Steroids from the Roots of *Nerium oleander*

M. Mostaqul Huq,[†] Abdul Jabbar,[†] Mohammad A. Rashid,*[,†] Choudhury M. Hasan,[†] Chihiro Ito,[‡] and Hiroshi Furukawa[‡]

Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh, and Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468, Japan

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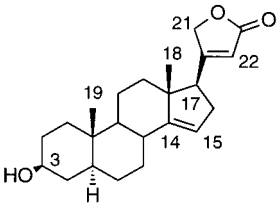
Two new cardenolides, 3β-hydroxy-5α-carda-14(15),20(22)-dienolide (β-anhydroepidigitoxigenin) (**1**) and 3β-*O*-(D-digitalosyl)-21-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide (neriumogenin-A-3β-D-digitaloside) (**2**), and two known compounds, proceragenin and neridienone A (**3**), have been isolated from the roots of *Nerium oleander*. The structures of **1** and **2** were elucidated on the basis of spectral data interpretation.

Nerium oleander L. (Apocynaceae), known as "white oleander" (flower, pale white and single), is an evergreen shrub cultivated as an ornamental plant in Bangladesh.[1] Various parts of the plant are used in Bengali traditional medicine because of their antibacterial, anticancer, antidote, antileprotic, and cardiotonic properties.[2] Previous investigations on *Nerium* species have revealed the presence of several glycosides, including cardenolides, triterpenes, and straight-chain aliphatic compounds.[3−13] In a search for bioactive compounds from medicinal plants of Bangladesh, we have studied *N. oleander* and recently reported odoroside B, 3β-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide, and 12β-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide.[14] This paper deals with the isolation and structure elucidation of four steroids, two of which are new cardenolides, 3β-hydroxy-5α-carda-14(15),20(22)-dienolide (β-anhydroepidigitoxigenin, **1**) and 3β-*O*-(D-digitalosyl)-21-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide (neriumogenin-A-3β-D-digitaloside, **2**), while two are known compounds identified as proceragenin,[15] and a pregnane, neridienone A (**3**).[16] Although proceragenin has been previously isolated from *Calotropis procera* (Asclepiadaceae),[15] this is the first report of its isolation from a plant in the Apocynaceae. The complete ^{1}H and ^{13}C NMR spectral assignments of **3** are reported here for the first time.



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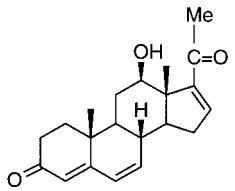

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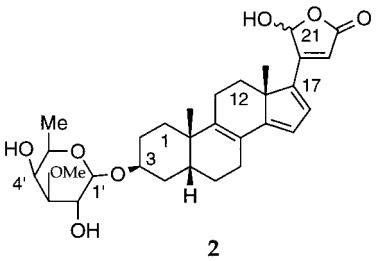

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Compound **1** was the major isolate obtained in this investigation and gave a positive test for cardenolides.[17] HREIMS showed a molecular ion peak at *m*/*z* 356.2351, which corresponds to $C_{23}H_{32}O_3$, suggesting eight double-bond equivalents in the molecule. The mass spectral fragments were characteristic of a cardenolide.[18,19] Additionally, it showed strong fragment ion peaks at *m*/*z* 338 ($C_{23}H_{30}O_2$) and 323 ($C_{22}H_{27}O_2$) due to the loss of water and water plus a methyl group, respectively, from the molecular ion. Although the fragment ion at *m*/*z* 264 is common to all cardenolides carrying a tertiary hydroxyl group at C-14 and a further hydroxyl group in ring A or B,[20] the absence of a peak at *m*/*z* 264 in the mass spectrum of **1** suggested that no hydroxyl group was present either at C-14 or in ring A or B in **1**. However, the daughter ion at *m*/*z* 246 demonstrated the presence of a double bond either in rings A or B or at C-14, which could be formed biogenetically by dehydration.

