Cardenolides from the Methanolic Extract of *Nerium oleander* Leaves Possessing Central Nervous System Depressant Activity in Mice

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Two new cardenolides, 3β-*O*-(D-2-*O*-methyldigitalosyl)-14β-hydroxy-5β-carda-16,20(22)-dienolide (**1**) and 3β-hydroxy-8,14-epoxy-5β-carda-16,20(22)-dienolide (**2**), and two known cardenolides, 3β-*O*-(D-digitalosyl)-14β-hydroxy-16β-acetoxy-5β-card-20(22)-enolide (**3**) and 3β-*O*-(D-digitalosyl)-14β-hydroxy-5β-card-20(22)-enolide (**4**), have been isolated from the leaves of *Nerium oleander* following a bioactivity-directed isolation of the MeOH extract, which showed central nervous system (CNS) depressant activity in mice at a dosage of 50 mg/kg ip. Their structures were established on the basis of chemical and spectral data. Compounds **1**, **3**, and **4** were found to exhibit sedation in mice at a dosage of 25 mg/kg, although **2** had no effect on the CNS of mice at a dosage of up to 50 mg/kg.

Nerium oleander L. (Syn. *N. odorum* Soland; *N. indicum* Mill)[1] (Apocynaceae), distributed in the Mediterranian region and in subtropical Asia, is indigenous to the Indo-Pakistan subcontinent. The plant is commonly known as "kaner", and its various parts are reputed as therapeutic agents in the treatment of swellings, leprosy, and eye and skin diseases. The leaves possess cardiotonic, antibacterial, anticancer, and antiplatelet aggregation activity and depress the central nervous system.[2−7] Investigations on the different parts of the plant have revealed the presence of several glycosides, triterpenes, and straight-chain compounds.[8−14] This paper deals with the isolation and structure elucidation of four cardenolides, two of which are new, namely nerizoside (**1**) and Δ^{16}-dehydroadynerigenin (**2**), while two are known compounds identified as neritaloside (**3**) and odoroside H (**4**). Compound **3** was previously not reported from the leaves. The structural studies are based on ^{1}H- and 2D NMR experiments (COSY-45, NOESY, *J* resolved, and HMQC). Structures of **1** and **2** have been elucidated as 3β-*O*-(D-2-*O*-methyldigitalosyl)-14β-hydroxy-5β-carda-16,20(22)-dienolide and 3β-hydroxy-8,14-epoxy-5β-carda-16,20(22)-dienolide.

