacteriostatic	170	Bactericidal
C. pseudodiph-thericum	250	Bacteriostatic	170	Bactericidal
B. anthracis	350	Bacteriostatic	150	Bacteriostatic
B. subtilis	250	Bacteriostatic	170	Bacteriostatic
Ps. aeruginosa	250	Bacteriostatic	150	Bacteriostatic
S. aureus	250	Bacteriostatic	150	Bactericidal
S. fecalis	250	Bacteriostatic	150	Bactericidal
S. agalactiae	+	+	170	Bacteriostatic

(+) = Bacterical growth.

The C-4H showed long-range correlations with C-2, C-3, C-5, C-10, and C-19. This helped to confirm the proton-carbon assignments of ring A in the HMBC spectrum. Other couplings appeared between the C-6H with C-4, C-5, C-7, C-8, and C-9. Similarly the C-22H showed cross-peaks with C-24, C-25, and C-26. These connectivities further confirmed various chemical shift assignments.

TABLE 4. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Chloroform Extract of *Withania somnifera* and Its Pure Compound 2,3-Dihydrowithaferine-A

Name of the fungus	Chloroform extract		2,3-dihydrowithaferine-A	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
A. boydill	500	Fungicidal	350	Fungicidal
T. mentagrophyte	500	Fungicidal	350	Fungicidal

TABLE 5. Determination of Antifungal Activity of the Pure Compound Quresimine-A

Name of the test organism	Concentration of Test Sample 400 μg/ml	Inference
C. albicans	-	No Activity
F. moniliformis	-	No Activity
D. rostrata	+	Low Activity
A. flavus	-	No Activity
E. floccosum	+++	Good Activity
A. niger	+	Low Activity
C. irregularis	+++	Good Activity
M. canis	+++	Good Activity
C. lunata	+	Low Activity
N. oryzae	+++	Good Activity

Medium used: Sabouraud Dextrose Agar.

TABLE 6. Determination of Antibacterial Activity of the Pure Compound Quresimine-A

Name of the test organism	Zone of Inhibition in mm		AMP 10 μg	TOB 10 μg
	100 μg/100 μl	200 μg/100 μl		
S. typhii	5,5	7	6,5	7
C. diphtheriae	5,5	6	11	11
V. cholarae	6	7	8	9
S. boydii	5,5	6	6	7
E. coli	5,5	7	7	8
K. pneumoniae	—	7	7	7
Staph. aureus	5,5	6	11	11
Ps. aeruginosa	6	7	6	10

mm) Antibacterial activity; AMP) ampicillin; TOB) tobramycin; —) bacterial growth.

Method: Agar well diffusion method.

Media used: Mueller Hinton Agar.

Size of borrer: 5 mm.

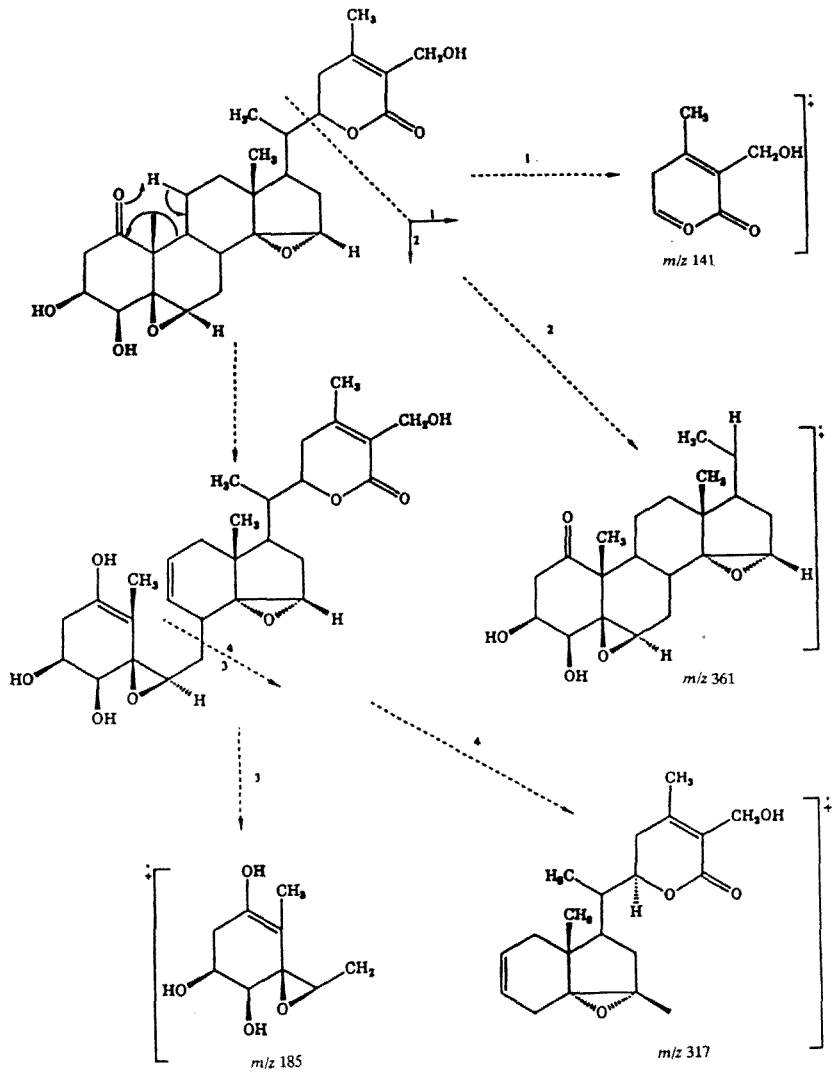
TABLE 7. ^{13}C and ^{1}H NMR Chemical Shifts of Compound (**1-4**)[a,b]

