WITHANIA SOMNIFERA - A SOURCE OF EXOTIC WITHANOLIDES

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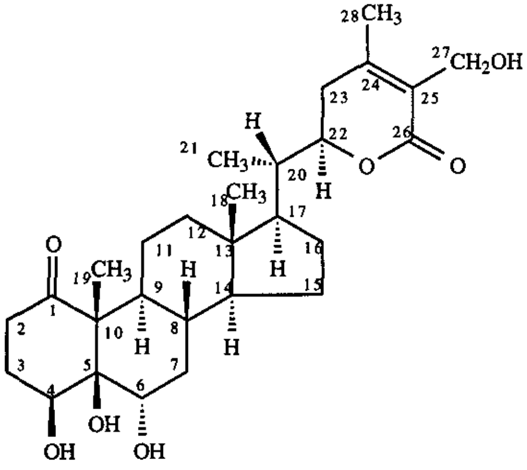
Abstract- *Withania somnifera* has yielded three new withanolides somniferícin (**1**), 2,3-dehydrosomniferícin (**2**), and withaoxylactone (**3**).

Withanolides are steroidal lactones, the first of which, withaferin A, was isolated 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.[1,2] Our studies have revealed that *W. somnifera* ramains a rich source of interesting new withanolides with potentially useful biological activities.[2]

During our investigations on withanolidal constituents of family Solanaceae, we examined the organic extracts of the plants of genus *Withania* and isolated several new withanolides.[2] In this paper we describe the isolation of three compounds (**1**, **2**, and **3**) from *W. somnifera*, whose structures were determined by using spectroscopic techniques.

Compound (**1**) was isolated from the defatted methanolic extract of the whole plant of *W. somnifera*. The high-resolution electron-impact mass spectrum (HREIms) afforded the molecular ion at m/z 490.2929 establishing the molecular formula as $C_{28}H_{42}O_7$ (calcd 490.2930) indicating eight degrees of unsaturation in the molecule. The M^+ was further confirmed by positive FAB ms data. The uv spectrum showed absorption at 225 nm indicating the presence of an α,β-unsaturated lactone.[3] The ir spectrum displayed bands at 3340, 1725, 1700, and 1665 cm^{-1} from which the presence of hydroxylic group, α,β-unsaturated lactone, ketone, and double bond in the molecule could be inferred.[4]

Dedicated to the memory of Dr. Yoshio Ban



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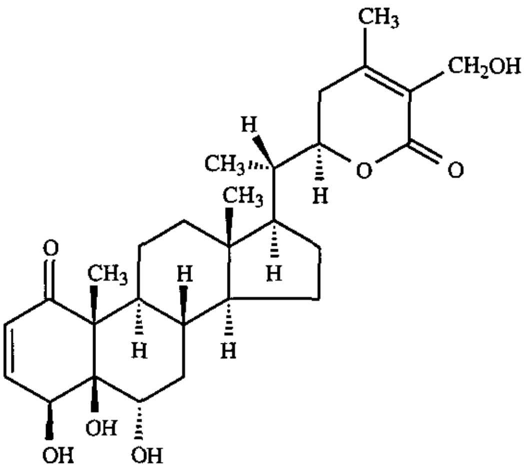