Results and Discussion

Nerizoside (**1**) tested positive for cardenolides (Legal and Raymond test.[15] Its molecular formula, $C_{31}H_{46}O_8$ was obtained by FAB +ve, EI, HRMS, and ^{13}C NMR. Its UV spectrum showed a maximum at 267 nm, indicating the presence of an α,β-unsaturated γ-lactone with further conjugation,[16] while the IR spectrum showed bands at 3450 (OH), 1780, 1740 (α,β-unsaturated γ-lactone), and 1620 (C=C) cm^{-1}. The ^{1}H-NMR spectrum showed two double doublets of one proton each at δ 5.13 and 5.06 for H-21a (J = 16.7, 1.6 Hz) and H-21b (J = 16.7, 1.6 Hz) and a one-proton broad singlet at δ 6.03 for H-22. Two singlets at δ 1.23 and 1.02 were attributed to H-18 and H-19, respectively. The double bond was placed at C-16, considering that the UV maximum was at 267 nm, of a one-proton triplet at δ 6.27 (J = 3.1 Hz, H-16) and two double doublets at δ 2.66, 2.55 (J = 20.0, 3.1 Hz, H-15a and H-15b). A broad singlet at δ 4.01 was assigned to the carbinylic proton H-3. Thus, the nine double-bond equivalents exhibited by the molecular formula were satisfied by the four rings of the steroidal skeleton, the α,β-unsaturated lactone ring, a C=C double bond at C-16, and the ring of the sugar moiety indicated by the NMR spectra. The ^{1}H- and ^{13}C-NMR (Table 1) data indicated the presence of one molecule of sugar because of an anomeric proton at δ 4.23 for H-1′ (d, J = 7.7 Hz) connected to the anomeric carbon at δ 101.36 in the HMQC spectrum. The chemical shift and coupling constants of H-1′ suggested β-linkage of the sugar. The ions at *m/z* 192.0942 ($C_8H_{16}O_5$), 175.0993 ($C_8H_{15}O_4$), 174.0870 ($C_8H_{14}O_4$), 157.0919 ($C_8H_{13}O_3$), 144.0835 ($C_7H_{12}O_3$), and 113.0631 ($C_6H_9O_2$) showed the composition of the sugar moiety as $C_8H_{16}O_5$. This was confirmed by $[M + H]^+$ and $[M - H]^-$ ions at *m/z* 547 and 545 in FAB +ve and FAB −ve mass spectra, respectively. The connectivity of H-1′ with H-2′, H-2′ with H-1′ and H-3′, of H-3′ with H-4′ and H-2′, of H-4′ with H-3′ and H-5′, and of H-5′ with H-4′ and H-6′ in the COSY-45 spectrum led to assignments of all protons of the sugar moiety. The relationship of various protons was confirmed by proton decoupling experiments. Their multiplicities and coupling constants were deduced from the normal ^{1}H-NMR spectrum as follows. H-2′ showed a double doublet at δ 3.53 (J = 9.7, 7.7 Hz); H-3′ appeared as a one-proton double doublet at δ 3.13 (J = 9.7, 3.3 Hz); H-4′ resonated as a one-proton double doublet at δ 3.82 (J = 3.3, 1.7 Hz), whereas H-5′ appeared as a doublet of quartet at δ 3.56 (J = 6.4, 1.7 Hz). H-6′ resonated as a three-proton doublet at δ 1.26 (J = 6.4 Hz), while the two OCH_3 protons resonated at δ 3.34 and 3.45 as two singlets. The NOESY interactions of OCH_3 protons at δ 3.34 with H-1′ (δ 4.26) and of OCH_3 protons at δ 3.45 with H-3′ (δ 3.13) and H-4′ (δ 3.82) were particularly helpful in assigning exactly the chemical shifts at δ 3.34 and 3.45 to OCH_3 groups at C-2′ and C-3′, respectively. An interaction between H-4′ (δ 3.82) and H-5′ (δ 3.56) was also present. These observations showed that the sugar was β-D-2-*O*-methyldigitalose. The carbon chemi-

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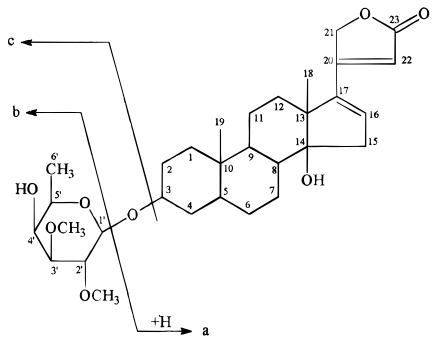
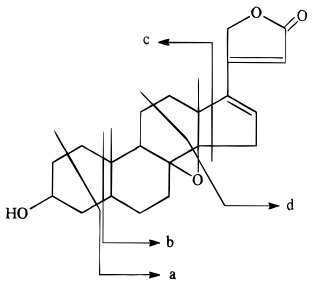
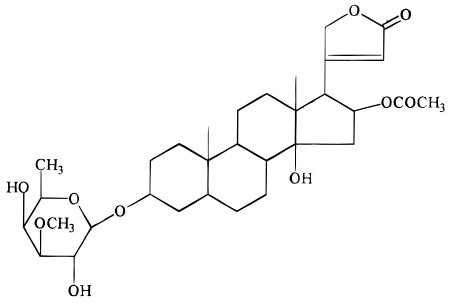


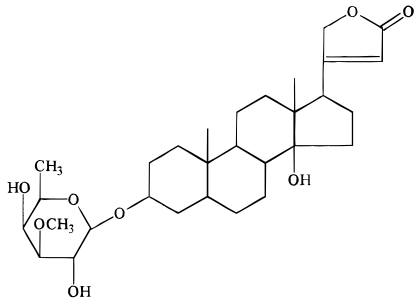
Significant EI-ms fragments of *nerizoside* (1)



Significant EI-ms fragments of *Δ 16 dehydroadynerigenin* (2)



Neritaloside (3)



Odoroside H (4)

cal shifts were conclusively assigned on the basis of ^{13}C-NMR (broad band and DEPT) and HMQC spectra and comparison with similar compounds.[17−20] A significant fragment at *m*/*z* 372 (EIMS), 373 (FAB +ve), a double bond at C-16, and a hydroxyl group at C-14 (δ_C 84.50) revealed that the aglycon is 14β-hydroxy-5β-carda-16,-20(22)-dienolide. Thus, compound **1** was assigned the structure 3β-*O*-(D-2-*O*-methyldigitalosyl)-14β-hydroxy-5β-carda-16,20(22)-dienolide.