C. No	1		2		3		4	
	^{13}C	$\delta^{1}H$	^{13}C	$\delta^{1}H$	^{13}C	$\delta^{1}H$	^{13}C	$\delta^{1}H$
1	203,1	—	210,6	—	209,7	—	209,7	—
2	129,0	5,82 dd, $J_{2,3}$ = 10,2, $J_{2,4}$ = 2,3	39,8	2,28 m, 2,58 m	39,2	2,86 m, 2,50 m	39,4	3,05 m, 2,67 m
3	139,7	6,57 ddd, $J_{3,2}$ = 10,2, $J_{3,4\alpha}$ = 5,0, $J_{3,4\beta}$ = 2,1	77,4	3,63 dd, $J_{2,3}$ = 7,4, $J_{3,4}$ = 7,4, $J_{3,4}$ = = 3,5	77,7	3,68 m	77,5	ddd
4	36,8	2,68 ddd, $J_{4\alpha,4\beta}$ = 20,1, $J_{4\beta,3}$ = 4,9, $J_{4\beta,2}$ = 0,3, 2,51 m	73,9	3,40 d, $J_{4,3}$ = 3,7	75,1	3,47 J = 3,2	75,2	3,4 d
5	73,3	—	64,3	—	64,9	—	65,1	—
6	56,3	3,03 d, $J_{6,7}$ = 3,8	55,7	3,14 brs	60,2	3,19 brs	60,7	3,21 nrs
7	57,2	3,30 dd, $J_{7,6}$ = 3,8, $J_{7,8}$ = 2,1	30,9	2,13 m, 1,35 m	31,2	2,15 m, 1,30 m	31,2	2,19 m, 1,40 m
8	36,0	1,75 m	42,6	1,10 m	29,4	1,40 m	29,7	1,70 m
9	35,5	2,05 m	29,3	1,35 m	42,8	1,18 m	42,9	1,18 m
10	50,0	—	50,3	—	50,4	—	50,4	—
11	23,2	1,55 m	26,9	1,50 m	21,6	1,40 m, 1,40 m	21,5	—
12	21,0	1,38 m	23,9	1,25 m	24,3	1,10 m, 1,65 m	23,7	—
13	48,8	—	49,0	—	42,7	—	47,9	—
14	45,9	2,00α	63,5	—	56,1	0,95 m	56,3	—
15	30,0	1,28 m	58,4	3,31 brs	27,3	1,66m, 1,40 m	32,8	—
16	32,0	2,25 m	38,7	2,18 m, 1,10 m	39,7	2,05 m, 1,95 m	36,5	—
17	36,8	1,60 m	51,5	1,20 m	52,0	1,09 m	50,3	1,09 m
18	14,7	0,83 s	11,1	0,63 s	11,6	0,65 s	9,5	0,75 s
19	15,1	1,22 s	14,2	1,22 s	15,6	1,29 s	14,7	1,30 s
20	43,0	2,28 m	38,5	1,64 m	38,6	1,89 m	36,1	1,90 m
21	9,5	1,01 d, $J_{21,20}$ = 7,0	12,8	0,95 d, $J_{21,20}$ = 6,6	13,7	0,97 d, $J_{1,2}$ = 6,7	12,3	0,99 d, J = 7,0
22	78,8	4,59 ddd, $J_{22,20}$ = 11,3, $J_{22,23\beta}$ = 5,3, $J_{21,23\alpha}$ = 2,7	78,6	4,38 ddd, $J_{22,20}$ = 13,3, $J_{22,23\beta}$ = =3,5, $J_{21,23\alpha}$ = 3,5	78,7	4,40 ddd, J_1 = J_2 = 3,5, J_3 = = 14,1	78,7	4,55 ddd, $J_{1,2}$ = 2,96, $J_{1,3}$ = 4,99, $J_{1,5}$ = = 11,60
23	32,9	2,45 m, 1,84 m	29,5	2,46 m, 1,90 m	29,7	2,45 m, 1,90 m	21,7	2,45 m, 1,90 m
24	150,4	—	154,9	—	125,8	—	121,9	—
25	121,5	—	124,9	—	152,8	—	147,0	—
26	167,1	—	167,1	—	166,7	—	167,0	—
27	20,5	1,91 s	55,7	4,35 d, J = 12,3,4,51 d, J = 12,3	57,4	4,36, J_{AB} = 12,5, 4,32, J_{AB} = =12,5	18,1	1,90 s
28	12,4	1,85 s	19,6	2,03 s	19,9	2,02 s	15,8	1,85 s
OCH_3	—	—	—	—	56,4	3,33 s	56,0	3,33 s