2

The ^{1}H-nmr spectrum of **1** exhibited three 3H singlets at δ 0.67, 1.24 and 2.03, assigned to the protons of the tertiary methyls. The doublet at δ 1.00 ($J_{21,20}$ = 6.5 Hz) was due to the protons of the C-21 secondary methyl group. Two AB doublets at δ 4.30 and 4.36 ($J_{27\alpha,27\beta}$ = 10.4 Hz) were due to the geminally coupled C-27 hydroxymethylenic protons. A downfield multiplet at δ 4.42 was ascribed to the C-22 methine proton geminal to an oxygen of an ester. Another multiplet at δ 2.00 was assigned to the C-20 methine proton. Two other downfield signals for hydroxy-bearing methine protons appeared at δ 4.87 ($J_{4,3a}$ = 10.8 Hz, $J_{4,3b}$ = 5.2 Hz) and 4.06 ($J_{6,7\alpha}$ = *11.1 Hz,* $J_{6,7\beta}$ *= 4.1 Hz) which were assigned to the C-4 and C-6 protons respectively.* No coupling between the two was seen. The presence of another hydroxy group was inferred by the appearance of a downfield quaternary carbon signal at δ 78.0 in the ^{13}C-nmr spectrum. The two secondary OH groups were tentatively placed on C-4 and C-6 based on literature precedents. The stereochemical assignments at C-4 and C-6 are based on the observed coupling constants. Assuming *cis*-decalin type chair conformations of rings A and B, the C-4 methine proton with β-oriented C-4 hydroxyl group should show one diaxial and one *axial-equatorial* couplings with the C-3 methylene protons. Coupling constants ($J_{a,a}$ = *10.8 Hz and* $J_{a,e}$ = 5.2 Hz) *of C-4H* signal, therefore, convincingly indicate β-orientation (*equatorial*) of the hydroxyl substituent. Similarly the C-6 methine proton exhibiting coupling constant $J_{a,a}$ = 11.1 Hz and $J_{a,e}$ = 4.1 Hz indicated an α-orientation (*equatorial*) of the C-6 hydroxyl and hence a β-orientation (*axial*) of the C-6 proton. The tertiary hydroxyl group was placed at C-5 because no other coupling was seen between C-4H or C-6H with a common coupled proton at C-5. The stereochemistry at C-5 was difficult to deduce from the spectroscopic data. The ^{13}C-nmr chemical shift (δ 78.0), however, indicatd a β-orientation of the hydroxyl group as in withaperuvin.[5] The β-orientation of the C-5 hydroxyl group can also be inferred from a *gauche* interaction between the C-19 and C-5β OH group, which results in a considerable shielding of the C-19 resonance (δ 9.8 instead of δ~15).[5] The C-2 methylenic protons appeared as multiplets at δ 2.50 and 2.30, while both the C-3 methylenic protons appeared as overlapping multiplets centered at ~ δ 2.00. The C-23 mehtylenic protons resonated as two multiplets at δ 2.00 and 2.50. The C-7 methylenic protons exhibited multiplets at ~ δ 2.00 and 1.50. The ^{1}H-nmr assignments to the various protons of rings A and B were based on a comparison with a previously reported compound, withaperuvin, isolated from the leaves of *Physalis peruviana*,[5] and further confirmed from the COSY-45° experiment.

The ^{1}H-^{1}H long-range correlations were determined by recording a series of HOHAHA (TOCSY) spectra with variable delays (20, 60, 100 ms). The C-21 methyl protons displayed interactions with the C-22 methine and C-23 α and β protons. The C-22H also exhibited long-range couplings with the C-21 methyl protons. The C-28 methyl protons showed homoallylic coupling with the C-27 methylene protons in HOHAHA spectra.

The ^{13}C-nmr spectra (broad-band decoupled and DEPT)[6] (Table **1**) showed resonances for all twenty eight carbons in the molecule. These experiments indicated that there were four methyl, nine methylene, eight methine, and seven quaternary carbons. The downfield signals at δ 210.8 aand 166.8 were assigned to the ketone and lactone carbonyls, respectively. The C-5 quaternary carbon was found to be resonating at δ 78.0, its downfield chemical shift was indicative of the presence of an oxygen function at this position. The three oxygen-bearing carbons appeared at δ 68.1 (C-4), 75.8 (C-6) and 78.1 (C-22). The ^{13}C-nmr assignments of various carbons in **1** is given in Table **1**.

To establish direct ^{1}H/^{13}C connectivities, the HMQC (*Heteronuclear Multiple Quantum Coherence*) experiment[6,7] was performed. The proton signals at δ 4.87 (C-4H) and 4.06 (C-6H) were found to be cross-linked with the carbons resonating at δ 68.1 and 75.8, assigned to C-4 and C-6 carbons respectively. The AB doublets of the C-27 methylene at δ 4.30 and 4.36 exhibited cross-peaks with the carbon signal at δ 57.4 (C-27). The one-bond heteronuclear interactions determined by HMQC are presented in Table **1**.

The ^{1}H/^{13}C long-range connectivities were determined by HMBC (*Heteronuclear Multiple Bond Connectivity*) experiment.[6,7] The C-19 methyl exhibited long-range interactions with C-1 carbonyl. The C-27 methylenic protons were found to be coupled with C-25, C-24 and C-26. The C-28 methyl protons showed long-range couplings with the C-24 and C-25 carbons.

The mass fragmentation pattern of **1** was typical of hydroxylated multifunctional withanolides. The ion at *m/z* 169.1008 of composition $C_9H_{13}O_3$ in the eims resulted by the cleavage of the C-17/C-20 bond. Another ion at *m/z* 212.1351 of composition $C_{11}H_{16}O_4$ may be formed by the cleavage of the C-9/C-11 and C-8/C-14 bonds, and thus indicated the presence of four oxygen functions in rings A and B.