Δ^{16}-Dehydroadynerigenin (**2**) showed the molecular ion peak at 370, an exact measurement of which gave 370.2120, corresponding to $C_{23}H_{30}O_4$. The IR spectrum showed peaks at 3425 (OH), 1780, 1740 (α,β-unsaturated γ-lactone), and 1620 (C=C) cm^{-1} and an UV absorption at 267 nm, indicating the presence of an α,β-unsaturated γ-lactone with further conjugation.[15] The double bond was placed at C-16, considering the UV maxima and the ^{1}H-NMR spectrum, which showed a one-proton triplet at δ 6.27 (J = 2.8 Hz, H-16) and two one-proton double doublets at δ 2.64 and 2.55 (J = 20.0, 2.8 Hz, H-15a and H-15b). The ^{1}H-NMR spectrum further showed two one-proton doublets at δ 5.02 and 5.12 for H-21a (J = 16.8, 1.6 Hz) and H-21b (J = 16.8, 1.6 Hz), and a one-proton broad singlet at δ 6.02 for H-22. The molecular formula of **2** showed nine double-bond equivalents in the molecule, eight of which were justified by the carbocyclic nucleus, the α,β-unsaturated lactone ring, and the carbon−carbon double bond. Placement of various functional groups in the steroidal skeleton left one oxygen function and a double-bond equivalent to be accounted for, which was interpreted in terms of an epoxy function between C_8 and C_{14}, and was corroborated by the absence of any other proton geminal to the oxygen function and the presence of two quaternary carbinylic carbons at δ 64.48 (C-8) and 71.84 (C-14) in the ^{13}C-NMR spectrum. Hence **2** was 3β-hydroxy-8,14β-epoxy-5β-carda-16,20(22)-dienolide. The stereochemistry as drawn in the structure was supported by NOESY interactions of H-19 (δ 1.03) with H-18 (δ 1.22) and H-5 (δ 1.51), showing that all three protons lie in the same plane, that is, β, and hence, the A/B ring junction is *cis*, as normally observed in cardenolides. Compound **2** has been reported earlier as a

Table 1. ^{1}H- and ^{13}C-NMR Data of **1**[a]

C	δ_C	H	δ_H	multiplicity	*J* (Hz)
1	26.50	1a	1.55	m	
		1b	1.95	m	
2	23.40	2a	1.75	m	
		2b	2.15	m	
3	77.00	3	4.01	br s	
4	30.55	4a	1.94	m	
		4b	1.46	m	
5	36.80	5	1.79	m	
6	25.50	6a	1.12	m	
		6b	1.19	m	
7	19.00	7a	1.39	m	
		7b	2.15	m	
8	37.50	8	1.82	m	
9	36.50	9	1.76	m	
10	33.70				
11	24.00	11a	1.85	m	
		11b	1.40	m	
12	37.00	12a	2.00	m	
		12b	2.10	m	
13	51.40				
14	84.50				
15	33.00	15a	2.66	dd	20.0, 3.1
		15b	2.55	dd	20.0, 3.1
16	136.00	16	6.27	t	3.1
17	160.70				
18	16.50	18	1.23	s	
19	24.20	19	1.02	s	
20	176.90				
21	73.50	21a	5.13	dd	16.7, 1.6
		21b	5.06	dd	16.7, 1.6
22	114.00	22	6.03	br s	
23	174.70				
1′	105.50	1′	4.26	d	7.7
2′	73.00	2′	3.53	dd	9.7, 7.7
3′	86.00	3′	3.13	dd	9.7, 3.3
4′	69.00	4′	3.82	dd	3.3, 1.7
5′	72.20	5′	3.56	qd	6.4, 1.7
6′	16.80	6′	1.26	d	6.4
−OCH_3	57.50		3.34	s	
−OCH_3	57.50		3.45	s	

[a] The assignments are based on COSY-45, *J*-resolved, and HMQC spectra.

reaction product,[21] but this is the first instance of its isolation as a natural product. It may be noted that compound **2** is not an artifact because fresh plant material was extracted with MeOH at room temperature. Furthermore, a co-TLC of **2** with the extract showed its presence in the extract, thus avoiding any chance of its formation further along in the isolation process.

