The Structure of a New Cardenolide Diglycoside and the Biological Activities of Eleven Cardenolide Diglycosides from *Nerium oleander*

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A new cardenolide diglycoside (1) was isolated from *Nerium oleander* together with ten known cardenolide diglycosides 2—11. The structure of compound 1 was established on the basis of their spectroscopic data. The *in vitro* anti-inflammatory activity of compounds 1—11 was examined on the basis of inhibitory activity against the induction of the intercellular adhesion molecule-1 (ICAM-1). Compounds 2—5 were active at an IC_{50} value of less than 0.8 μM. The cytotoxicity of compounds 1—11 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). Compound 3 was active toward VA-13 cells, and compounds 2—5 were active toward HepG2 cells at IC_{50} values of less than 1.3 μM. The multidrug resistance (MDR)-reversal activity of compounds 1—11 was evaluated on the basis of the amount of calcein in MDR human ovarian cancer 2780AD cells in the presence of each compound. Compounds 1 and 8 showed moderate effects on calcein accumulation.

Key words *Nerium oleander*; cardenolide diglycoside; *in vitro* anti-inflammatory activity; cytotoxicity; multidrug resistance-reversal activity

Nerium oleander L. 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, sub-tropical Asia, and the Indo–Pakistan subcontinent. Cardenolides in the leaves,[1—8] roots, and root bark[9—12] of this plant were investigated because of the interests in their biological activities.[13] The cardiac glycoside digitoxin and digoxin have been used in treatment of cardiac diseases for many years,[13,14] 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.[15,16] We recently reinvestigated the cardenolide monoglycosides[17,18] from *N. oleander*. Further chemical investigation of cardenolide diglycosides from *N. oleander* has led to the isolation of a new cardenolides diglycoside (**1**) together with ten known cardenolide diglycosides **2**—**11**.

Results and Discussion

A methanol extract of air-dried leaves of the plant was partitioned with hexane, ethyl acetate, and butanol. Methanol-soluble portion of butanol extract was separated by silica gel column chromatography and reversed-phase HPLC. A new compound **1** was isolated together with ten known cardenolide diglycosides **2**—**11** (Fig. 1).

Compound **1** has the composition $C_{36}H_{56}O_{13}$, which was determined by high resolution (HR)-FAB-MS analysis. The IR spectrum of compound **1** indicated the existence of hydroxyl (3467 cm^{-1}), α,β-unsaturated-γ-lactone (1736 cm^{-1}), and carbonyl (1698 cm^{-1}) groups. The ^{13}C-NMR displayed 36 carbon signals (Table 1). Two carbonyl carbons resonated at δ 216.2 and 174.1, and two olefin carbons were located at δ 172.4 (qC) and 116.8 (CH). Three signals for carbons bearing oxygen were observed at δ 74.0 (CH_2), 72.8 (CH), and 79.5 (CH) in addition to one methoxy methyl and ten oxygenated carbon signals of one 2,6-dideoxyhexose sugar and one hexose sugar moiety. From the distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum coherence (HMQC) spectra, the remaining carbon resonances were three methyl, ten methylene, three methine, and two quaternary carbons. The ^{1}H-NMR spectra showed two methyl singlets (δ 0.68, 0.74) and one additional methyl doublet from sugar portion at δ 1.51 (d, J=6.4 Hz). The connectivity of the protonated carbons was determined by the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum (Fig. 2). Heteronuclear multiple bond connectivity (HMBC) experiment was used to determine the carbon–carbon connection through the nonprotonated carbon atoms (Fig. 2). Interpretation of these results suggests that compound **1** has the 8,14-secocardenolide skeleton bearing a carbonyl group at C-8, a hydroxyl group at C-14, and an α,β-unsaturated γ-lactone moiety at C-17. The HMBC cor-

