Bioactive Cardenolides from the Stems and Twigs of *Nerium oleander*

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Four new cardenolide monoglycosides, cardenolides N-1 (**1**), N-2 (**2**), N-3 (**3**), and N-4 (**4**), were isolated from *Nerium oleander*, together with two known cardenolides, **5** and **12**, and seven cardenolide monoglycosides, **6**−**11** and **13**. The structures of compounds **1**−**4** were established on the basis of their spectroscopic data. The *in vitro* anti-inflammatory activity of compounds **1**−**13** was examined on the basis of inhibitory activity against the induction of the intercellular adhesion molecule-1 (ICAM-1). Compounds **1**, **5**, **6**, and **11**−**13** were active at an IC_{50} value of less than 1 μM. The cytotoxicity of compounds **1**−**13** was evaluated against three human cell lines, normal human fibroblast cells (WI-38), malignant tumor cells induced from WI-38 (VA-13), and human liver tumor cells (HepG2). Compounds **1**, **4**, **6**, and **11**−**13** were active toward V-13 cells, and compounds **1**, **11**, and **12** were active toward HepG2 cells at IC_{50} values of less than 1 μM. Compounds **4**, **5**, **10**, and **12** showed selective cell growth inhibitory activity toward V-13 tumor cells compared with that of parental normal WI-38 cells. The MDR-reversal activity of compounds **1**−**13** was evaluated on the basis of the amount of calcein accumulated in MDR human ovarian cancer 2780AD cells in the presence of each compound. Compounds **4**, **9**, and **10** showed significant effects on calcein accumulation, compound **4** showing stronger activity than that of verapamil.

Nerium oleander L. (synonyms: *Nerium indicum*; *Nerium odorum*) (Apocynaceae) is a medium-sized evergreen flowering tree of 2−5 m in height and is planted throughout Japan as a garden and roadside tree. This species was distributed originally in the Mediterranean region, subtropical Asia, and the Indo-Pakistan subcontinent. Cardenolides in the leaves,[1−8] roots, and root bark[9−11] have been investigated because of interest in their biological activity.[12] The cardiac glycosides digitoxin and digoxin have been used in the treatment of cardiac diseases for many years,[12,13] but they have a narrow therapeutic window because of arrhythmia and disturbance of atrio-ventricular contraction. Anticancer utilization of digitoxin, digoxin, and related cardenolides has been also investigated.[14,15] These reports prompted us to reinvestigate cardenolides in *N. oleander* and their biological activities.

Results and Discussion

A methanol extract of air-dried stems and twigs of *N. oleander* was partitioned successively with hexane, EtOAc, and *n*-BuOH. From the EtOAc-soluble portion, four new cardenolide monoglycosides, cardenolides N-1 (**1**), N-2 (**2**), N-3 (**3**), and N-4 (**4**) were isolated together with nine known cardenolides and cardenolide monoglycosides (**5**−**13**) using silica gel column chromatography and reversed-phase HPLC.

Cardenolide N-1 (**1**) gave the elemental composition $C_{30}H_{46}O_7$, which was determined by HRESIMS analysis. The IR spectrum indicated the presence of hydroxy (3613 and 3591 cm^{-1}) and α,β-unsaturated-γ-lactone (1784 and 1745 cm^{-1}) groups. The ^{13}C NMR spectrum displayed 30 carbon resonances (Table 1). A carbonyl carbon resonated at δ 174.4, and two olefin carbon resonances were

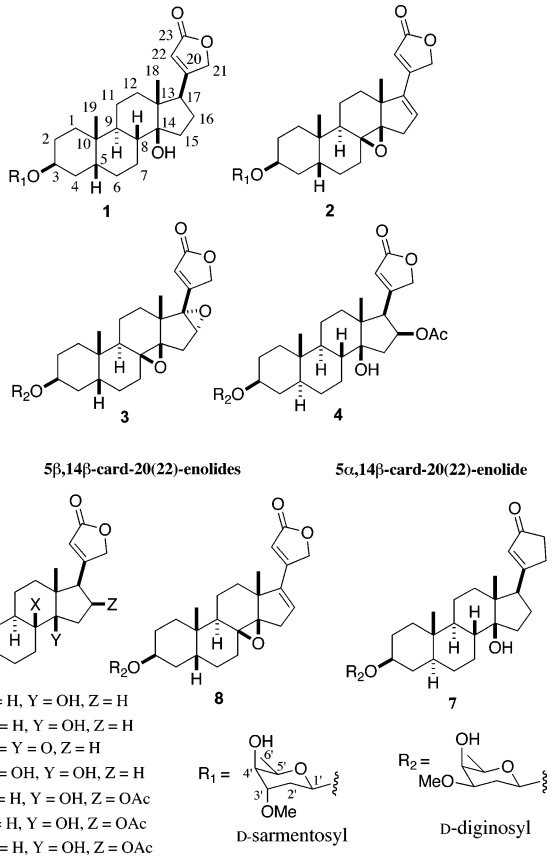

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Table 1. ^{13}C and ^{1}H NMR Data of **1**−**4** ($CDCl_3$, 125 MHz for ^{13}C NMR and 500 Hz for ^{1}H NMR, δ in ppm J in Hz)[a]