[a]Multiplicities were confirmed by DEPT.

[b]Proton-carbon correlations were based on the HMQC experiment.

Scheme 2

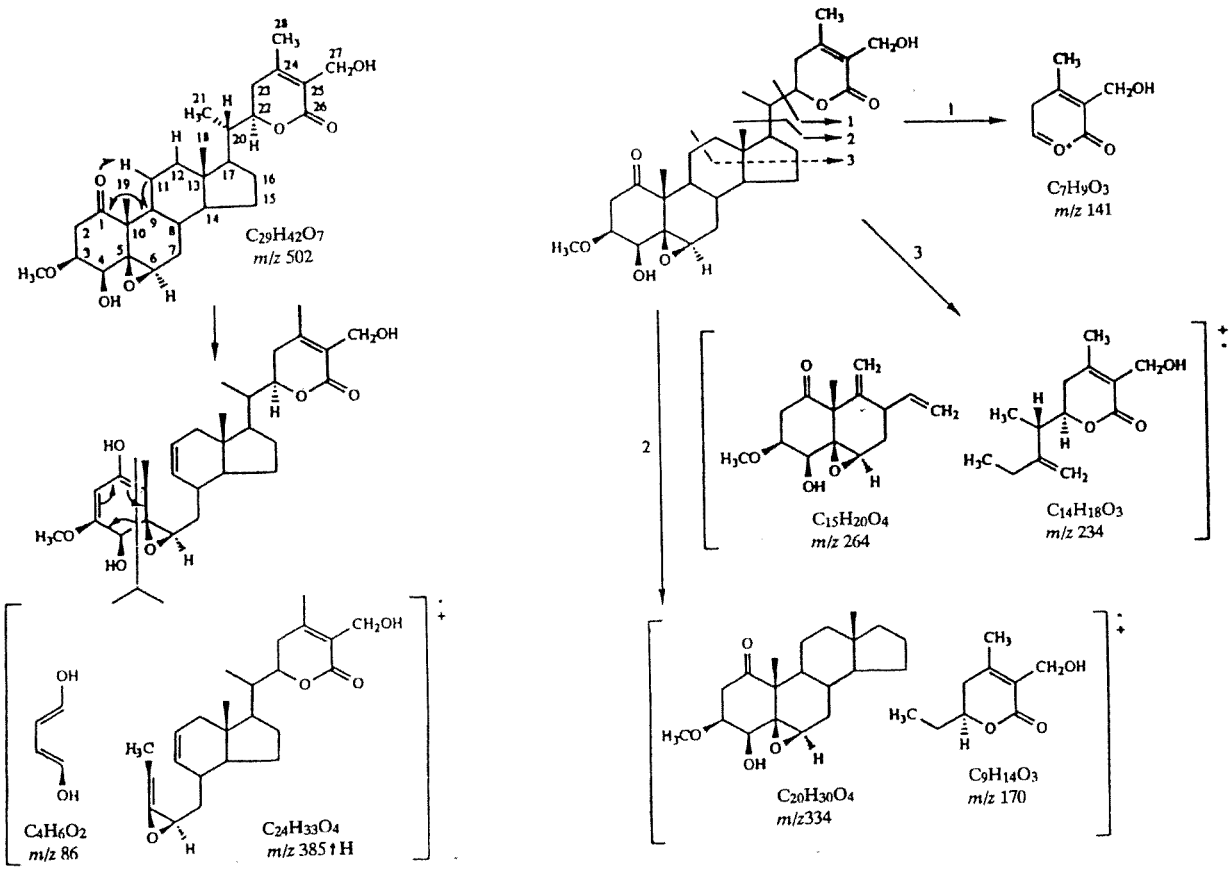


Mass fragmentation pattern of withaoxylactone **1**

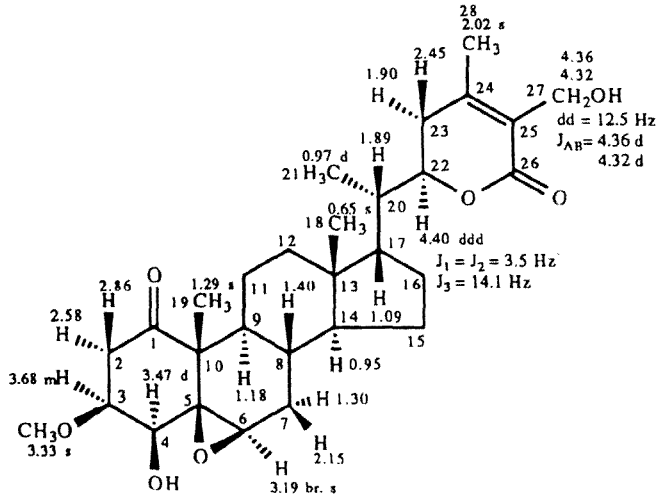
The HREI mass spectrum of **2** showed the M^+ at *m/z* 502.2560 corresponding to the molecular formula, $C_{28}H_{38}O_8$ (calcd 502.2566), indicating ten degrees of unsaturation in the molecule. The ion at *m/z* 141.0552 of composition $C_7H_9O_3$ could result by the cleavage of the C-20/C-22 bond, whereas the ion at *m/z* 169.0865 ($C_9H_{13}O_3$) may arise by the cleavage of the C-17/C-20 bond [15, 25]. The fragment at *m/z* 333.1702 ($C_{19}H_{25}O_5$) in turn represented the remaining half of molecule. The peak at *m/z* 185.0812 ($C_9H_{13}O_4$) may arise by the cleavage of rings B and C. This fragment has four oxygen atoms and therefore supports the proposed substitution at rings A and B of the molecule [26]. The fragment at *m/z* 317.1756 ($C_{19}H_{25}O_4$) represented the remaining portion of molecule. These spectroscopic studies led to the structure **2** [3β,4β-dihydroxy-5α,6α,14α,15α-diepoxy-1-oxo(22R)-witha-24-enolide] for this withanolide.