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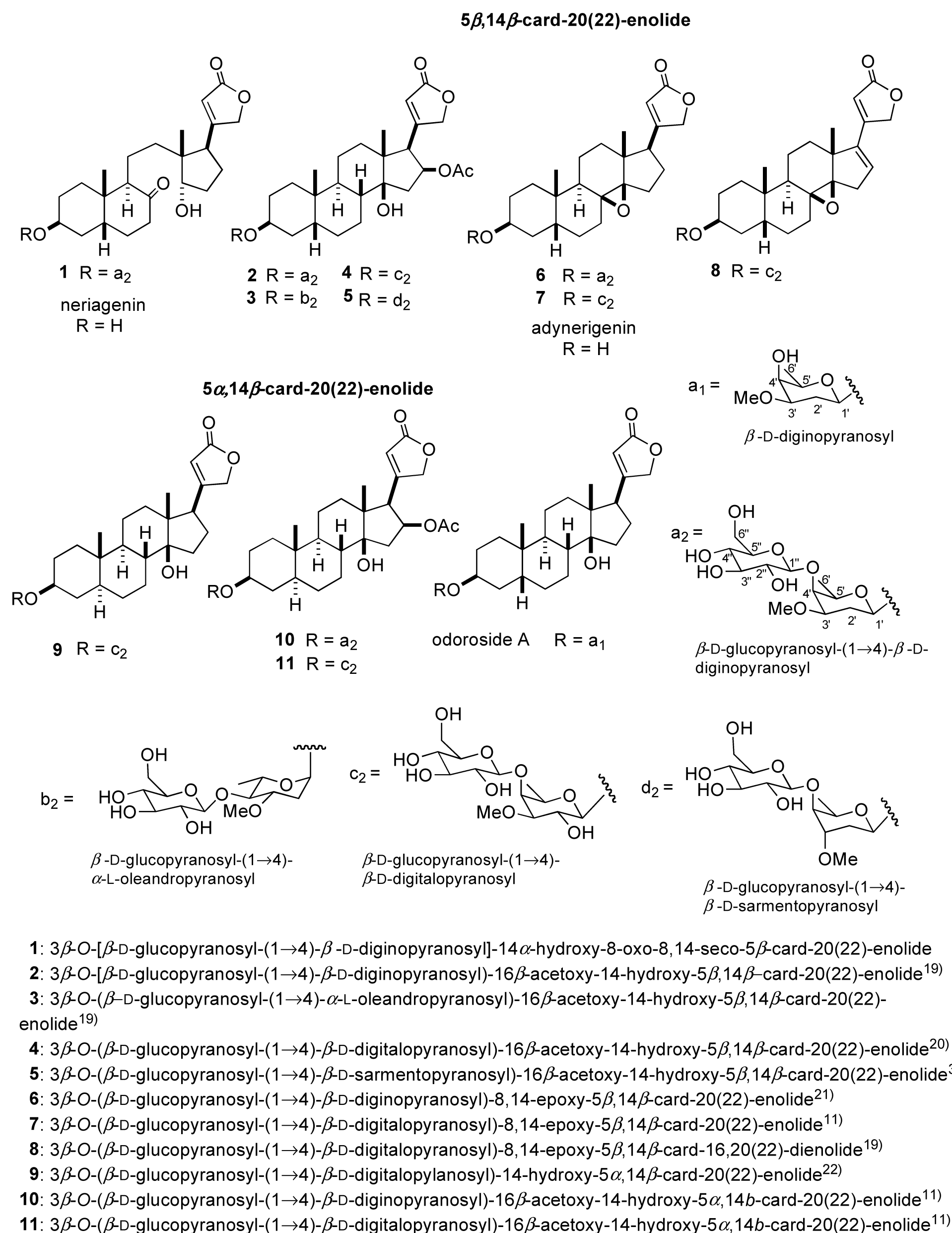

Fig. 1. Structure of Compounds **1**—**11**, Neriagenin, Adynerigenin, Odoroside A, and Digitoxigenin

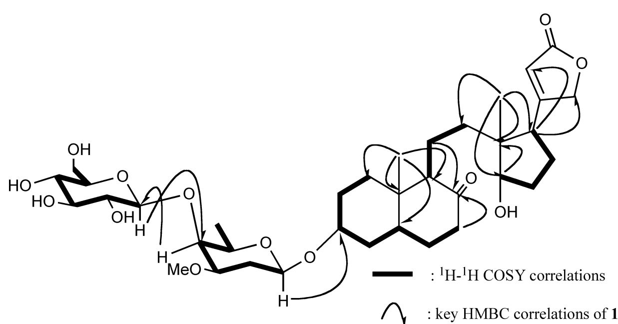

Fig. 2. ^{1}H–^{1}H COSY and Key HMBC Correlations of **1**

relations [H-1′ with C-3] and the nuclear Overhauser effect spectroscopy (NOESY) correlation [H-1′ with H-3] were used to place an *O*-glycosyl bond at C-3. The HMBC correlation [H-1″ to C-4′ and H-4′ to C-1″] indicated that the second glycosyl bond exist at C-4′. The sugar portion of **1** was assigned to β-glucopyranosyl-(1→4)-β-diginopyranose on the basis of comparison of the ^{13}C-NMR data of **1** with those of known analogous compound such as **2**[19] and **6**.[21] The ^{13}C- and ^{1}H-NMR data of the sugar moiety of **2** and the ^{13}C-NMR data of the sugar moiety of **6** were actually superimposable with those of 3β-*O*-[β-D-glucopyranosyl-(1→4)-β-diginopyranosyl] moiety of **1** (Table 2). This is supported by the NOESY correlation (Fig. 3) and the coupling constants of diginosyl moiety ($J_{1',2'\beta}$=9.8 Hz, $J_{2'\beta,3'}$=12.2 Hz, $J_{2'\alpha,3'}$=4.4 Hz, $J_{3',4'}$=2.7 Hz) and gulcosyl moiety ($J_{1'',2''}$=7.8 Hz, $J_{2',3''}$=8.8 Hz, $J_{3'',4''}$=8.8 Hz). The small coupling constant of H-3 ($W_{1/2\,\mathrm{h}}$=8.0 Hz) was in good agreement with that of α(eq)-H at C-3 of 5β-steriods. NOESY correlations confirmed AB-*cis* ring fusion, α-configurations of 14-hydroxyl group, and β-configurations of unsaturated γ-lactone moiety at C_{17} (Fig. 3). Although the relative stereochemistry between CH_3-19 and CH_3-18 was not determined by the