Compounds **3** and **4** were identified as neritaloside and odoroside H based on comparison of ^{1}H- and ^{13}C-NMR data with literature reports.[22]

Compounds **1**−**4** were obtained after a bioassay-directed isolation of leaf MeOH extract that showed central nervous system (CNS) depressant activity in mice at a dosage of 50 mg/kg ip. Compound **1** produced a decrease in locomotor activity at 25 mg/kg, but no other signs of depression were observed. At a dosage of 50 mg/kg, the animals showed other signs in addition to decreased motor activity, such as a decrease in touch response and a staggering gait, which lasted for 40 min. The animals regained normal activity in 1.5 h.

Compound **3** also showed a decrease in motor activity of the animals at 25 mg/kg ip; again, there was no decrease in touch response. Some staggering was, however, observed after 30 min. At 50 mg/kg, the animals exhibited signs of fast respiration, abduction of limbs, slight tremor on movement, passivity after 1 h, and a decrease in body and limb tone, but returned to normal after 4 h.

The common signs observed on injecting compound **4** at a 25-mg/kg dose were decreased activity, touch response, ptosis, staggering gait, and passivity. At a dosage of 50 mg/kg, the animals remained motionless, with decrease in body and limb tone, and two out of five animals died.

Experimental Section

General Experimental Procedures. Melting points were determined on a Gallenkamp melting-point apparatus and are uncorrected. UV and IR spectra were recorded on Hitachi-u-3200 and JASCO A-302 spectrometers, respectively. The EIMS, FABMS, and HRMS were recorded on Finnigan MAT-112, MAT-312, and JMS HX-110 spectrometers. The ^{1}H-NMR spectra were taken in CD_3OD (for compounds **1** and **2**) and $CDCl_3$ (for compounds **3** and **4**) on a Bruker Aspect AM-300 and Bruker AM-500 FT-NMR spectrometers operating at 300 and 500 MHz, respectively, while the ^{13}C-NMR (broad band and DEPT) spectra were obtained in CD_3OD and $CDCl_3$ on the same instruments operating at 75 and 125 MHz, respectively. The spectra were referenced to the residual solvent signals. The chemical shifts are in parts per million (δ), and coupling constants (J) are in Hz. The ^{13}C-NMR (Tables 1 and 2) spectral assignments were made partly through DEPT and HMQC and partly through a comparison of the chemical shifts with the published data for similar compounds.[17−22] Assignments of protons were based on COSY-45 and NOESY experiments. The purity of compounds was monitored on TLC with Si gel PF_{254}: mobile phase $CHCl_3$−MeOH (9.80:0.20). Si gel 9385 was used for flash (column chromatography Eyela).

Biological Activity. Mice of NMRI strain, weighing between 18 and 22 g, were used. They were maintained under standard colony conditions in our animal house. The volume of injection was 10 mL/kg of body weight. The samples were dissolved in 5% Tween 80 and given intraperitoneally. The animals were observed continuously for 0.5 h and then every 30 min for 6 h. The behavior was scored according to the modified procedure as described by Irwin.[23]

Table 2. ^{1}H- and ^{13}C-NMR Data of **2**[a]

C	δ C	H	δ H	multiplicity	J(Hz)
1	27.90	1a	1.67	m	
		1b	1.94	m	
2	25.70	2a	1.89	m	
		2b	2.00	m	
3	67.38	3	4.05	quintet	2.7
4	28.90	4a	1.61	m	
		4b	1.54	m	
5	34.08	5	1.51	m	
6	24.54	6	1.57	m	
7	24.15	7	1.88	m	
8	64.40				
9	37.40	9	1.85	m	
10	33.80				
11	19.50	11	1.55	m	
12	39.80	12a	1.62	m	
		12b	1.61	m	
13	47.60				
14	71.84				
15	34.30	15a	2.64	dd	20.0, 2.8
		15b	2.55	dd	20.0, 2.8
16	134.30	16	6.27	t	2.8
17	160.70				
18	16.20	18	1.22	s	
19	15.80	19	1.03	s	
20	176.90				
21	73.20	21a	5.02	dd	16.8, 1.6
		21b	5.12	dd	16.8, 1.6
22	113.00	22	6.02	br s	
23	160.70				

[a] Coupling constants, in Hz, were calculated from ^{1}H-NMR and 2D J-resolved spectra.