	1		**2**		**3**		**4**	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	30.2 (t)	1.48 (1H, m) 1.46 (1H, m)	30.2 (t)	1.46 (1H, m) 1.43 (1H, m)	30.2 (t)	1.44 (1H, m) 1.41 (1H, m)	37.1 (t)	β: 1.70 (1H, m) α: 0.97 (1H, m)
2	26.7 (t)	α: 1.66 (1H, m) β: 1.46 (1H, m)	26.9 (t)	1.71 (1H, m) 1.46 (1H, m)	26.8 (t)	1.74 (1H, m) 1.44 (1H, m)	29.1 (t)	α: 1.92 (1H, m) β: 1.48 (1H, m)
3	72.6 (d)	4.03 (1H, br s, $W_{h/2}$=7.5)	72.5 (d)	4.03 (1H, br s, $W_{h/2}$=7.5)	72.6 (d)	4.05 (1H, br s, $W_{h/2}$=7.5)	76.6 (d)	3.67 (1H, m)
4	29.9 (t)	α: 1.73 (1H, m) β: 1.43 (1H, m)	29.9 (t)	α: 1.73 (1H, m) β: 1.52 (1H, brd, 13.4)	29.9 (t)	α: 1.76 (1H, m) β: 1.50 (1H, m)	34.1 (t)	α: 1.63 (1H, m) β: 1.28 (1H, m)
5	36.3 (d)	1.65 (1H, m)	36.4 (d)	1.79 (1H, m)	36.3 (d)	1.77 (1H, m)	44.2 (d)	1.06 (1H, m)
6	26.6 (t)	β: 1.87 (1H, m) α: 1.26 (1H, m)	24.7 (t)	β: 2.17 (1H, tt, 13.9, 4.6) α: 1.30 (1H, m)	24.6 (t)	β: 2.11 (1H, tt, 14.0, 4.2) α: 1.26 (1H, m)	28.4 (t)	β: 1.37 (1H, m) α: 1.24 (1H, m)
7	21.4 (t)	β: 1.73 (1H, m) α: 1.66 (1H, m)	26.9 (t)	α: 1.83 (1H, m) β: 1.16 (1H, brd, 14.0)	26.3 (t)	α: 1.82 (1H, td, 14.0, 5.1) β: 1.12 (1H, brd, 14.0)	27.0 (t)	β: 1.97 (1H, m) α: 1.03 (1H, m)
8	41.9 (d)	1.56 (1H, m)	65.2 (s)		65.6 (s)		41.6 (d)	1.49 (1H, m)
9	35.8 (d)	1.60 (1H, m)	36.2 (d)	1.94 (1H, brdd, 10.5, 5.1)	36.3 (d)	1.90 (1H, dd, 11.7, 4.2)	49.7 (d)	0.87 (1H, m)
10	35.2 (s)		36.8 (s)		36.7 (s)		35.8 (s)	
11	21.2 (t)	α: 1.43 (1H, m) β: 1.20 (1H, m)	15.7 (t)	1.30 (2H, m)	14.7 (t)	α: 1.30 (1H, m) β: 1.18 (1H, m)	20.8 (t)	α: 1.55 (1H, m) β: 1.26 (1H, m)
12	40.1 (t)	β: 1.52 (1H, m) α: 1.39 (1H, m)	33.4 (t)	β: 1.84 (1H, m) α: 1.28 (1H, m)	30.0 (t)	β: 1.51 (1H, m) α: 1.48 (1H, m)	39.1 (t)	β: 1.52 (1H, m) α: 1.31 (1H, m)
13	49.6 (s)		44.8 (s)		41.9 (s)		49.9 (s)	
14	85.6 (s)		70.1 (s)		67.4 (s)		84.2 (s)	
15	33.2 (t)	α: 2.12 (1H, m) β: 1.68 (1H, m)	33.1 (t)	α: 2.61 (1H, dd, 20.0, 2.8) β: 2.57 (1H, dd, 20.0, 2.8)	29.5 (t)	α: 2.23 (1H, brd, 15.1) β: 2.07 (1H, brd, 15.1)	41.0 (t)	α: 2.67 (1H, dd, 15.6, 9.8) β: 1.75 (1H, dd, 15.6, 2.5)
16	26.9 (t)	α: 2.15 (1H, m) β: 1.87 (1H, m)	132.1 (d)	6.07 (1H, br t, 2.8)	63.2 (d)	3.69 (1H, brs)	73.9 (d)	5.45 (1H, ddd, 9.8, 8.5, 2.5)
17	50.9 (d)	2.77 (1H, brd, 8.3, 4.9)	143.1 (s)		66.7 (s)		56.0 (d)	3.17 (1H, d, 8.5)
18	15.8 (q)	0.87 (3H, s)	19.9 (q)	1.22 (3H, s)	18.0 (q)	1.21 (3H, s)	15.9 (q)	0.93 (3H, s)
19	23.6 (q)	0.93 (3H, s)	24.5 (q)	1.03 (3H, s)	24.6 (q)	0.98 (3H, s)	12.1 (q)	0.79 (3H, s)
20	174.4 (s)		157.6 (s)		162.6 (s)		167.5 (s)	
21	73.4 (t)	α: 4.97 (1H, brd, 18.1) β: 4.81 (1H, brd, 18.1)	71.4 (t)	α: 4.97 (1H, dd, 16.2, 1.6) β: 4.91 (1H, dd, 16.2, 1.6)	71.7 (t)	α: 4.79 (1H, dd, 17.8, 1.7) β: 4.71 (1H, dd, 17.8, 1.7)	75.6 (t)	α: 4.95 (1H, dd, 18.1, 1.7) β: 4.84 (1H, dd, 18.1, 1.7)
22	117.7 (d)	5.87 (1H, brs)	112.9 (d)	5.95 (1H, br s)	119.5 (d)	6.23 (1H, t, 1.7)	121.4 (d)	5.97 (1H, t, 1.7)
23	174.4 (s)		174.2 (s)		172.8 (s)		174.0 (s)	
16-OAc							21.0 (q) 170.4 (s)	1.96 (3H, s)
1′	96.5 (d)	4.71 (1H, dd, 9.5, 2.4)	96.9 (d)	4.72 (1H, dd, 9.5, 2.4)	98.0 (d)	4.46 (1H, dd, 9.8, 2.0)	97.5 (d)	4.53 (1H, dd, 9.8, 2.2)
2′	31.5 (t)	α: 1.84 (1H, m) β: 1.76 (1H, m)	31.5 (t)	α: 1.86 (1H, m) β: 1.78 (1H, m)	32.1 (t)	α: 1.95 (1H, br, dd, 12.2, 4.9) β: 1.71 (1H, ddd, 12.2, 12.2, 9.8)	32.0 (t)	α: 1.94 (1 H, m) β: 1.68 (1 H, ddd, 12.0, 12.0, 9.8)
3′	78.5 (d)	3.58 (1H, q, 2.9)	78.5 (d)	3.58 (1H, q, 3.2)	78.0 (d)	3.34 (1H, ddd, 12.2, 4.9, 3.2)	77.9 (d)	3.33 (1 H, ddd, 12.0, 4.9, 3.2)
4′	67.9 (d)	3.39 (1H, m)	67.9 (d)	3.40 (1H, m)	67.2 (d)	3.68 (1H, m)	67.1 (d)	3.69 (1 H, brs)
5′	69. (d)	3.91 (1 H, q, 6.6)	69.1 (d)	3.91 (1h, br q, 6.6)	70.4 (d)	3.43 (1H, q, 6.3)	70.4 (d)	3.44 (1 H, q, 6.6)
6′	16.6 (q)	1.23 (3H, d, 6.6)	16.6 (q)	1.23 (3H, d, 6.6)	16.8 (q)	1.34 (3H, d, 6.3)	16.8 (q)	1.35 (3H, d, 6.6)
3′-OMe	57.1 (q)	3.38 (3H, s)	57.1 (q)	3.38 (3H, s)	55.7	3.40 (3H, s)	55.7 (q)	3.39 (3H, s)