Quresimine-A (**3**) $C_{29}H_{42}O_7$ showed a UV absorption maximum at 202 nm, characteristic of an α,β-unsaturated lactone chromophore [14]. The IR spectrum displayed intense absorption at 1683 for α,β-unsaturated lactone and a broad peak at 3455 cm^{-1} for O−H). The high resolution mass spectrum of **3** showed the molecular ion peak at *m/z* 502.2914 corresponding to the molecular formula $C_{29}H_{42}O_7$ which indicated the presence of nine double bond equivalents in the molecule. The peak appearing at *m/z* 484.2804 and 470.2667 showed the loss of one water and methylene group respectively. The peak appeared at *m/z* 452.2530 again exhibited the loss of water molecule. The peak at *m/z* 387.2522 ($C_{24}H_{35}O_4$) and 86 ($C_4H_6O_2$) resulted by the cleavage of the C-1 (10)/C-4 (5) bond which further indicated that four oxygen groups were present in major portion. The mass fragment at *m/z* 331.1979 ($C_{20}H_{27}O_4$) formed by the cleavage of ring C was indicative of the presence of four oxygen functions and the remaining fragment appeared at *m/z* 169.0810 ($C_9H_{13}O_3$) indicated the presence of a six-membered

Scheme 3



Mass Fragmentation of Quresimine-A3



3

lactone substituent at the C-20 side chain, the prominent peak at *m/z* 141.571 ($C_7H_9O_3$) originated by cleavage of the C-20/C-22 bonds. The remaining mass peak was shown in Scheme 3.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **3** showed three three-proton singlets for the quaternary methyls at δ 0.65, 1.29, and 2.02 for the C-18, C-19, and C-28 protons respectively, while a three-proton doublet at δ 0.97 (J = 6.7 Hz) was due to the secondary methyl protons. A doublet of double doublets appeared at δ 4.40 ($J_1 = J_2 = 3.5$, $J_3 = 14.1$ Hz) was assigned to the C-22 proton of the lactone moiety [19]. A two proton AB doublet at δ 4.36 and 4.32 ($J_{AB} = 12.5$ Hz) ascribed to the C-27 methylenic protons. The observations conveniently supported a *tetracyclic steroidal* skeleton with a *lactone substituent* as *terminal* in a number of common withanolides [18]. A broad singlet appeared at δ 3.19 was assigned to the C-6α proton of oxirane ring. A doublet appeared at δ 3.47 (J = 3.2 Hz) due to the C-4α proton. The C-3α proton at δ 3.68 multiplet was coupled with C-4α methine proton while C-2 methylene protons appeared as a multiplet at δ 3.69. A three proton singlet appeared at δ 3.33 due to the C-3 methoxy group.

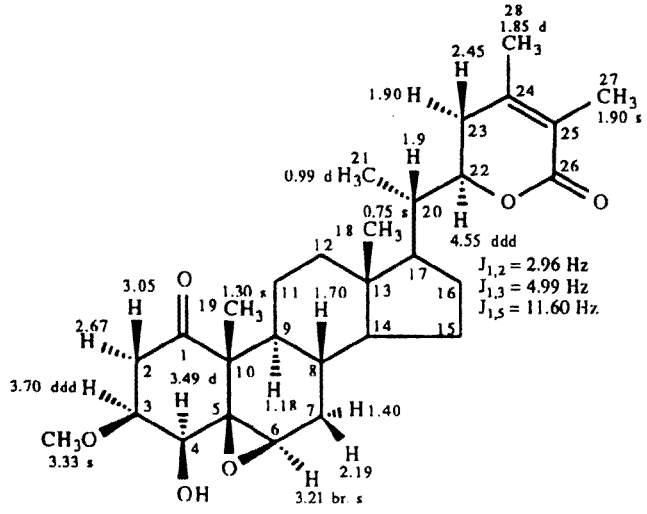
The COSY-45° spectrum of **3** served to establish proton-proton connectivities. The peak at δ 3.68 (C-3α proton) showed strong connectivities with δ 2.58 and 2.86 (C-2 methylene protons) and δ 3.47 (C-4α methine proton). The proton at δ 3.19 (C-6α proton) showed connectivities with δ 1.30 and δ 2.15 (C-7 methylene protons). A peak at δ 4.40 (C-22α methine proton) showed connectivities with δ 1.89 (C-20β methine proton) and also with δ 1.90 and 2.45 (C-22 methylene protons).

Heteronuclear multiple quantum coherence (HMQC) [16, 17] spectrum of quresimine-A (Table 7) exhibited one-bond heteronuclear connectivities of various proton-carbon nuclei. This showed that the methine carbon at δ 38.6 (C-20) was coupled with its β methine proton at δ 1.89. Similarly the carbons at δ 77.7 (C-3), 75.1 (C-4), 60.2 (C-6), 78.7 (C-22) were coupled with the protons resonating at δ 3.60 (H-3α), 3.47 (H-4α), 3.19 (H-6α), and 4.40 (H-22α) respectively [18]. Likewise the methylene carbon at δ 29.7 (C-23) showed a cross-peak with its protons at δ 1.90 and 2.45. The peak at δ 39.2 (C-2) showed coupling with the protons at δ 2.58 and 2.86.