Table 1. ^{13}C- and ^{1}H-NMR Data of **1** (Pyridine-d_5, 125 MHz for ^{13}C-NMR and 500 MHz for ^{1}H-NMR, δ in ppm J in Hz)

Position	^{13}C$^{a)}$	Connected ^{1}H$^{b)}$
1	31.0 (CH_2)	β 1.56 (1H, m)
		α 1.70 (1H, m)
2	27.7 (CH_2)	β 1.93 (1H, m)
		α 1.68 (1H, m)
3	72.8 (CH)	4.28 (1H, br s, $W_{h/2}$=8.0)
4	31.0 (CH_2)	α 2.08 (1H, m)
		β 1.73 (1H, m)
5	36.8 (CH)	1.90 (1H, m)
6	28.7 (CH_2)	β 2.05 (1H, m)
		α 1.50 (1H, m)
7	38.2 (CH_2)	β 2.50 (1H, dddd, 13.4, 13.4, 6.8)
		α 2.28 (1H, ddd, 13.4, 4.9, 2.7)
8	216.2 (qC)	
9	52.0 (CH)	2.79 (1H, d, 10.0)
10	42.7 (qC)	
11	18.4 (CH_2)	a 1.76 (1H, m)
		b 1.45 (1H, m)
12	35.3 (CH_2)	a 1.65 (1H, m)
		b 1.29 (1H, ddd, 12.5, 12.5, 6.1)
13	51.3 (qC)	
14	79.5 (CH)	4.15 (1H, m)
15	27.3 (CH_2)	β 2.03 (1H, m)
		α 1.70 (1H, m)
16	30.8 (CH_2)	α 2.03 (1H, m)
		β 1.84 (1H, m)
17	46.3 (CH)	3.00 (1H, t, 9.3)
18	17.7 (CH_3)	0.68 (3H, s)
19	23.9 (CH_3)	0.74 (3H, s)
20	172.4 (qC)	
21	74.0 (CH_2)	a 4.89 (1H, dd, 17.6, 2.0)
		b 4.71 (1H, dd, 17.6, 1.5)
22	116.8 (CH)	6.01 (1H, br d, 1.0)
23	174.1 (qC)	
1′	99.1 (CH)	4.71 (1H, dd, 9.8, 2.2)
2′	33.2 (CH_2)	β 2.34 (1H, td, 12.2, 9.8)
		α 2.13 (1H, dd, 12.2, 4.4)
3′	80.1 (CH)	3.45 (1H, ddd, 12.2, 4.4, 2.7)
4′	74.1 (CH)	4.14 (1H, m)
5′	70.9 (CH)	3.52 (1H, br q, 6.4)
6′	17.9 (CH_3)	1.51 (3H, d, 6.4)
3′-OMe	56.2 (CH_3)	3.36 (3H, s)
1″	105.0 (CH)	5.11 (1H, d, 7.8)
2″	76.0 (CH)	3.91 (1H, dd, 8.8, 7.8)
3″	78.4 (CH)	4.17 (1H, t, 8.8)
4″	72.0 (CH)	4.11 (1H, t, 8.8)
5″	78.5 (CH)	3.90 (1H, m)
6″	63.2 (CH_2)	b 4.51 (1H, dd, 11.7, 2.4)
		a 4.30 (1H, dd, 11.7, 5.6)

a) Multiplicity were determined by DEPT. *b*) Connection were determined by HMQC.

NOESY experiment, the chemical correlation of the genin of **6**, adynerigenine[23] with the genin of **1**, neriagenin was reported.[7] Thus, the relative stereochemistry of **1** is 3β-*O*-(β-*O*-glucopyranosyl-(1→4)-β-diginopyranosyl)-14α-hydroxy-8-oxo-8,14-*sec*-5β-card-20(22)-enolide.