The spectroscopic evidence presented above led to structure (**1**) for this new withanolide. The stereochemistry at various asymmetric centers was assigned on the basis of chemical shift comparisons with known withanolides and on biogenetic arguments.[8,9]

Compound (**2**) was found to be the 2,3-dehydro derivative of **1**. The uv spectrum showed a characteristic absorption at 219 nm for an α,β-unsaturated lactone.[3] In the ir spectrum the absorption bands for hydroxyls, lactone and α,β-unsaturated ketone appeared at 3425, 1720 and 1681 cm^{-1}, respectively.[4]

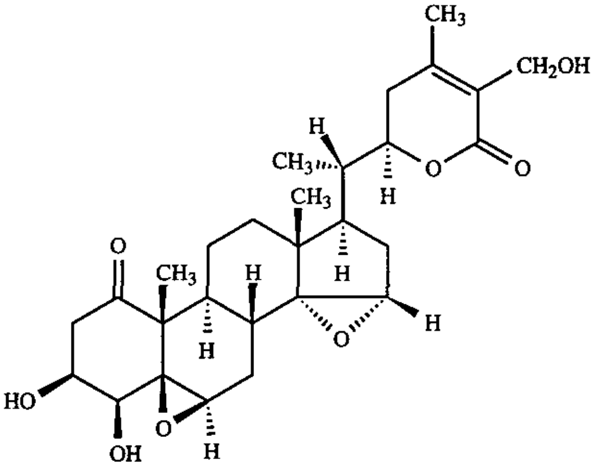
The ^{1}H-nmr spectrum of **2** showed distinct resemblance to that of **1**. The appearance of two downfield doublet of doublets at δ 5.90 and 6.48 was however the only notable difference between **2** and **1**. These signals were assigned to the C-2 and C-3 vinylic protons in ring A. The vinylic C-2 and C-3 protons exhibited COSY-45° interactions with the oxygen-bearing C-4H (δ 5.10). The C-6H resonated as a doublet of doublet at δ 4.09 showed COSY interactions with the two multiplets at δ 2.10 and 1.40 which were assigned to the C-7 protons. In the HOHAHA spectra, the C-2H showed interactions with the C-3 and C-4 protons. the remaining COSY and TOCSY patterns were same as **1**.

The ^{13}C-nmr spectra (Table **1**) of **2** was distinctly similar to that of **1**. Two downfield signals at δ 128.0 and 143.8 representing vinylic C-2 and C-3 respectively, further confirmed an α,β-unsaturated ketone function in ring A. The one-bond $^1H/^{13}C$ connectivities in **2** were established on the basis of HMQC experiment and are summarized in Table **1**.

In the HMBC spectrum, the C-2H (δ 5.90) showed cross-peaks with C-4 and C-10, while C-3H (δ 6.48) exhibited cross-peaks with C-5 and C-1. The C-4H at δ 5.10 showed cross-peaks with C-3, C-2 and C-5. The C-6H in ring B showed interactions with C-5 and C-4.

The mass spectrum of **2** showed the M^+ at *m/z* 488.2785 which is 2 a.m.u. less than that of **1** corresponding to the molecular formula $C_{28}H_{40}O_7$ (calcd 488.2783). The overall mass spectral behaviour of **2** distinctly resembled to that of **1**. The above mentioned spectroscopic observations led to structure (**2**) for this new withanolide.

The third compound, named withaoxylactone (**3**), $C_{28}H_{38}O_8$, showed uv absorption at 215 nm, characteristic of an α,β-unsaturated lactone chromophore.[3] The ir spectrum showed peaks at 1701 (α,β-unsaturated lactone) and 3500 (OH) cm^{-1}.[4]



3

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-18, C-19 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, $J_{3\alpha,2\alpha}$ = 7.4 Hz, $J_{3\alpha,4\alpha}$ = 3.7 Hz) was assigned to the C-3 methine proton. Another methine proton resonating as a doublet at δ 3.40 ($J_{4,3}$ = 3.7 Hz) was assigned to C-4 methine proton. A downfield doublet of double doublets centered at δ 4.38 ($J_{22,20}$ = 13.3 Hz, $J_{22,23\beta}$ = 3.5 Hz, $J_{22,23\alpha}$ = 3.5 Hz) was assigned to the C-22 methine proton geminal to the lactone oxygen. Two AB doublets at δ 4.32 and 4.25 ($J_{27\alpha,\beta}$ = 12.3 Hz) were ascribed to the hydroxymethylene protons. 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 (H-6) and 3.31 (H-15) 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 quarternary carbon and the other end was tertiary carbon. The β stereochemistry of C-5/C-6 epoxide was assigned on the basis of comparison between the coupling constants of H-6/H-7 in **3** to those reported earlier in the literature.[8-11] The compounds having C-5/C-6 β epoxide give J-values in the range between 0.8-2.3 Hz.[9] While in case of C-5/C-6 α epoxide the J-values ($J_{6,7}$) are in the range of 4-5 Hz.[10-11] In many cases it was also seen that the C-6H appeared as a broad singlet at δ 3.14-3.18, very similar to **3**.[9] The α-sterechemistry of C-14/C-15 epoxide was deduced on the basis of chemical shift comparison of H-15 in **3**, to those reported in the literature.[12] The H-15 in withanolides containing C-14/C-15 epoxide resonate in the range of δ 3.26 - 3.58 as broad singlets.[12] The C-14/C-15 β epoxides have not been reported, presumably due to to the steric hindrance preventing the oxidizing enzyme from approaching the C-14/C-15 olefin from the same side as the bulky C-17 side chain.[13]