Heteronuclear multiple bond connectivity (HMBC) spectrum of quresimine-A showed that the H-4α (δ 3.47) interacted with the carbons at δ 209.7 (C-1), 39.2 (C-2), 77.7 (C-3), 64.9 (C-5), and 50.4 (C-10). This helped to confirm the assignment of ring-A. Similarly H-6α (δ 3.19) showed cross peak with carbon signals at δ 31.2 (C-7), 64.9 (C-5), and 29.4 (C-8), thereby confirming the assignments of ring B respectively [14]. Another important signal at δ 4.40 (H-22α) showed interactions with carbons at δ 38.6 (C-20), 29.7 (C-23), 166.7 (C-26), 152.8 (C-25), and 125.8 (C-24) which confirmed various assignments in the ring containing lactone moiety.

The C-4α proton resonating at δ 3.47 showed homonuclear long range coupling with C-2 at δ 2.58 and 2.86 methylene protons in the HOHAHA spectrum, while the C-6α proton (δ 3.19) exhibited interaction with the protons at δ 1.18 (C-9) and 0.95 (C-14). Thus all above mentioned spectroscopic evidence led to the elucidation of structure **3** for this new withanolide quresimine-A.

Quresimine-B (**4**) $C_{29}H_{42}O_6$ showed a UV absorption at 215 nm, characteristic of an α,β-unsaturated lactone chromophore. The IR spectrum displayed intense absorption at 1692 (α,β-unsaturated lactone) 1700 (C=O)[3] and 3555 cm^{-1} (O−H). The high resolution mass spectrum of **4** showed the molecular ion peak at *m/z* 486.2981 corresponding to the



4

molecular formula $C_{29}H_{42}O_6$ (calcd. 486.3470) which indicated the presence of nine double bond equivalents in the molecule.

Two sharp peaks at *m/z* 125.0602 of composition $C_7H_9O_2$ (Scheme 4) resulted in the cleavage of six-membered lactone substituent at the C-20 side chain, while the fragment ion at *m/z* 361.2379 ($C_{22}H_{33}O_4$) in turn represented the remaining half of the molecule. The mass fragment at *m/z* 187.0907 ($C_{90}H_{15}O_4$), formed by the cleavage of ring B, indicated the presence of four oxygen groups in ring A.

The ^1H NMR spectrum (400 MHz, $CDCl_3$) of **4** (Table 7) displayed four three-proton singlets at δ 0.75, 1.30, 1.85, and 1.90 which were assigned to the quaternary methyl C-18, C-19, C-28, and C-27 protons respectively, while a three-proton doublet at δ 0.99 (J = 7.0 Hz) due to the C-20 secondary methyl protons [15]. A sharp singlet for three-proton integration, appeared at δ 3.33 which was due to the methoxy group. A broad singlet appeared at δ 3.21 which was due to the C-6α proton. A one-proton doublet resonated at δ 3.70 ($J_{1,2}$ = 3.3 Hz) which indicated the C-4α proton, whereas doublet of doublets resonated at δ 3.70 ($J_{1,2}$ = 3.3 Hz, $J_{1,3}$ = 4.2 Hz, and $J_{1,5}$ = 7.5 Hz) which was due to the C-3α methine proton. A downfield doublet of double doublets appeared at δ 4.55 ($J_{1,2}$ = 2.96, $J_{1,3}$ = 4.99, $J_{1,5}$ = 11.60 Hz) was due to the C-22α methine proton [17]. The downfield value of C-22α proton indicated the attachment of oxygen with the molecule.