Acid hydrolysis of **1** with 1 : 1 mixture of 0.04 M H_2SO_4 and MeOH at 60 °C for 12 h gave β-D-glucopyranosyl-(1→4)-D-diginopyranose {$[\alpha]_D^{20}$ +50.77 (c=1.30, MeOH)} and neriagenine {$[\alpha]_D^{20}$ −8.93 (c=3.03, MeOH)}, whose structure had already been established by the chemical correlation with adynerigenin.[5] Thus, the structure of **1** was regarded as 3β-*O*-[β-D-glucopyranosyl-(1→4)-β-D-diginopyranosyl]-14α-hydroxy-8-oxo-8,14-*sec*-5β-card-20(22)-enolide

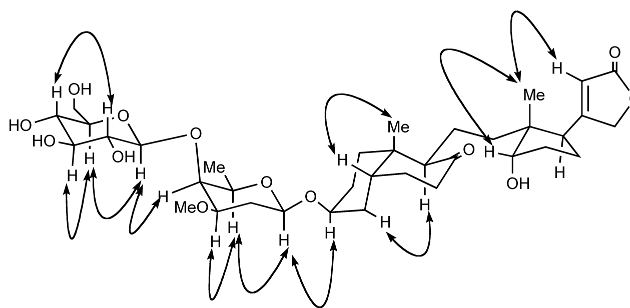

Fig. 3. The Key NOESY Correlations of Compound **1**

(**1**) with (3*S*, 5*R*, 9*R*, 10*S*, 13*R*, 14*S*, 17*R*) configuration.

The *in vitro* anti-inflammatory activity of the isolated compounds **1**—**11** was estimated by inhibition of the induction of ICAM-1 in the presence of interleukin (IL)-1α and tumor necrosis factor (TNF)-α[24—27] for 8 h using human lung carcinoma A549 cells (Table 3). Cell viability was also measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for 24 h incubation time (Table 3). The assay results of **1**—**11** are summarized as follows: (1) 3β-*O*-(diglycosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide structure is important for the inhibitory activity on the induction of intercellular adhesion molecule-1 (ICAM-1) as shown in those of **2**—**5**. (2) Compound **3** is the most effective compound. Since **3** showed very weak cytotoxic activity (IC_{50}>320 μM) during an incubation time up to 24 h, it is a desirable compound as an anti-inflammatory agents. (3) The structural change of diglycosyl moiety at C-3 of **3** from the 3β-*O*-(b_2)-group to the 3β-*O*-(a_2)-, 3β-*O*-(c_2), or 3β-*O*-(d_2) groups showed a decrease in the activities as that shown for **2**, **4**, and **5**. (4) Structural changes of **2** and **4** in genin moiety from 16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide to 8,14-epoxy-5β,14β-card-20(22)-enolide such as **6** and **7** or 8,14-epoxy-5β,14β-card-16,20(22)-dienolide such as **8** induced a large decrease of the activity. (5) The change of the 5β,14β-card-20(22)-enolide structure of **2** and **4** to the corresponding 5α,14β-card-20(22)-enolide structure of **10** and **11** led to a large decrease in activity. (6) Compounds **2**, **3**, **5**, **7**—**11** 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 nuclear factor (NF)-κB activation. Consistent with this, we have recently shown that odoroside A and oubanin inhibit Na^+/K^+ ATPase and prevent NF-κB-inducible protein expression by blocking Na^+-dependent amino acid transport.[28]

The structure of compounds **2**—**11** were confirmed by full analysis of their NMR spectra at 500 MHz (^{1}H-NMR, ^{1}H–^{1}H COSY, NOESY, HMQC, HMBC) and at 125 MHz (^{13}C-NMR, DEPT) as well as HR-FAB-MS and $[\alpha]_D$.

Cytotoxic activities of **1**—**11** were evaluted against three cell lines, WI-38, VA-13, and HepG2 cells for longer incubation time (48 h) (Table 4). The results of **1**—**11** are summarized as follows: (1) 16β-Acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide structure is important for the cell growth inhibitory activity of cardenolides. Thus, compounds **2** and **4** with 16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide structure showed stronger activity toward WI-38, VA-13 and, HepG2 cells than those of the corresponding compounds **10**