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-16α and β protons respectively.

The ^{13}C-nmr spectra of withaoxylactone (**3**) exhibited twenty eight carbon resonances. The signals at δ 154.9, 124.9 and 167.1 were assigned to the vinylic C-24, C-25 and carboxylic C-26 respectively.[4] 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 **1**.

The HMQC spectrum of **3** established one-bond ^{1}H/^{13}C couplings. 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 αH). The downfield methine protons at δ 3.63 (H-3), 3.40 (H-4), 3.14 (H-6), 3.31 (H-15β) and 4.38 (H-22) showed one-bond interactions with the carbons resonating at δ 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 **3** is presented in Table **1**.

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 shifts assignments.

The HREIms spectrum of **3** 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 $C_{7}H_{9}O_{3}$ could result by the cleavage of the C-20/C-22 bond, whereas the ion at *m/z* 169.0865 ($C_{9}H_{13}O_{3}$) may arise by the cleavage of the C-17/C-20 bond. The peak at *m/z* 185.0812 ($C_{9}H_{13}O_{4}$) may arise by the cleavage of rings B and C. This fragment has four oxygen atoms and therefore supports the proposed substitutions at rings A and B of the molecule. These spectroscopic studies supported structure (**3**) [3β,4β-dihydroxy-5β,6β,14α,15α-diepoxy-1-oxowitha-24-enolide] for this new withanolide.

Table 1: ^{13}C- and ^{1}H-nmr chemical shifts of compounds (**1**, **2**, and **3**) #,†.

C	**1**		**2**		**3**	
No.	$\delta^{13}C$	$\delta^{1}H$ [J(Hz)]	$\delta^{13}C$	$\delta^{1}H$ [J(Hz)]	$\delta^{13}C$	$\delta^{1}H$ [J(Hz)]
1	210.8	-	200.4	-	210.6	-
2	29.9	2.30 m 2.50 m	128.0	5.90 dd, $J_{2,3}$= 10.4, $J_{2,4}$= 2.3	39.8	2.58 m 2.84 m
3	28.8	2.00 m	143.8	6.48 dd, $J_{3,2}$= 10.4, $J_{3,4}$= 2.2	77.4	3.63 ddd, $J_{3,2\beta}$= 7.4, $J_{3,2\alpha}$= 7.4, $J_{3,4}$ = 3.7
4	68.1	4.87 dd,$J_{4,3\alpha}$=10.8, $J_{4,3\beta}$=5.2	66.9	5.10 br t, $J_{4,3/2}$= 2.2	73.9	3.40 d, $J_{4,3}$= 3.7
5	78.0	-	78.7	-	64.3	-
6	75.8	4.06 dd,$J_{6,7\alpha}$= 11.1 $J_{4,7\beta}$= 4.1	74.9	4.09 dd, $J_{6,7\alpha}$= 11.8, $J_{4,7\beta}$= 5.8	55.7	3.14 br s
7	29.6	2.00 m 1.50 m	29.9	2.10 m 1.40 m	30.9	2.13 m 1.35 m
8	38.7	1.62 m	33.8	1.65 m	42.6	1.10 m
9	35.1	1.97 m	39.2	1.98 m	29.3	1.35 m
10	57.3	-	55.7	-	50.3	-
11	22.1	1.46 m	22.7	1.43 m	26.9	1.50 m
12	36.1	1.37 m	36.6	1.38 m	23.9	1.25 m
13	43.1	-	43.1		49.0	-
14	51.9	2.34 m	45.2	2.10 m	63.5	-
15	24.12	1.29 m	24.1	1.29 m	58.4	3.31 br s
16	39.4	1.95 m	38.8	1.93 m	38.7	2.18 m 1.10 m
17	46.5	1.56 m	55.7	1.60 m	51.5	1.20 m
18	11.8	0.67 s	11.9	0.68 s	11.1	0.63 s
19	9.8	1.24 s	10.1	1.20 s	14.2	1.22 s
20	39.4	2.00 m	40.0	2.00 m	38.5	1.64 m
21	13.3	1.00 d, $J_{21,20}$= 6.5	13.3	0.96 d, $J_{21,20}$= 6.6	12.8	0.95 d, $J_{21,20}$= 7.0
22	78.1	4.42 m	78.8	4.40 m	78.6	4.38 ddd, $J_{22,20}$= 13.3, $J_{22,23\beta}$= 3.5, $J_{21,23\alpha}$= 3.5
23	35.0	2.00 m 2.50 m	30.0	2.00 m 2.50 m	32.9	1.90 m 2.46 m
24	152.5	-	152.6	-	154.9	-
25	125.6	-	125.8	-	124.9	-
26	166.8	-	166.3	-	167.1	-
27	57.4	4.30 d, J= 10.4 4.36 d, J= 10.4	57.4	4.30 d, $J_{27a,27b}$= 10.4 4.39 d, $J_{27a,27b}$= 10.4	55.7	4.35 d, $J_{27a,27b}$= 12.3 4.51 d, $J_{27a,27b}$= 12.3
28	19.9	2.03 s	19.9	2.02 s	19.6	2.03 s