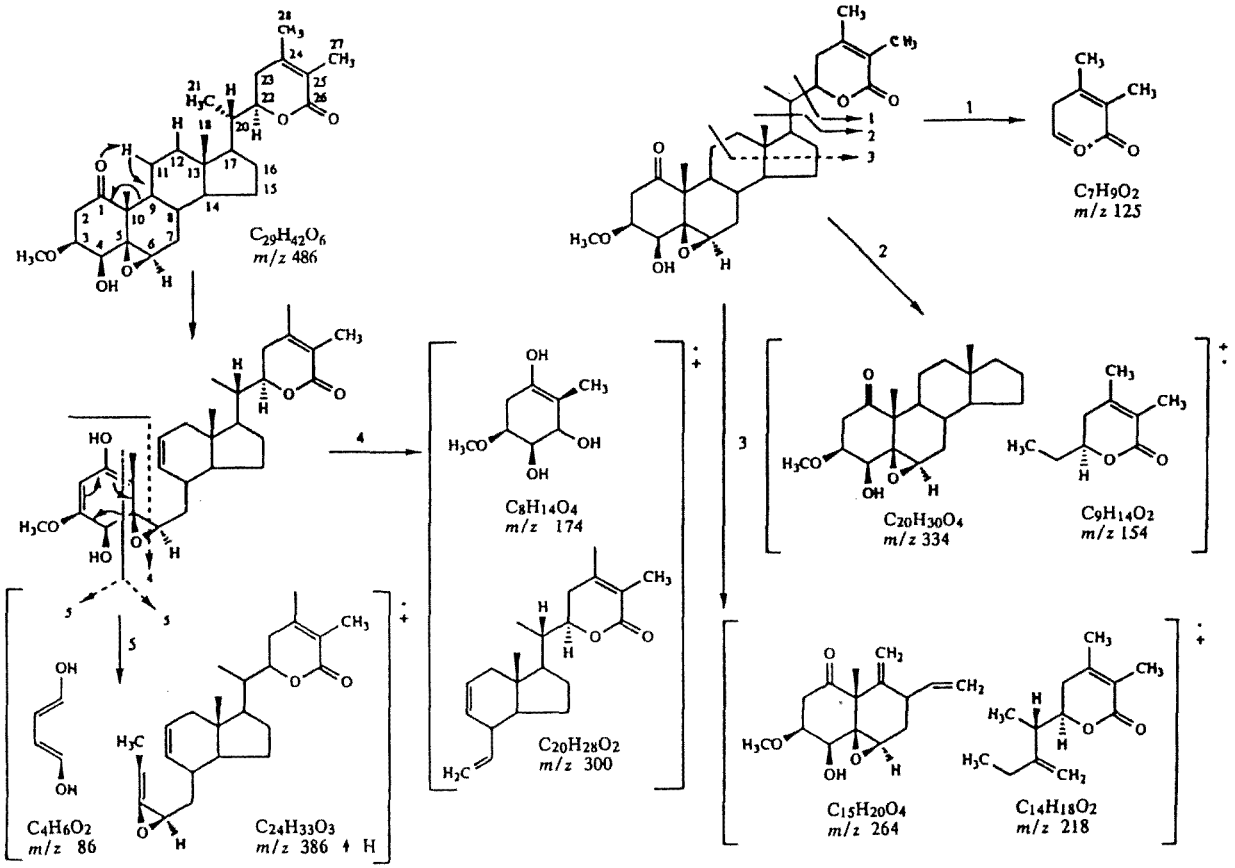
The two-dimensional COSY-45° experiment of **4** was performed to determine the ^1H connectivities. The COSY-45° spectrum exhibited three spin systems which are described as follows. The C-3α proton at δ 3.70 showed strong connectivities with the C-4α (δ 3.49) and (C-2) methylene protons at (δ 3.05 and 2.67). The proton of the oxirane (C-6α) showed connectivities with the C-7 metylene protons resonating at δ 2.19 and 1.40. The C-22α proton at δ 4.52 showed connectivities with δ 2.45 and 1.90 (C-23) methylene protons. Structure **4** was assigned on the basis of the above mentioned spectroscopic observations to this new withanolide, named as quresimine-B.

EXPERIMENTAL

Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a JASCO 302-A spectrophotometer. The UV spectra were recorded on a Hitachi U 3200 spectrophotometer. EI and HREI mass spectra (80 eV) were recorded on a JMS-HX 110 mass spectrometer with a JMA-DA 5000 data system. The ^1H and ^{13}C NMR spectra were recorded on Bruker AM 400 and AM 300 NMR spectrometers.