Table 2. NMR Data of the Sugar Moiety **1**, **2**, and **6** in C_5D_5N

Position	**1**		**2**		**6**
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1′	99.1 (CH)	4.71 (1H, dd, 9.8, 2.2)	98.9 (CH)	4.69 (1H, dd, 9.8, 1.7)	98.8 (CH)
2′	33.2 (CH_2)	β 2.34 (1H, ddd, 12.2, 12.2, 9.8) α 2.13 (1H, dd, 12.2, 4.4)	33.2 (CH_2)	2.33 (1H, ddd, 12.2, 12.0, 9.8) 2.11 (1H, m)	33.3 (CH_3)
3′	80.1 (CH)	3.45 (1H, ddd, 12.2, 4.4, 2.7)	80.1 (CH)	3.42 (1H, ddd, 12.2, 4.4, 2.7)	80.2 (CH)
4′	74.1 (CH)	4.14 (1H, m)	74.2 (CH)	4.13 (1H, m)	73.3 (CH)
5′	70.9 (CH)	3.52 (1H, br q, 6.4)	70.8 (CH)	3.50 (1H, br q, 6.3)	70.9 (CH)
6′	17.9 (CH_3)	1.51 (3H, d, 6.4)	17.9 (CH_3)	1.51 (3H, d, 6.3)	18.2 (CH_3)
3′-OMe	56.2 (CH_3)	3.36 (3H, s)	56.1 (CH_3)	3.35 (3H, s)	56.2 (CH_3)
1″	105.0 (CH)	5.11 (1H, d, 7.8)	105.0 (CH)	5.09 (1H, d, 7.8)	104.6 (CH)
2″	76.0 (CH)	3.91 (1H, dd, 8.8, 7.8)	76.0 (CH)	3.91 (1H, m)	75.7 (CH)
3″	78.4 (CH)	4.17 (1H, t, 8.8)	78.4 (CH)	4.17 (1H, t, 8.8)	78.6 (CH)
4″	72.0 (CH)	4.11 (1H, t, 8.8)	72.0 (CH)	4.12 (1H, m)	71.9 (CH)
5″	78.5 (CH)	3.90 (1H, m)	78.5 (CH)	3.89 (1H, m)	78.4 (CH)
6″	63.2 (CH_3)	β 4.51 (1H, dd, 11.7, 2.4) α 4.30 (1H, dd, 11.7, 5.6)	63.2 (CH_3)	4.51 (1H, dd, 11.5, 2.4) 4.30 (1H, dd, 11.5, 5.6)	62.8 (CH_3)

Table 3. Effect of Compounds on Induction of ICAM-1 and Cell Viability

Compound	ICAM-1[a] [IC_{50} (μM)][b]		Cell viability by MTT assay[c]
	IL-1[d]	TNF-α[d]	[IC_{50} (μM)]
1	>320	>150	>320
2	0.63	0.62	>320
3	0.34	0.25	>320
4	0.65	NT[e]	>320
5	0.72	0.6	>320
6	>320	300	>320
7	28	16	>320
8	35	21	>320
9	34	28	>320
10	29	22	>320
11	28	19	>320
Odoroside A	0.27[f]	0.42[f]	>10[f]

a) A549 cells were pretreated with various concentrations of the compound for 1 h and then incubated in the presence of IL-1α or TNF-α for 6 h. Absorbance of 415 nm was assayed after treatment of the cells with primary and secondary antibodies and addition of the enzyme substrate. *b*) The experiment were carried out in triplicate cultures. Absolute value for non-stimulated cells was 0.072—0.093, compared with 0.516—0.668 for IL-1-stimulated cells and 0.377—0.521 for TNF-α-stimulated cells as positive control. *c*) A549 cells were incubated with serial dilutions of the compounds for 24 h. Cell viability (%) was measured by MTT assay and used for determination of IC_{50}. The experiments were carried out in triplicate cultures. Absolute values for background and non-treated cells were 0.047—0.092 and 0.679—1.181, respectively. *d*) IC_{50} represent the means of two independent experiment except for **4** and **10**. *e*) Not tested. *f*) For comparison, IC_{50} values described in ref. 29 were cited.

Table 4. Cell Growth Inhibitory Activities of Compounds **1**—**11** against WI-38, VA-13, and HepG2 Cells

Compound	IC_{50} (μM)[a]		
	WI-38	VA-13	HepG2
1	117	>143	130
2	0.95	9.2	0.80
3	0.38	1.2	0.41
4	0.94	8.1	1.3
5	0.39	3.1	0.80
6	>147	>147	>147
7	57.7	114	24.8
8	46.6	87.3	87.9
9	22.0	125	17.9
10	36.9	71.2	9.3
11	23.2	88.5	12.8
Paclitaxel	0.19	0.04	0.78
Adriamycin	1.10	0.37	0.03

a) IC_{50} represents the mean of duplicate determination.

and **11** with 16β-acetoxy-14-hydroxy-5α,14β-card-20(22)-enolide structure. (2) Compound **3** is the most effective compound toward WI-38, VA-13, and HepG2 cells. Its 3β-*O*-(a_2)-, 3β-*O*-(c_2)-, or 3β-*O*-(d_2)-derivatives (**2**, **4**, **5**) also showed strong activity toward WI-38 and HepG2 cells. Their IC_{50} values were less than 1.3 μM. (3) Structural changes of **2** and **4** in genin moiety from 16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide to 8,14-epoxy-5β,14β-card-20(22)-enolide such as **6** and **7** or 8,14-epoxy-5β,14β-card-16,20(22)-dienolide such as **8** led to a 10 to 100 fold decrease in the activity as shown by the increase in the IC_{50} values of **6**—**8**. Thus, the 14β-hydroxy group in **2**—**5** is the essential functional group for expression of strong cytotoxic activities.