† Multiplicities were confirmed by DEPT.

Proton-carbon correlations are based on the HMQC experiment.

EXPERIMENTAL

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

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

Extraction and Isolation.- The air dried whole plant was soaked in MeOH (250 l) for fifteen days at room temperature. The extract obtained (200 l) was evaporated to a gum (C-20 kg). The gum was dissolved in MeOH (10 l) and defatted with pet. ether (10 l) (40-60°) for two days. The defatted MeOH extract was again evaporated and the residue (1.78 kg) was dissolved in water (10 1). The aqueous extract was extracted with $CHCl_3$ at different pH values. The fraction (500 mg) obtained at pH 7 was loaded on a silica gel column (3.13 kg) and eluted first with hexane and then with haxane-chloroform, chloroform, and chloroform-methanol mixtures. The fractions collected on elution with chloroform-methanol mixture were combined and subjected to column and preparative tlc (precoated silica gel, 0.25 mm) in acetone-pet. ether (4:6) to afford **1** (6 mg, yield $6x10^{-6}$ %) and **2** (10 mg, yield $1x10^{-5}$ %) while the fraction collected with hexane-chloroform (3:7) was subjected to preparative tlc (precoated silica gel, 0.25 mm) in methanol-chloroform (2.5 : 7.5) to afford **3** (31 mg, yield $3.1x10^{-5}$ %).

Somniferícin (**1**).- Colorless gum, $[\alpha]_D^{20}$= 162° (c = 0.024, $CHCl_3$). Uv λ_{max} (MeOH) 225 nm. Ir ν_{max} ($CHCl_3$) 3340, 2850, 1725, 1700, 1665 cm^{-1}. ^1H-Nmr ($CDCl_3$, 300 MHz) δ see Table **1**. Eims m/z (rel. int.%) [M^+], 490.2929 (31), 349 (6), 212 (4). 169 (7), 149 (9), 141 (100), 117 (57). ^{13}C-Nmr ($CDCl_3$, 100 MHz) δ see Table **1**.

2,3-Dehydrosomniferícin (**2**).- Colorless amorphous powder, $[\alpha]_D^{20}$ = 272° (c = 0.01, $CHCl_3$). Uv (MeOH) 219 nm. Ir ν_{max} ($CHCl_3$) 3425, 1681 cm^{-1}. ^1H-Nmr ($CDCl_3$, 300 MHz) δ see Table **1**. Eims m/z (rel. int. %), [M^+] 488.2785 (15), 405 (26.5), 347 (11.6), 169 (5.8), 144 (22), 141 (100), 83 (55). ^{13}C-nmr ($CDCl_3$, 100 MHz) δ see Table **1**.

Withaoxylactone (**3**).- Colorless amorphous powder, $[\alpha]_D^{20}$ = -26° (c = 0.6, $CHCl_3$). Uv λ_{max} (MeOH) 215 nm. Ir ν_{max} ($CHCl_3$) 3500, 1701 cm^{-1}. ^1H-Nmr ($CDCl_3$, 400 MHz) δ see Table **1**. Eims 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 **1**.

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