located at δ 174.4 (s) and 117.7 (d). Three resonances for carbons bearing oxygen were observed at δ 73.4 (t), 72.6 (d), and 85.6 (s) in addition to one methoxy methyl and four oxygenated carbon resonances of a 2,6-dideoxyhexose sugar. From the DEPT and HMQC spectra, the remaining carbon resonances were three methyl, 11 methylene, four methine, and two quaternary carbons. The ^{1}H NMR spectra showed two methyl singlets (δ 0.93 and 0.87) and one additional methyl doublet from the sugar portion at δ 1.23 (d, J = 6.6 Hz). The connectivity of the protonated carbons (C-1 through C-9; C-9, C-11, and C-12; C-15 through C-17) was determined from the $^{1}H-^{1}H$ COSY spectrum. An HMBC experiment was used to determine the carbon−carbon connection through the nonprotonated carbon atoms [HMBC correlations: C-10 (δ 35.2) with H-6α and CH_3-19; C-13 (δ 49.6) with H-11α, H-15α, H-17, and CH_3-18; C-14 (δ 85.6) with H-12β, H-15α, H-17, and CH_3-18]. Interpretation of these results suggests that compound **1** has steroidal A, B, C, and D rings[16] bearing a hydroxy group at C-14.[10] The HMBC correlation of the methine carbon bearing a glycosyl oxygen at δ 72.6 with H-1′ and H-2β and COSY correlation of H-3 with H_2-2 and H_2-4 were used to place an *O*-glycosyl bond at C-3. The HMBC correlations [carbonyl carbon at δ 174.4 with olefinic H-22 (δ 5.87); olefinic methine carbon C-22 (δ, 117.7) with H-17 and H_2-21; quaternary olefinic C-20 (δ 174.4) with H-17

and H_2-21] showed the structure of the γ-lactone moiety and the connection of its C-20 position at C-17 of the steroid D ring.

The sugar portion of **1** was assigned as sarmentose on the basis of comparisons of the ^{13}C and ^1H NMR data with those of analogous compounds.[1,6,10] This is supported by the NOESY correlations (H-1′ with H-5′ and H-2′α; H-3′ with H-4′; H-4′ with H-3′, H-5′, and CH_3-6′) as well as a small coupling constant of H-3′ (q, J = 2.9 Hz). Since only D-sarmentose is known in *N. oleander*, the sugar in **1** is regarded as D-sarmentose.