The ^{1}H NMR (Table 1) spectrum of **1** showed a doublet (J = 17.4) and a double doublets (J = 17.4, 1.8 Hz) of one proton intensity each at δ 4.90 and 4.86 for H-21a and H-21b, respectively, and a one-proton doublet (J = 1.8 Hz) at δ 6.04 assignable to H-22 of an α,β-unsaturated-γ-lactone ring in a cardenolide skeleton.[11] The signal at δ 5.18 (1H, d, J = 1.8 Hz) could be assigned to an olefinic proton, which was coupled to the protons at δ 2.35 (d, J = 9.0 Hz) and 2.49 (m) in the COSY spectrum of **1**. The H_2-16 methylene proton signals also showed COSY coupling to a proton at δ 2.78 (1H, t, J = 9.0 Hz, H-17). Key HMBC correlations from the latter proton to C-16, C18, C-21, and C-22 (Table 1) confirmed these assignments and required a Δ^{14} olefinic moiety. Two sharp singlets at δ 0.75 and 0.73 were attributed to the methyl protons at C-13 and C-10, respectively. The one-proton multiplet at δ 3.38 indicated the presence of an oxymethine proton. The large half width (26 Hz) of this signal required it to have an axial configuration (a small width for the signal is indicative of an equatorial orientation[21]), thus suggesting the hydroxyl group was equatorial.

The ^{13}C NMR spectrum of **1** displayed 23 carbon resonances, while the HMQC experiment confirmed that 18 out of the 23 carbons were attached to protons. The ^{13}C NMR spectrum of **1** showed a close correspondence to that of uzarigenin [3β,14β-dihydroxy-5α-carda-20(22)-enolide, the C-5 epimer of digitoxigenin],[15,22] particularly the chemical shifts for C-1 to C-7 and the lactone ring carbons suggestive of a close structural similarity. The upfield resonance of one of the angular methyls at δ_C 11.8 (C-19) suggested a 5α-cardenolide-type carbon skeleton in **1**.[13]

The multiplicities of the carbon signals were determined by performing DEPT experiments[23] using last pulse angles

* To whom correspondence should be addressed. Present address: SAIC Frederick, NCI−Frederick Cancer Research and Development Center, Bldg. 560, Rm. 32-63B, Post Box B, Frederick, MD 21702. Tel: (301) 846-5966. Fax: (301) 846-6157. E-mail: rashid@mail.ncifcrf.gov.
[†] University of Dhaka.
[‡] Meijo University.

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Table 1. NMR Assignments for β-Anhydroepidigitoxigenin (**1**) in DMSO-d_6

			HMBC (C→H)	
position	^{13}C	^{1}H (J in Hz)	^2J	^3J
1	36.6	0.90 m	C-2, C-10	C-19
		1.68 m	C-10	C-3, C-5
2	31.2	1.62	C-3	
		1.28 m		
3	69.3	3.38 m ($W_{1/2}$=26)		
3-OH		4.45 br s	C-3	C-2, C-4
4	38.0	1.18 m	C-3, C-5	
		1.42 m		
5	43.8	1.02 m		
6	28.0	1.25 m		
		1.25 m		
7	29.6	1.85 m		C-5, C-9
		1.40 m		
8	34.7	1.96 m		
9	53.3	0.65 t (11.0)	C-11	C-5, C-19
10	35.3			
11	21.3	1.60 m		C-8, C-13
		1.28 m		
12	40.3	1.90 m		C-8, C-13
		1.23 m		
13	47.9			
14	153.6			
15	116.5	5.18 d (1.8)	C-14, C-16	C-13, C-17
16	33.1	2.35 d (9.0)	C-15, C-17	C-13, C-14
		2.49 m		
17	51.6	2.78 t (9.0)	C-13, C-16, C-20	C-12, C-18, C-21, C-22
18	17.9	0.75 s		
19	11.8	0.73 s		
20	172.1			
21	73.3	4.90 dd (17.4)	C-20	C-22, C-23
		4.86 dd (17.4, 1.8)	C-20	C-22, C-23
22	115.2	6.04 d (1.8)	C-20, C-23	C-17, C-21
23	173.7			