Plant Material. The leaves of *N. oleander* were collected from the Karachi region. The plant was authenticated by Prof. Dr. Syed Irtifaq Ali of the Department of Botany, University of Karachi, and a voucher specimen (N.ol-1) was deposited in the herbarium of the same department.

Extraction and Isolation. Fresh and uncrushed leaves (40 kg) were extracted with MeOH at room temperature. The concentrated syrupy residue obtained on removal of the solvent from the combined extracts under reduced pressure showed CNS depressant effects in mice. It was shaken out with EtOAc and H_2O. The EtOAc layer was extracted with 4% aqueous Na_2CO_3 solution to separate the acidic fraction from the neutral fraction. The EtOAc layer containing the neutral fraction was washed, dried over anhydrous Na_2SO_4, and charcoaled. The charcoal bed was washed successively with EtOAc and MeOH−C_6H_6 (1:1). The solvent from the combined EtOAc filtrate and washings was evaporated, and the fraction was marked as N-1. The residue from the MeOH−C_6H_6 eluate was marked as N-2. Both of these fractions were tested for their effects on the CNS. N-1 was found to be active and showed a CNS depressant activity at a dosage of 50 mg/kg, whereas N-2 was inactive at this dosage. N-1 was divided into petroleum ether-soluble (NPE) and -insoluble (NIPE) fractions. The NPE fraction was inactive up to 50 mg/kg and was not pursued further in the present studies. The active NIPE fraction was dissolved in a minimal quantity of MeOH and kept cold overnight. A white crystalline residue precipitated, which was filtered, and

the filtrate was again kept for crystallization. Several crystalline crops thus obtained were combined and recrystallized from the same solvent. The colorless flowers of needles ultimately obtained were identified as ursolic acid through comparison of its spectral data with those reported in the literature.[24,25] The residue from the filtrate of ursolic acid was marked as B-1. Both ursolic acid and B-1 were tested for their activity on the CNS. B-1, the active fraction, was again treated with petroleum ether to give petroleum ether-soluble (B-3) and -insoluble (B-2) fractions. These were again tested for their activity on the CNS. B-2 showed CNS depressant activity with convulsions, while B-3 exhibited depressant activity with hypnosis. B-2 was subjected to further separation using VLC (petroleum ether−EtOAc, followed by $CHCl_3$−MeOH, in order of increasing polarity). On combining the eluates on the basis of TLC, 14 fractions (fr.1−fr.14) were ultimately obtained. The main fraction (fr.2) possessing significant sedative−hypnotic activity was subjected to further purification through VLC (petroleum ether−EtOAc, in increasing order of polarity), which afforded 20 fractions (NO-1−NO-20) on combining the eluates on the basis of TLC. NO-15, NO-17, and NO-18 were pure constituents subjected for determination of their effect on the CNS. NO-15 showed sedation at 50 mg/kg, while NO-17 and NO-18 showed sedation at 25 mg/kg. NO-15 was characterized as 3*β*-*O*-(D-2-*O*-methyldigitalosyl)-14*β*-hydroxy-5*β*-carda-16,20(22)-dienolide (**1**), NO-17 as 3*β*-*O*-(D-digitalosyl)-14*β*-hydroxy-5*β*-card-20(22)-enolide (**4**), and NO-18 as 3*β*-*O*-(D-digitalosyl)-14*β*-hydroxy-16*β*-acetoxy-5*β*-card-20(22)-enolide (**3**).