Plant Material. The fresh plant material (100 kg) was collected in November, 1989 from suburban areas of Karachi (Pakistan) and identified by the plant taxonomist at the Botany Department, University of Karachi. A voucher specimen (KUH number 46259) was deposited in the herbarium of the University of Karachi.

Scheme 4



Mass Fragmentation of Quresimine-B **4**

Extraction and Isolation. The methanolic extract (2001) of the air-dried whole plant was evaporated to a gum (2.70 kg). The gum was dissolved in MeOH (10l) and defatted with petroleum ether (40-60°). The defatted MeOH extract was again evaporated and the residue (1.78 kg) was dissolved in water (10l). The aqueous extract was extracted with $CHCl_3$ at different pH values. The fraction (500 g) obtained at pH 7 was loaded on a silica gel column (3.13 kg) and eluted first with hexane and then with hexane−chloroform, chloroform, and chloroform−methanol mixtures. The fractions collected on elution with hexane−chloroform (3:7) were subjected to preparative TLC (precoated silica gel, 0.25 mm) in methanol−chloroform (2.5:7.5) to afford **1** (31 mg, yield $3.1 \cdot 10^{-5}$%) and **2** (60 mg, yield $6 \cdot 10^{-5}$%). The chloroform fraction (12.03 gms) was loaded on a 3.5 cm diametercolumn packed with silica. The fraction obtained with methanol-chloroform (4:96) system, (43 mg) was again subjected to a 14.5 cm long and 1.5 cm in diameter flash column chromatography, which resulted in the isolation of the compounds **3** and **4** by the preparative TLC in acetone-hexane system (30:70) and (35:65) affording **3** (20 mg) and **4** (10 mg) respectively.

Withalactone (1). Colorless amorphous powder, $[\alpha]D^{20} = +61°$ (c = 0.31, $CHCl_3$). HREI MS *m/z* 454.2617 (M^+, calcd for $C_{28}H_{38}O_5$, 454.2719). UV λ_{max} (MeOH) 224 nm. IR ν_{max} ($CHCl_3$) 3500, 1700, 1685, 1600 cm^{-1}.

^1H NMR ($CDCl_3$, 400 MHz) δ see Table 7. EI MS *m/z* (rel. int., %); [M^+] 454.2767 (5), 383 (12), 301 (11), 262 (14), 193 (15), 153 (29), 71 (100). ^{13}C NMR ($CDCl_3$, 100 MHz) δ see Table 7.

Withaoxylactone (2). Colorless amorphous powder, $[\alpha]D^{20} = -26°$ (c = 0.6, $CHCl_3$). UV λ_{max} (MeOH) 21.5 nm. IR ν_{max} ($CHCl_3$), 3500, 1701 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ see Table 7. EI MS *m/z* (rel. int., %); [M^+] 502 (31), 361 (27), 317 (11), 185 (53), 141 (100). ^{13}C NMR ($CDCl_3$, 100 MHz) δ see Table 7.

Quresimine-A (3). Colorless gum, $[\alpha]_D^{20} = +10$ (C = 0.8 g/100 ml $CHCl_3$). UV λ_{max} MeOH 202 nm. IR ν_{max} ($CHCl_3$) 1683, 3455 cm^{-1}. ^1H NMR ($CDCl_3$ 400 MHz) δ see Table 7. For EI MS *m/z*, (rel. int., %), M^+ 484.2804, (3.17), 470.2667 (2.99), 452.2530 (3.15), 387.2522 (3.42), 386.2456 (2.97), 331.1979 (3.46), 169.0810 (12.20), and 141.0571 (13.65). ^{13}C NMR ($CDCl_3$, 100 MHz) δ see Table 7.

Quresimine-B (4). Colorless gum, $[\alpha]D^{20} = +0.476190$ (C = 4.2 g/100 ml $CHCl_3$). UV λ_{max} (MeOH 215 nm). IR ν_{max} ($CHCl_3$) 1692, 1700, and 3555 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ see Table 7. For EI MS *m/z* (ret. int., %) cm^{-1}, [M^+] 486.3470 (2.31), 125.06025 (100), 361.2378, (4.50), and 187.0970 (13.61). ^{13}C NMR ($CDCl_3$, 100 MHz) δ see Table 7.

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