(θ) 45°, 90°, and 135°, which revealed the presence of two methyls, nine methylenes, seven methines, and five quaternary carbons. Therefore, the unsaturation index exhibited by the molecular formula ($C_{23}H_{32}O_3$) of **1** was satisfied by the four rings of a steroidal skeleton, the α,β-unsaturated γ-lactone ring, and a carbon−carbon double bond between C-14 and C-15. However, the location of the secondary equatorial hydroxyl group in ring A or B remained to be defined. The hydroxyl proton at δ 4.45 (br s) exhibited a ^2J correlation with the oxymethine carbon (δ_C 69.3) and three bond interactions with the methylene carbons (δ_C 31.2 and 38.0) in the HMBC spectrum (Table 1). This required the hydroxyl group to be situated between two methylene groups, that is, either at C-2 or C-3. In the HMBC spectrum one of the C-1 methylene protons (δ 0.90) showed two bond correlations with C-2 (δ_C 31.2) and C-10 (δ_C 35.3) in addition to a ^3J correlation with the methyl carbon (C-19). This allowed the assignment of the oxymethine proton at C-3.

On the basis of the above spectral data, the structure of the compound **1** was elucidated as 3β-hydroxy-5α-carda-14(15),20(22)-dienolide (β-anhydroepidigitoxigenin). The C-5 epimer of **1**, β-anhydrodigitoxigenin, has previously been reported as an artifact in the acidic hydrolysate of *Elytropus chilensis* (Apocynaceae).[24] However, no $[\alpha]_D$ value or NMR data were published, and the structure of the reported compound was determined only on the evidence of UV, IR, and MS data.[24]

HRFABMS of **2** showed the pseudomolecular ion peak at m/z 529.2814 $[M + H]^+$, compatible with the molecular formula $C_{30}H_{40}O_8$. The fragment ion at m/z 369 in the FABMS could be attributed to the aglycon moiety $[C_{23}H_{28}O_4+H]^+$. The ^{1}H NMR spectrum showed olefinic proton signals at δ 7.11 (1H, br s), 6.22 (1H, d, J = 2.4 Hz), and 5.79 (1H, s) for H-16, H-15, and H-22, respectively, of a cardenolide-type carbon skeleton having the 8,14,16,20(22)-tetraene (conjugated) system.[25] The signals at δ 6.26 (1H, s) and 3.89 (1H, m) were assigned to the oxymethine protons at C-21 and C-3, respectively. The sharp singlets at δ 1.22 and 1.10 were ascribed, in turn, to the angular methyl protons at C-13 and C-10, respectively. The above ^{1}H NMR spectral features of compound **2** were in good agreement with those reported for the aglycon of neriumosides, 3β,21-dihydroxy-5β-carda-8,14,16,20(22)-tetraenolide.[26] The ^{1}H NMR spectrum of **2** also exhibited a one-proton doublet (J = 7.8 Hz) at δ 4.30 assignable to the anomeric proton, H-1' of a sugar moiety. The chemical shift and coupling constant of H-1' suggested the β-linkage of the sugar molecule with the aglycon. The sugar was identified as β-D-digitalose by comparison with previous reports.[13,27,28] On this basis, compound **2** was identified as 3β-*O*-(D-digitalosyl)-21-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide (neriumogenin-A-3β-D-digitaloside). Compound **2** was reportedly isolated from *N. odorum* by Handa et al.,[13] who claimed it was a known compound from earlier work on the root bark of the same species.[26] Although the latter reference describes the isolation of seven glycosides, four of which contain the aglycon of **2**, no mention is made of **2** itself. We therefore conclude that **2** is not a known natural product and present here its previously unrecorded spectral data.