In cancer chemotherapy, 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)[29,30] and their transport of anticancer agents from inside to the outside of cancer cells. We estimated the effects of cardenolides, **1**—**11** 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**—**11** on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. Compounds **1** and **8** showed moderate effects on the accumulation of calcein in MDR 2780AD cells by comparison with a control (Tables 5-1—5-4). It is interesting to note that the cytotoxic activity of compounds **1** and **8** toward WI-38, VA-13, and HepG2 cells is relatively weak in addition to the moderate effect on the accumulation of calcein in MDR 2780AD, because cytotoxicity is unnecessary for MDR cancer reversal agents. Thus, compounds **1** and **8** are possible lead compounds for an MDR cancer reversal agent.

Experimental

Melting points are uncorrected. Optical rotations were measured using a Horiba Sepa-200 polarimeter. IR spectra were recorded on a Shimadzu

Table 5-1. Effect of Compounds **1** and **2** on Accumulation of Calcein in MDR 2780AD

Compound	Concentration[a] (μg/ml)	Average of flouorescence/well[b]	SD[c]	% of control[d]
Control	0	3395	±955	
1	0.25	3486	±636	103
	2.5	3140	±399	93
	25	3996	±723	118
2	0.25	2953	±318	87
	2.5	2974	±216	88
	25	2597	±564	76
Verapamil[e]	0.25	3298	±100	97
	2.5	3701	±236	109
	25	5034	±387	148

Table 5-2. Effect of Compounds **3**, **5**, **7**, **8**, and **10** on Accumulation of Calcein in MDR 2780AD

Compound	Concentration[a] (μg/ml)	Average of flouorescence/well[b]	SD[c]	% of control[d]
Control	0	3456	±352	
3	0.25	3349	±263	97
	2.5	3198	±127	93
	25	3195	±282	92
5	0.25	3379	±355	98
	2.5	3221	±261	93
	25	3328	±713	96
7	0.25	3578	±350	104
	2.5	3816	±237	110
	25	2955	±66	86
8	0.25	4360	±832	126
	2.5	3819	±373	110
	25	3827	±410	111
10	0.25	3283	±423	95
	2.5	2860	±192	83
	25	3045	±169	88
Verapamil[e]	0.25	3252	±14	94
	2.5	3870	±781	112
	25	4826	±903	140

Table 5-3. Effect of Compounds **4**, **9**, and **11** on Accumulation of Calcein in MDR 2780AD

Compound	Concentration[a] (μg/ml)	Average of flouorescence/well[b]	SD[c]	% of control[d]
Control	0	2211	±516	
4	0.25	2110	±422	95
	2.5	1553	±126	70
	25	1827	±163	83
9	0.25	2319	±378	105
	2.5	2071	±288	94
	25	1855	±322	84
11	0.25	2042	±486	92
	2.5	2021	±332	91
	25	1404	±120	64
Verapamil[e]	0.25	2236	±405	101
	2.5	2165	±291	98
	25	3191	±308	144

Table 5-4. Effect of Compound **6** on Accumulation of Calcein in MDR 2780AD

Compound	Concentration[a] (μg/ml)	Average of flouorescence/well[b]	SD[c]	% of control[d]
Control	0	2448	±66	
6	0.25	2202	±665	90
	2.5	2281	±67	93
	25	2320	±369	95
Verapamil[e]	0.25	2551	±304	104
	2.5	2835	±501	116
	25	3570	±277	146

a) The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5, and 25 μg/ml of compounds. *b, c*) The values represent means of tripricate and the S.D. (standard deviations). *d*) The values are relative amount of calcein accumulated in the cell compared with the control the experiment. *e*) Positive control.