NOESY corelations [CH_3-19 with H-5 and H-12α with H-15α] suggested AB-*cis* and CD-*cis* ring junctions of **1**. In the ^1H NMR spectra, the small coupling constant of H-3 ($W_{h/2}$ = 7.5 Hz) was in good agreement with that of α(eq)-H at C-3 of 5β-steroids. The spectroscopic analyses and NOESY correlations [CH_3-19 with H-6β, H-8, and H-11β; H-12β with CH_3-18; CH_3-18 with H-22; H-12α with H-17; H-17 with H-16α; H-16β with H-22] indicated the structure and relative configuration of **1** as 3β-*O*-(β-D-sarmentosyl)-14-hydroxy-5β,14β-card-20(22)-enolide.

Cardenolide N-2 (**2**) had the composition $C_{30}H_{42}O_7$, which was determined by HRFABMS analysis. The IR spectrum indicated the presence of hydroxy (3572 cm^{-1}) and α,β-unsaturated-γ-lactone (1749 cm^{-1}) groups. The ^{13}C NMR spectrum displayed 30 carbon resonances. ^1H and ^{13}C NMR data of the sugar part and A ring are in good accordance with those of **1**. Thus, the partial structures of the sugar moiety and the A ring of **1** and **2** are the same. The 3β-*O*-(β-D-sarmentosyl)-5β,14β-card-20(22)-enolide structure of **2** was confirmed by analogous NMR analysis of **2** with that of **1**. Since only D-sarmentose is known in *N. oleander* as mentioned above, the sugar moiety in **2** is also regarded as D-sarmentose. The unsaturated γ-lactone structure, its position at C-17, and the existence of an additional double bond between C-16 and C-17 were confirmed by HMBC correlations [C-23 (δ 174.2) with H_2-21 and H-22; C-20 (δ 157.6) with H_2-15, H-16 (δ 6.07), H_2-21, and H-22]. HMBC correlations of two oxymethine carbons [C-8 (δ 65.2) with H-7β, H-9, and H_2-15; C-14 (δ 70.1) with H-7α, H_2-15, H-16, and CH_3-18] indicated the existence of an 8,14-epoxide ring. Analysis of NOESY correlations [H-3 with H-1′; H-4α with H-7α and H-9; H-9 with H-12α; CH_3-19 with H-5 and H-6β; H-12β with H-22; H-15α with H-7β and H-16; CH_3-18 with H-22] indicated the structure and relative configuration of **2** to be 3β-*O*-(β-D-sarmentosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide. The β-orientation of the 8,14-epoxide ring was supported by NOE correlation of H-15α with H-7β[17] and the observed 0.35 ppm downfield shift of CH_3-18 of **2**[18] compared with that of **1**.

Cardenolide N-3 (**3**) had the composition $C_{30}H_{42}O_8$, as determined by HRFABMS. Similar IR data were obtained for compound **3** as compared to compounds **1** and **2**. The ^{13}C NMR spectrum displayed 30 carbon resonances. ^1H and ^{13}C NMR spectra of the sugar part of **3** are different from those of **1** and **2**. Chemical shifts in the ^{13}C NMR spectrum of the sugar portion, C-1′−C-6′, and OCH_3 of **3** in $CDCl_3$ are in good accordance with those of neridiginoside,[8] oleandrigenin 3-*O*-β-D-diginoside,[22] and digitoxigenin 3-*O*-β-D-diginoside.[22] The coupling constants ($J_{3',2'\beta}$ = 12.2 Hz, $J_{3',2'\alpha}$ = 4.9 Hz, and $J_{3',4'}$ = 3.2 Hz) of H-3′ and NOESY correlations (H-3 with H-1′; H-1′ with H-3′ and 5′) of the sugar part of **3** indicated it to be diginose. Since only D-diginose is known in *N. oleander*, the sugar moiety in **3** is regarded as D-diginose.

Chemical shifts in the ^1H and ^{13}C NMR spectra of the A and B rings of **3** are in good accordance with those of **2**. Thus, **3** is suggested to possess a 3β-*O*-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide structure, which was confirmed by analysis of ^1H and ^{13}C NMR spectra by a method analogous with that of **2**. The unsaturated γ-lactone structural moiety, its position at C-17, and existence of an additional epoxide ring between C-16 and C-17 were confirmed by HMBC correlations [C-23 (δ 172.8) with H-22; C-20 (δ 162.6) with H_2-21 and H-22; C-17 (δ 66.7) with H_2-15, CH_3-18, and H-22; C-16 (δ 63.2) with H_2-15]. NOESY correlations (H-12β with CH_3-18 and H-22; CH_3-18 with H-22 and H-15β) indicated the existence of a 17β-unsaturated γ-lactone and 16α,-17α-epoxide ring. Thus, the full structure and relative configuration of **3** is 3β-*O*-(β-D-diginosyl)-8,14;16α,17-diepoxy-5β,14β-card-20-(22)-enolide.