Proceragenin[15] and neridienone A (**3**)[16] were identified by comparison of their spectral data with previously reported values. The high resolution ^{1}H and ^{13}C NMR assignments for neridienone A (**3**) are reported here for the first time, using ^{1}H−^{1}H COSY and by comparing their data with data from structurally similar compounds.[11,13,29,30]

Experimental Section

General Experimental Procedures. The optical rotations were measured on a JASCO DIP-370 polarimeter using a sodium lamp (589 nm). UV and IR spectra were obtained on a V-500 UV/vis (JASCO) and IR-230 (JASCO) spectrophotometers, respectively. The ^{1}H NMR spectra were obtained in $CDCl_3$ for all compounds and were repeated in DMSO-d_6 for **1** on a FG 2H×54 T=25 A600 instrument operating at 600 MHz, while the ^{13}C NMR spectra were obtained on the same instrument at 150 MHz using TMS as an internal standard. The chemical shifts (δ) and coupling constants (J) are expressed in parts per million and Hertz, respectively. Inverse-detected heteronuclear correlations were measured using the HMQC (optimized for $^1J_{CH}$ = 145 Hz) and HMBC (optimized for $^nJ_{CH}$ = 8.3 Hz) pulse sequences with a pulsed-field gradient. The EIMS and FABMS were recorded on Hitachi-U and Harata-013 spectrometers, respectively.

Plant Material. The roots of *N. oleander* were collected from an ornamental garden in Khulna, Bangladesh, in September 1996. The plant was identified by Mr. Monzurul Qader Mian, Principal Scientific Officer, Bangladesh National Herbarium, Dhaka, where a voucher specimen has been deposited (DACB accession no. 27,962).

Extraction and Isolation. The air-dried and pulverized roots (500 g) were extracted with EtOH at room temperature. After evaporation of solvent the yellow gum was partitioned between petroleum ether (bp 60−80 °C) and H_2O, followed by EtOAc and H_2O. The EtOAc-soluble fraction (1.4 g) was subjected to vacuum liquid chromatography over Si gel 60H, and the column was eluted with petroleum ether−EtOAc mixtures of increasing polarity, with 13 fractions collected. Evaporation of solvent from fraction 7, followed by repeated washings of the crystalline deposits with *n*-hexane and EtOAc mixtures, gave 25.4 mg of **1**, while similar treatment of fraction 8 afforded proceragenin (10.2 mg). Compounds **2** (2.8 mg) and **3** (5.8 mg) were obtained by preparative TLC of fractions 12

and 6 over Si gel PF_{254}, using $CHCl_3$−MeOH (9.5:0.5) and toluene−EtOAc (1:1) as the developing solvents, respectively.

***β*-Anhydroepidigitoxigenin (1):** white amorphous powder; $[\alpha]_D$ −89.5° (*c* 0.044, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 210 nm (4.48); IR (KBr) ν_{max} 3567, 2929, 2856, 1784, 1749, 1627, 1456, 1035, 895 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.90 (1H, d, J = 1.8 Hz, H-22), 5.24 (1H, d, J = 1.8 Hz, H-15), 4.81 (1H, dd, J = 17.4, 1.8 Hz, H-21a), 4.72 (1H, d, J = 17.4, Hz, H-21b), 3.63 (1H, m, $W_{1/2}$= 26 Hz, H-3), 2.75 (1H, t, J = 9.0 Hz, H-17), 2.47 (2H, d, J = 9.0 Hz, H-16), 0.84 (3H, s, H-18), 0.82 (3H, s, H-19); ^1H and ^{13}C NMR (DMSO-d_6) data, see Table 1; EIMS *m*/*z* 356 (10), 338 (40), 323 (35), 246 (20), 228 (20), 213 (25), 199 (10), 176 (70), 107 (100), 91 (95), 79 (60), 56 (73); HREIMS *m*/*z* 356.2351 (calcd for $C_{23}H_{32}O_3$, 356.2351).