FTIR-4200 infrared spectrometer. UV spectrum was recorded on a Nihon-bunko V-550 UV/vis 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. HR-FAB-MS was recorded on a JEOL JMS-HX110 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-octadecyl functionalized silica gel (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 leaves, 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 1—11 Air-dried leaves (9.91 kg) were extracted two times with MeOH (66, 39 l) for 3 and 4 d. The MeOH extract was concentrated to 10 l and extracted with hexane (5×5 l). Water (4×10 l) was added to the MeOH layer, extracted with EtOAc (5×5 l). Then saturated NaCl aqueous solution (10 l) was added to the MeOH layer and extracted with *n*-BuOH (4×10 l). The *n*-BuOH extracts were dried and concentrated to give an oily material (528 g). To a part of the *n*-BuOH extract (53.76 g), 150 ml of MeOH was added, stirred for 1 h, and filtered. The filtrate was concentrated to give on drying a viscous oil, which was dissolved with 110 ml of MeOH, and added 990 ml of $CHCl_3$, stirred for 1 h, and filtered. The filtrate was concentrated to give a viscous oil, NB1 (18.75 g), which was dissolved with 200 ml of water, and extracted with $CHCl_3$ (5×200 ml). The $CHCl_3$ extracts were concentrated to give a semisolid, BC (5.54 g), which was separated by column chromatography on silica gel (275 g) in 11 fractions, BC-1—BC-11, using a gradient of $CHCl_3$ and MeOH. Fraction BC-3 [$CHCl_3$–MeOH (9 : 1)] gave 606.7 mg of a semisolid, which was further separated by HPLC [ODS, MeOH–H_2O (9 : 1)] into three fractions (BC3-1—BC3-3). Fraction BC3-1 gave a powder (542.8 mg), which was separated by HPLC [ODS, MeOH–H_2O (65 : 35)] into five fractions (BC31-1—BC31-5). Fraction BC31-2 (143.3 mg) was separated by HPLC [ODS, MeOH–H_2O (5 : 5)] to give **1** [23.9 mg (0.0024%)], **2** [67.5 mg (0.0067%)] and **10** [16.5 mg (0.0016%)]. Fraction BC31-3 (71.7 mg) was separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 6 : 12)] to give **5** [41.2 mg (0.0041%)] and **3** [16.8 mg (0.0017%)]. Fraction BC-5 [$CHCl_3$–MeOH (9 : 1 and 8 : 2)] gave 288.8 mg of a semisolid, which was further separated by HPLC [ODS, MeOH–H_2O (8 : 2)] into three fractions (BC5-1—BC5-3). Fraction BC5-1 gave a powder (228.0 mg), which was separated by HPLC [ODS, MeOH–H_2O (6 : 4)] into five fractions (BC51-1—BC51-5). Fraction BC51-3 (29.2 mg) was separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 2 : 7)] to give **4** [14.9 mg (0.0015%)] and **11** [3.2 mg (0.0003%)]. Fraction BC51-4 (36.1 mg) was separated by HPLC [ODS, MeCN–H_2O (28 : 72)] to give **7** [9.0 mg (0.0009%)] and **8** [10.6 mg (0.0010%)]. The yield of each compound in parentheses is based on the weight of air-dried leaves.

Air-dried stems and twigs (19.46 kg) were combined and extracted with MeOH (85 l) for 20 d. The MeOH extract was concentrated to 4 l and extracted with hexane (8×1 l). Water (1 l) was added to the MeOH layer, and extracted with EtOAc (3×3 l) and *n*-BuOH (4×2 l), successively. The *n*-BuOH extracts were dried and concentrated to give an oily residue (244 g). A part of the *n*-BuOH extract (6.02) was separated by column chromatography on silica gel (539 g) in 14 fractions, A—N, using a gradient of $CHCl_3$ and MeOH. Fraction D [$CHCl_3$–MeOH (8 : 2)] gave on drying a viscous oil (374.6 mg), which was further separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 2 : 5)] into five fractions (D-1—D-5). Fraction D-2 gave 42.4 mg of

a semisolid, which was separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 2 : 7)] to give **4** [14.7 mg (0.0030%)] and **11** [(4.5 mg (0.0009%)). Fraction D-5 (30.3 mg) was separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 2 : 6)] to give **9** [14.5 mg (0.0030%)]. Fraction E gave on drying a viscous oil (91.8 mg), which was separated by HPLC [ODS, MeOH–MeCN–H_2O (1 : 3 : 9)] to give **4** [2.5 mg (0.0005%)], **9** [4.4 mg (0.0009%)], and **6** [3.8 mg (0.0008%)]. The yield of each compound in parentheses is based on the weight of air-dried stems and twigs.

3*β*-*O*-[*β*-D-Glucopyranosyl-(1→4)-*β*-D-diginopyranosyl]-14*α*-hydroxy-8-oxo-8,14-*sec*-5*β*-card-20(22)-enolide (1) Colorless microcrystals, mp 138—143 °C (acetone–hexane); $[\alpha]_D^{20}$ −28.56 (c=1.362, MeOH); IR (KBr) cm^{-1}: 3467, 2938, 1736, 1698, 1626, 1453, 1192; UV (MeOH) nm (log ε): 215 (4.05); ^1H- and ^{13}C-NMR data are shown in Table 1; HR-FAB-MS m/z 719.3615 $[M+Na]^+$ (Calcd for $C_{36}H_{56}O_{13}Na$, 719.3619).