Fraction B-3 (30 g), referred to above, was also subjected to further separation using VLC (petroleum ether−EtOAc, followed by $CHCl_3$−MeOH, in order of increasing polarity). On combining the eluates on the basis of TLC, four fractions (fr. 1−fr. 4) were ultimately obtained. The main fraction (fr. 1; 14 g), possessing significant sedative−hypnotic activity, was subjected to further purification through VLC (petroleum ether−EtOAc, in increasing order of polarity), which afforded nine fractions (fr. 1-I−fr. 1-IX) on pooling together the eluates on the basis of TLC. All these fractions were tested for CNS activity, and Fr. 1-VI (4.2 g), which was found active at a dose of 12.5 mg/kg, was further purified through flash column chromatography ($CHCl_3$, $CHCl_3$−MeOH, in increasing order of polarity). On usual follow up, 11 fractions (fr. 1-VI-1−fr. 1-VI-11) were ultimately obtained. Fr. 1-VI-2 and Fr. 1-VI-3 were the major components, showing more or less similar TLC pattern. These were combined (1.43 g) and resolved on aluminum cards precoated with silica using the solvent system $CHCl_3$−MeOH (9.80:0.20) into six components. Of these, the third band (89.8 mg) was the major one and further resolved over precoated silica cards with $CHCl_3$−MeOH (9.80:0.20) into five components. The fifth band (13.5 mg) consisted of a single compound characterized as 3*β*-hydroxy-8,14*β*-epoxy-5*β*-carda-16,20-(22)-dienolide (**2**). It was inactive up to a dosage of 50 mg/kg. The remaining bands could not be purified in a workable amount.

Nerizoside (1): fine needles from MeOH (10 mg); mp 223−225 °C; UV (MeOH) λ_{max} 267 nm; IR (KBr) ν_{max} 3450 (OH), 2900 (CH, aliphatic), 1780, 1740 (α,*β*-unsaturated *γ*-lactone), 1620 (C═C) cm^{-1}; negative FABMS *m/z* $[M - H]^-$ 545 (10); HREIMS *m/z* 372.2231 (fragment a, $C_{23}H_{32}O_4)^\bullet$ (60.62), (calcd for $C_{23}H_{32}O_4$, 372.2300), 354.2138 (fragment a-H_2O, $C_{23}H_{30}O_3$) (83.17), 192.0942 (fragment c, $C_8H_{16}O_5$) (25.78), 175.0993 (fragment b, $C_8H_{15}O_4$) (50.59), 174.0891 (fragment c-H_2O, $C_8H_{14}O_4$) (22.99), 157.0919 (fragment b-H_2O, $C_8H_{13}O_3$) (17.77), 144.0835 (fragment b-OCH_3, $C_7H_{12}O_3$, fragment d) (10.51), 113.0631 (fragment d-OCH_3, $C_6H_9O_2$) (12.22); ^{1}H- and ^{13}C-NMR data are shown in Table 1.

Δ^{16}-Dehydroadynerigenin (2): fine needles from MeOH (13.5 mg); mp 220 °C; UV (MeOH) λ_{max} 267 nm; IR ($CHCl_3$) ν_{max} 3425 (OH), 2925 (CH, aliphatic), 1780, 1740 (α,*β*-unsaturated *γ*-lactone) and 1620 (C═C) cm^{-1}; HREIMS *m/z* 370.2120 $[M^+]$ (calcd for $C_{23}H_{30}O_4$, 370.2143) (100), 312.1668 (fragment a, $C_{20}H_{24}O_3$) (13.14), 298.1569 (fragment b, $C_{19}H_{22}O_3$) (12.13), 248.1719 (fragment c, $C_{16}H_{24}O_2$) (16.19), 175.0791 (fragment d, $C_{11}H_{11}O_2$) (12.25); ^{1}H- and ^{13}C-NMR data are shown in Table 2.

Neritaloside (3): fine needles from MeOH (930 mg); mp 138−140 °C (lit.[26] mp 135−140 °C); UV (MeOH) λ_{max} 217 nm; IR (KBr) ν_{max} 3040 (OH), 2975 (CH, aliphatic), 1740 (α,*β*-unsaturated *γ*-lactone) and 1100 (C−O) cm^{-1}; positive FABMS *m/z* $[M + H]^+$ 593 (10); HREIMS *m/z* 372.2250 (22), 355.2227 (100), 337.2090 (40), and 161 (11); ^{1}H- and ^{13}C-NMR data are comparable with those reported in literature.[22]

Odoroside-H (4): prisms from MeOH (290 mg); mp 236−239 °C (lit. mp 231−238°);[27] UV (MeOH) λ_{max} 217 nm; IR (KBr) ν_{max} 3400 (OH), 2885 (CH, aliphatic), 1778, 1735 (α, *β* unsaturated *γ*-lactone) and 1670 cm^{-1}; positive FABMS *m/z* $[M + H]^+$ 535 (100); HREIMS *m/z* 356.2284 (31), 341.2067 (6), 178 (7), and 161 (20); ^{1}H- and ^{13}C-NMR data are comparable with those reported in literature.[22]

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