Cardenolide N-4 (**4**) had the composition $C_{32}H_{48}O_9$, as determined by HRESIMS. Similar IR data were obtained for compound **4** when compared to compounds **1**, **2**, and **3**. The ^{13}C NMR displayed 32 carbon resonances. Chemical shifts in the ^1H and ^{13}C NMR spectra of the sugar part of **4** are in good accordance with those of **3** but different from those of **1** and **2**. Thus, the sugar part of **4** is diginose. Chemical shifts in the ^1H and ^{13}C NMR spectra of the A ring of **4** are different from those of **1**−**3**. NOESY correlations (H-1′ with H-3′, H-5′, and H-3; H-5 with H-3 and H-9; CH_3-19 with H-8) indicated a 5α-H orientation, A,B-*trans* ring fusion, and a 3β(eq)-*O*-glycosyl bond in **4**. Thus, **4** possesses a 3β-*O*-(β-D-diginosyl)-5α-card-20(22)-enolide structure. The A,B-*trans* and A,B-*cis* ring junctions in **4** and in **1**−**3**, respectively, are also indicated by the chemical shifts of C-19 (δ 12.1 for *trans*-derivative **4** and δ 23.6, 24.5, 24.6 for *cis*-derivatives **1**−**3**, respectively).[19] The structure of the unsaturated γ-lactone moiety and its attachment position at C-17 were confirmed by HMBC correlation for **4** similar to those for **1**−**3**. Existence of an acetoxy group at C-16 was confirmed by HMBC correlation [C-16 (δ 73.9) with H-15β and H-17] as well as the chemical shift and coupling constants of H-16 [δ 5.45 ($J_{16,15\alpha}$ = 9.8 Hz, $J_{16,15\beta}$ = 2.5 Hz, and $J_{16,17}$ = 8.5 Hz)]. HMBC correlation of tertiary carbons bearing a hydroxy group [C-14 (δ 84.2) with H-15α, H-16, CH_3-18] indicated the existence of a C-14 hydroxy group. *Cis*-fusion in the C and D rings, β-orientations of 14-hydroxy and 16-acetoxy groups, and the β-orientation of the unsaturated γ-lactone moiety at C-17 in **4** were confirmed by NOESY correlations (H-9 with H-15α; H-12α with H-16; CH_3-18 with H-12β and H− 22). Since only D-diginose is known in *N. oleander*, the sugar in **4** is also regarded as D-digiose. Thus, the full structure of **4** is 3β-*O*-(β-D-diginosyl)-16β-acetoxy-14-hydroxy-5α,14β-card-20(22)-enolide.

We isolated a further nine related cardenolides:[20] 3β,14-dihydroxy-5β,14β-card-20(22)-enolide (**5**),[21] 3β-*O*-(β-D-diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide (**6**),[22] 3β-*O*-(β-D-diginosyl)-14-hydroxy-5α,14β-card-20(22)-enolide (**7**),[1,23] 3β-*O*-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide (**8**),[24] 3β-*O*-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide (**9**),[6,25] 3β-*O*-(β-D-diginosyl)-8,14-dihydroxy-5β,14β-card-20(22)-dienolide (**10**),[6] 3β-*O*-(β-D-sarmentosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20-(22)-enolide (**11**),[26] 16β-acetoxy-3β,14-dihydroxy-5β,14β-card-20-(22)-enolide (**12**),[27] and 3β-*O*-(β-D-diginosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide (**13**).[22]

The *in vitro* anti-inflammatory activity of the isolated compounds **1**−**13** was estimated by inhibition of the induction of ICAM-1 in the presence of IL-1α and TNF-α[28−31] using human cultured cell line A549 cells, an *in vitro* human endothelial cell model. Cell viability was measured by an MTT assay (Table 2). The assay results of **1**−**13** are summarized as follows: (1) The 5β,14β-card-20(22)-enolide structure is important for the inhibitory activity on the induction of ICAM-1. (2) Cardenolide N-1 (**1**) is the most effective compound. Since **1** showed very weak cytotoxic activity (IC_{50} > 100 μM), it is a desirable compound as an anti-inflammatory agent. (3) The structural change at C-3 of **1** from the 3β-*O*-(β-D-sarmentosyl) group to the 3β-*O*-(β-D-diginosyl) or 3β-hydroxyl groups did not show as great a change in the activities as that shown for **5** and **6**. (4) Introduction of an acetoxy group at C-16 in **1**, **5**, and **6** did not induce as great a change in the activities as shown in the corresponding compounds **11**−**13**. (5) Introduction of an additional hydroxy group at C-8 in **6** to **10** or a change of the 14-hydroxy group of **6** to the 8,14-epoxide ring of **9** decreased the activity. (6) Introduction of a double bond or an epoxide ring between C-16 and C-17 in **9** induced a further decrease of activity

Table 2. Effect of Cardenolides **1**−**13** on Induction of ICAM-1 and on Cell Viability

assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
						IC_{50} (μM)[a]							
ICAM-1[b]													
IL-1α	0.20	69.3	62.1	21.5	0.62	0.20	2.1	133	13.9	6.0	0.57	0.52	0.51
TNF-α	0.16	55.4	45.6	16.9	NT	0.48	1.8	NT	NT	5.9	NT	0.36	0.31
						IC_{50} (μM)[c]							
cell viability by MTT assay[d]	>100	>100	>100	>100	>316	>316	>316	>316	>316	>100	>1000	>100	>316

[a] IC_{50} values were calculated by using the following equation. Expression of ICAM-1 (% of control) = [(absorbance with sample and IL-1α/TNF-α treatment − absorbance without IL-1α/TNF-α treatment)/(absorbance with IL-1α/TNF-α treatment − absorbance without IL-1α/TNF-α treatment)] × 100. [b] A549 cells (2×10^4 cells/well) were pretreated with various concentrations of the compounds for 1 h and then incubated in the presence of IL-1α or TNF-α for 6 h. Absorbance of 415 nm was measured after treatment of the cells with primary and secondary antibodies and addition of the enzyme substrate as described in the Experimental Section. The experiments were carried out in triplicate cultures. [c] IC_{50} values were determined by using the following equation. Cell viability (%) = [(experimental absorbance − background absorbance)/(control absorbance − background absorbance)] × 100. [d] A549 cells were incubated with serial dilutions of the compounds for 24 h. Cell viability (%) was measured by the MTT assay. The experiments were carried out in triplicate cultures.