3*β*-*O*-(D-Digitalosyl)-21-hydroxy-5*β*-carda-8,14,16,20-(22)-tetraenolide (2): yellow gum; $[\alpha]_D$ +89.5° (*c* 0.019, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 226 (3.63), 392 (3.31) nm; IR (KBr) ν_{max} 3586, 2927, 2854, 1731, 1602, 1457, 1375, 1170, 1108, 997 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.11 (1H, br s, H-16), 6.26 (1H, s, H-21), 6.22 (1H, d, J = 2.4 Hz, H-15), 5.79 (1H, s, H-22), 4.30 (1H, d, J = 7.8 Hz, H-1'), 3.89 (1H, m, H-3), 3.86 (1H, d, J = 3.6 Hz, H-4'), 3.64 (1H, dd, J = 9.6, 7.8 Hz, H-2'), 3.59 (1H, q, J = 6.6 Hz, H-5'), 3.53 (3H, s, OMe-3'), 3.22 (1H, dd, J = 9.6, 3.6 Hz, H-3'), 1.36 (3H, d, J = 6.6 Hz, H-6'), 1.22 (3H, s, H-18), 1.10 (3H, s, H-19); FABMS *m*/*z* 529 $[M + H]^+$, 369; HRFABMS *m*/*z* 529.2814 (calcd for $C_{30}H_{41}O_8$, 529.2801).

Proceragenin (3): white amorphous; $[\alpha]_D$ +25.0° (*c* 0.052, EtOH) [lit.[15]+6.0°, EtOH]; EIMS *m*/*z* 374.2468 (calcd for $C_{23}H_{34}O_4$, 374.2457); ^1H and ^{13}C NMR data, comparable to those reported in the literature.[15]

Neridienone A (4): white gum; $[\alpha]_D$ +92.1° (*c* 0.038, $CHCl_3$) [lit.[16] +71.5°, MeOH]; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.00 (1H, dd, J = 3.5, 1.8 Hz, H-16), 6.18 (1H, dd, J = 9.6, 3.0 Hz, H-7), 6.12 (1H, d, J = 9.6 Hz, H-6), 5.84 (1H, br s, OH-12), 5.71 (1H, d, J = 3.5 Hz, H-4), 3.71 (1H, dd, J = 10.5, 5 Hz, H-12), 2.60 (1H, dd, J = 10.8, 3.6 Hz, H-15a), 2.58 (1H, dd, J = 10.8, 3.6 Hz, H-15b), 2.41 (1H, dd, J = 15.5, 2.0 Hz, H-2a), 2.38 (3H, s, H-21), 2.35 (1H, dd, J = 15.5, 2.0 Hz, H-2b), 2.03 (1H, ddd, J = 13.2, 3.6, 1.8 Hz, H-14), 1.95 (1H, dd, J = 14, 5 Hz, H-11a), 1.75 (1H, dd, J = 13.2, 4.8 Hz, H-8), 1.47 (1H, dd, J = 14, 5 Hz, H-11b), 1.15 (3H, s, H-19), 0.98 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 33.9 (C-1), 33.6 (C-2), 199.4 (C-3), 124.3 (C-4), 162.7 (C-5), 129.1 (C-6), 138.6 (C-7), 36.1 (C-8), 48.9 (C-9), 34.8 (C-10), 26.8 (C-11), 73.2 (C-12), 51.4 (C-13), 52.8 (C-14), 28.6 (C-15), 155.2 (C-16), 148.9 (C-17), 11.6 (C-18), 16.1 (C-19), 198.8 (C-20), 31.7 (C-21); EIMS *m*/*z* 326 $[M]^+$, 308 (12), 293 (10), 265 (12), 204 (60), 176 (40).

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