Table 3. Cell Growth Inhibitory Activities of Cardenolides **1**−**13** against WI-38, VA-13, and HepG2 Cells

	IC_{50} (mM)[a]		
compd	WI-38	VA-13	HepG2
1	<0.02	0.80	0.14
2	16.3	85.7	81.4
3	40.9	178	74.1
4	23.0	0.72	140
5	11.8	1.9	18
6	0.07	0.18	1.5
7	0.37	1.3	10.2
8	102	161	151
9	33.1	13.4	76.4
10	9.4	1.6	4.7
11	0.08	0.24	0.82
12	1.7	0.16	0.20
13	0.09	0.17	1.5
Taxol	0.04	0.005	8.1
ADM	0.70	0.40	1.3

[a] IC_{50} represents the mean of duplicate determinations.

as shown in **8** and **3**. (7) The change of the 5β,14β-card-20(22)-enolide structure of **6** and **13** to the corresponding 5α,14β-card-20(22)-enolide structure of **7** and **4** led to a large decrease in activity. (8) Compounds **1**−**4**, **6**, **7**, **10**, **12**, and **13** showed inhibitory activities on the induction of ICAM-1 induced by IL-1α and TNF-α at the same level. The results suggest that these compounds block the common signaling pathway of NF-κB activation downstream of IκB kinase activation, *de novo* RNA/protein synthesis of ICAM-1, or its intracellular transport to the plasma membrane.

Cytotoxic activities of **1**−**13** were evaluted against three cell lines, WI-38, VA-13, and HepG2 cells (Table 3). The results of **1**−**13** are summarized as follows: (1) The 5β,14β-card-20(22)-enolide structure is important for the cell growth inhibitory activity of cardenolides. Thus, compounds **6** and **13**, with 5β,14β-card-20-(22)-enolide functionalities, showed stronger activity toward VA-13 and HepG2 cells than those of the corresponding compounds **7** and **4**, with 5α,14β-card-20(22)-enolide functions. (2) Cardenolide N-1 (**1**) is the most effective compound toward HepG2 cells. Its 3β-*O*-(β-D-diginosyl) derivative (**6**) also showed strong activity toward VA-13 cells. Their IC_{50} values were less than 0.2 μM. Compound **13**, the C-16 acetoxy derivative of **6**, and its aglycone (**12**) showed strong activity toward VA-13. Their IC_{50} values were also less than 0.2 μM. (3) Compound **11**, the 3β-*O*-(β-D-sarmentosyl) derivative of **13**, also showed effective cytotoxic activity toward VA-13 and HepG2 cells at IC_{50} values of less than 1 μM. In conclusion, the compounds possessing the 3β-*O*-(β-D-sarmentosyl)- or 3β-*O*-(β-D-diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide structure with or without an acetoxy group at C-16 are effective for expression of cytotoxicity toward VA-13 and HepG2 cells. (4) Change of the functional group of **6** from the 14-hydroxy group to the 8,14-epoxy or 8,14-dihydroxy group led to a 10- to 100-fold decrease in the activity, as shown by the increase in the IC_{50} values of **9** and **10**. (5) Introduction of a double bond or an α-epoxide ring into the C-16,17 position of **9** induced a further decrease in the activity, as shown by the ca. 10-fold increase in the IC_{50} values of compounds **8** and **3**. Thus, the 14-hydroxy group in **1**, **6**, **11**, **12**, and **13** is the essential functional group for expression of strong cytotoxic activities toward VA-13 and HepG2 cells. (6) IC_{50} values of compounds **4**, **5**, **10**, and **12** toward WI-38 cells are 6−30-fold higher concentrations than those toward VA-13. The assay results indicated that compounds **4**, **5**, **10**, and **12** showed stronger cytotoxicity toward VA-13 malignant tumor cells than those toward parental WI-38 normal cells. Thus, they are desirable compounds as antitumor agents toward VA-13 because the side reaction toward WI-38 normal cells is expected to be low *in vivo*. Compound **4** is especially interesting because it exhibited significant cytotoxicity, with an IC_{50} value of less than 1 μM and more than 30-fold lower cytotoxic activity toward the parental WI-38 cells. Although compounds **1**, **6**, **11**, and **13** also exhibited significant cytotoxic activity toward VA-13 cells, with IC_{50} values of less than 1 μM, they also exhibited 2−40-fold stronger cytotoxic activity toward WI-38 cells. Thus, compounds **1**, **6**, **11**, and **13** are undesirable compounds as antitumor agents toward VA-13, because their cytotoxicities toward parental WI-38 normal cells are stronger than those toward VA-13 malignant tumor cells.

In cancer chemotherapy, the occurrence of multidrug resistance (MDR) of cancer cells caused by repeated administration of anticancer agents is a serious problem. One of the mechanisms of MDR is overexpression of P-glycoprotein (P-gp)[32,33] and the transport of anticancer agents from inside to the outside of cancer cells. We estimated the effects of cardenolides **1**−**13** as MDR reversal agents by the increase of cellular accumulation of the fluorogenic dye calcein, which was derived from calcein AM in the course of assay by enzymatic hydrolysis inside the cells and was used as an easily operated functional fluorescent probe for the drug efflux protein. We assayed the increase of cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells. The effects of cardenolide derivatives **1**−**13** on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. Compounds **4**, **9**, and **10** showed effects on the accumulation of calcein in MDR A2780AD cells by comparison with a control (Table 4). It is interesting to note that the cytotoxic activity of compound **9** toward WI-38, VA-13, and HepG2 cells is relatively weak in addition to the significant effect on the accumulation of calcein in MDR A2789AD, because cytotoxicity is unnecessary for MDR cancer reversal agents. On the contrary, compounds **4** and **10** showed significant to moderate cytotoxicity toward V-13 or V-13 and HepG2. Thus, compound **9** is a possible lead compound for an MDR cancer reversal agent and compounds **4** and **10** are expected to be lead compounds in development of anti-MDR cancer agents. Compound **4** exhibited 128 maximum verapamil %, at 2.5 μg/mL, using verapamil as a positive control (Table 5).

Table 4. Effect of Compounds on the Accumulation of Calcein in MDR 2780 AD Cells

	calcein accumulation (% of control)[a,b]		
compound	0.25 μg/mL	2.5 μg/mL	25 μg/mL
1	89	83	86
2	99	100	102
3	104	96	97
4	105	126	109
5	86	74	66
6	93	89	96
7	78	67	58
8	88	89	99
9	92	102	112
10	112	107	112
11	90	81	91
12	92	100	99
13	67	77	75

[a] The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780 AD cells was determined relative to a control in the presence of 0.25, 2.5, and 25 μg/mL of test compounds. [b]Values are the relative amount of calcein accumulated in the cell compared with the control experiment and represent the means of triplicate determinations.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. Optical rotations were measured using a Horiba Sepa-200 polarimeter. IR spectra were recorded on a Shimadzu FTIR-4200 infrared spectrometer. ^{1}H and ^{13}C NMR spectra were measured with a Varian Unity-plus instrument at 500 and 125 MHz. ^{1}H NMR assignments were determined by ^{1}H−^{1}H COSY experiments. ^{13}C NMR assignments were determined using DEPT, HMQC, and HMBC experiments. HRFABMS were recorded on a JEOL JMS-HX110 instrument, and HREIMS and HRESIMS were recorded on a JEOL JMS-700TZ instrument. Silica gel 70−230 mesh was employed for column chromatography and silica gel 230−400 mesh for flash column chromatography. HPLC separations were performed on a Hitachi L-6200 HPLC instrument with an Inertsil Prep-sil GL 10 × 250 mm stainless steel column and an Inertsil Prep-ODS GL 10 × 250 mm stainless steel column and monitored by a Hitachi L-7400 UV detector and a Shodex SE-61 RI detector.

Plant Material. The stems and twigs of *N. oleander* were collected in Niigata City, Niigata Province, Japan, in November 2001. The plant was identified by Dr. K. Yonekura, Department of Biology, Faculty of Science, Tohoku University, Sendai, Japan. A voucher specimen (2001-11-10) was deposited at the Department of Chemistry and Chemical Engineering, Niigata University.

Extraction and Isolation. The air-dried stems and twigs (19.5 kg) were combined and extracted with MeOH (85 L) for 20 days. The MeOH extract was concentrated to 4 L and extracted with hexane (8 × 1.0 L). Water (1.3 L) was added to the MeOH layer, extracted with EtOAc (3 × 3.0 L), dried (Na_2SO_4), and concentrated to give an oily residue (96.5 g). This material was separated by column chromatography [silica gel (1.1 kg), gradient mixture of hexane, EtOAc, and MeOH] into five fractions, A−E. Fraction B [hexane−EtOAc (1:1), EtOAc] and fraction C (EtOAc) gave on drying viscous oils, 29.58 and 23.33 g, respectively. Fraction B was dissolved in EtOAc (200 mL), stirred for 1 h, filtered, and concentrated to give 19.86 g of a viscous oil, which was further separated by column chromatography [silica gel (1 kg), gradient mixture of hexane, EtOAc, and MeOH] into nine fractions, B1−B9. Fractions B5 [hexane−EtOAc (40:60)] and B6 and B7 [hexane−EtOAc (0:100)] gave on drying viscous oils (B5, 0.451 g; B6, 9.00 g; B7, 1.76 g). B5 afforded compounds **5*** (12.6 mg, 0.00006%), **1** (8.9 mg, 0.00005%), and **2** (23.5 mg, 0.00012%) by separation using silica gel HPLC [hexane−EtOAc (4:6)], followed by ODS HPLC [MeOH−MeCN−H_2O (1:9:10)]. B6 was subjected to silica gel column chromatography [silica gel (1 kg), a gradient of hexane, EtOAc, and MeOH] to give seven fractions, B61−B67. B64 (7.73 g) was further separated by HPLC [ODS, MeOH−MeCN−H_2O (1:6:9)] to give subfractions B643 (5.310 g), B644 [**6****, 1.880 g (0.0096%)], B645 [**9**, (392 mg, 0.0020%)], and B646 (623 mg). B643 was further separated by HPLC [ODS, MeOH−MeCN−H_2O (1:9:10)] to give **5*** [31.1 mg (0.00016%)], **6**** [231.2 mg (0.00119%)], **11** [52.7 mg (0.00027%)], and **12** [39.5 mg (0.00020%)]. B646 was further separated by HPLC [ODS, MeOH−MeCN−H_2O (1:9:10)] to give compounds **3** [46.7 mg (0.00024%)] and **8** [295.9 mg (0.00152%)]. **B7** was separated by column chromatography [silica gel (300 g), gradient mixture of hexane, EtOAc, and MeOH] into five fractions, B71−B75. B72 (157 mg) was further separated by HPLC [ODS, MeOH−MeCN−H_2O (1:6:9)] to give compound **6**** [21.0 mg (0.00011%)]. B73 (1.31 g) was separated by HPLC [ODS, MeOH−MeCN−H_2O (1:6:9)] to give compounds **7** [338.4 mg (0.00173%)], **10** [27.8 mg (0.00014%)], and **13** [308 mg (0.00158%)]. B74 (127 mg) was separated by HPLC [ODS, MeOH−MeCN−H_2O (1:6:8)] to give three fractions (B741, B742, and B743). B742 (73.5 mg) was further purified by HPLC [ODS, MeOH−MeCN−H_2O (1:6:9)] to give compound **4** (18.7 mg, 0.00010%)]. Fraction C was separated by flash column chromatography [silica gel, hexane−EtOAc (1:59)] into six fractions, C1−C6. Fraction C3 (9.32 g) was separated further by flash column chromatography [silica gel, hexane−EtOAc (3:7)] into four fractions, C31−C34. Fraction C31 (1.410 g, 0.00723%) was identified as compound **6****. *The combined yield of **5** is 43.7 mg (0.00022%). **The combined yield of **6** is 3.542 g (0.01816%).

3*β*-*O*-(*β*-D-Sarmentosyl)-14-hydroxy-5*β*,14*β*-card-20(22)-enolide (1): colorless microcrystals; mp 110 °C (acetone−hexane); $[\alpha]^{20}_D$ −1.3 (*c* 0.231, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3613, 3591, 3009, 1784, 1745 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI *m*/*z* 541.3156 (calcd for $C_{30}H_{46}O_7Na$ 541.3156). Since only D-sarmentose is known in *N. oleander*, the sugar moiety in **1** and **2** is regarded as D-sarmentose.

3*β*-*O*-(*β*-D-Sarmentosyl)-8,14-epoxy-5*β*,14*β*-card-16,20(22)-dienolide (2): colorless microcrystals; mp 114 °C (acetone−hexane); $[\alpha]^{20}_D$ +52.9 (*c* 0.662, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3572, 3011, 1749, 1626 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 515.3005 (calcd for $C_{30}H_{43}O_7$ $[M + 1]^+$, 515.3009).

3*β*-*O*-(*β*-D-Diginosyl)-8,14,16α,17-diepoxy-5*β*,14*β*-card-20(22)-enolide (3): colorless microcrystals; mp 123 °C (acetone−hexane); $[\alpha]^{20}_D$ +95.7 (*c* 0.277, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3555, 3030, 1788, 1755 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 531.2968 [calcd for $C_{30}H_{43}O_8$ $[M + 1]^+$, 531.2958]. Since only D-diginose is known in *N. oleander*, the sugar moiety in **3** and **4** is regarded as D-diginose.

Table 5. Effect of Compound **4** on the Accumulation of Calcein in Multidrug-Resistant 2780 AD Cells

		calcein accumulation[a]				evaluation
compound	concentration (μg/mL)	average[b] (count/well)	% of control[c]	activities[d]	verapamil (%)[e]	max. verapamil % concentration
4	0.25	2559	105	±	113	P[f]
	2.5	3081	126	+	128	128
	25	2665	109	±	82	2.5 μg/mL
verapamil	0 (control)	2445				
	0.25	2268	93	±	100	
	2.5	2407	98	±	100	
	25	3249	133	++	100	

[a] The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780 AD cells was determined relative to the control in the presence of 0.25, 2.5, and 25 μg/mL of the pregnanes. [b]Values represent the mean of triplicate determinations. [c]Values are the relative amount of calcein accumulated in the cell compared with the control experiment. [d]± indices are expressed on a scale of four by the range of the relative amount of calcein accumluation as compared with a control experiment (%): +++, >151; ++, 131−150%; +, 111−130%; ±, 91−110%; −, <90%. [e]Values are expressed as the relative amount of calcein accumulation in the cell as compared with that of verapamil. [f]P, positive: the activity was more potent than that of verapamil (verapamil % >100%).

3*β*-*O*-(*β*-D-Diginosyl)-16*β*-acetoxy-14-hydroxy-5α,14*β*-card-20-(22)-enolide (4): colorless microcrystals; mp 201 °C (acetone−hexane); $[\alpha]^{20}_D$ −21.4 (*c* 0.42, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3516, 3439, 1743 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESI *m*/*z* 599.3197 (calcd for $C_{32}H_{48}O_9Na$ 599.3196).

Inhibitory Activity on Induction of ICAM-1. Experimental details were described in previous papers.[30,31,34]

Cell Growth Inhibitory Activity to WI-38, VA-13, and HepG2 *in Vitro*. Experimental details were described in previous papers.[34−36]

Cellular Accumulation of Calcein. Experimental details were described in previous papers.[35,36]

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