Acid Hydrolysis of 1 Compound **1** (16.4 mg) was refluxed with 0.04 M H_2SO_4 (1.2 ml) in MeOH (1.2 ml) at 60 °C for 12 h. After cooling, the reaction mixture was extracted with $CHCl_3$ (3×3 ml). The $CHCl_3$ extracts were washed with aqueous $NaHCO_3$ and water, dried over anhydrous Na_2SO_4, and concentrated to give crude neriagenin, which was purified by column chromatography [silica gel (1 g, $CHCl_3$–MeOH (20 : 1)] to give pure neriagenin {7.1 mg, *Rf* 0.45 [silica gel TLC, thickness 0.25 mm, $CHCl_3$–MeOH (9 : 1)]}. The aqueous phase was neutralized with saturated $NaHCO_3$ aqueous solution and evaporated. The residue was dissolved in a small amount of a mixture of $CHCl_3$ and MeOH (1 : 1) and separated by silica gel column chromatography [silica gel 1 g, $CHCl_3$–MeOH (9 : 1)] to give *β*-D-glucopyranosyl-(1→4)-D-diginopyranose {4.2 mg, *Rf* 0.14 [silica gel TLC, thickness 0.25 mm, $CHCl_3$–MeOH (9 : 1)]}.

Neriagenin Colorless microcrystals, mp 225—227 °C; $[\alpha]_D^{20}$ −8.93 (c=3.03, MeOH); IR (KBr) cm^{-1}: 3460, 2930, 1740, 1630; UV (MeOH) nm (log ε): 215 (4.1); ^1H-NMR (500 MHz, δ in ppm, J in Hz, $CDCl_3$) δ: 5.82 (1H, s, H-22), 4.79 (1H, d, J=17.6, H-21a), 4.70 (1H, d, J=17.6, H-21b), 4.29 (1H, br s, $W_{h/2}$=8.0, H-3), 4.13 (1H, d, J=7.2, H-14), 2.89 (1H, dd, J=9.3, 9.3 Hz, H-17), 2,54 (1H, d, J=10.8, H-9), 2.45 (1H, ddd, J=13.4, 13.4, 6.8, H-7b), 2.34 (1H, dd, J=13.4, 4.9, 3.2, H-7a), 0.81 (3H, s, H-19), 0.76 (3H, s, H-18). ^{13}C-NMR (125 MHz, δ in ppm, J in Hz, $CDCl_3$) δ: 216.6 (C-8), 174.1 (C-23), 171.5 (C-20), 116.8 (C-22), 78.9 (C-14), 73.8 (C-21), 66.1 (C-3), 51.5 (C-13), 50.9 (C-9), 45.9 (C-17), 42.6 (C-10), 37.8, (C-7), 35.7 (C-5), 34.8 (C-12), 33.6 (C-4), 29.8 (C-1), 29.7 (C-16), 28.6 (C-2), 28.1 (C-6), 26.9 (C-15), 24.1 (C-19), 17.5 (C-11), 17.3 (C-18).

β-D-Glucopyranosyl-(1→4)-D-diginopyranose $[\alpha]_D^{20}$ +50.8° (c=1.30, MeOH).

Inhibitory Activity on Induction of ICAM-1 A549 cells were seeded in a microtiter plate at 2×10^4 cell/well the day before the assay. After A549 cells were pretreated with or without test compounds in 75 μl for 1 h, 25 μl of IL-1α (1 ng/ml) or TNF-α (10 ng/ml) were added to the culture, and the cells were further incubated for 6 h. The cells were washed once with phosphate-buffered saline (PBS) and fixed by incubation with 1% paraformaldehyde–PBS for 15 min and then washed once with PBS. After blocking with 1% bovine serum albumin–PBS overnight, the fixed cells were treated with mouse anti-human ICAM-1 antibody (clone 15.2) for 60 min. After being washed three times with 0.02% Tween 20-PBS, the cells were treated with horseradish peroxidase-linked anti-mouse immunoglobulin G (IgG) antibody for 60 min. The cells were washed three times with 0.02% Tween 20–PBS. The cells were incubated with the substrate (0.1% *o*-phenylenediamine dihydrochloride and 0.02% H_2O_2 in 0.2 M sodium citrate buffer, pH 5.3) for 20 min at 37 °C in the dark and assayed for the absorbance at 415 nm by using a microplate reader. Expression of ICAM-1 was calculated as follows:

expression of ICAM-1 (% of control)
=[(absorbance with sample and cytokine treatment−absorbance without cytokine treatment)/(absorbance with cytokine treatment−absorbance without cytokine treatment)]×100

A549 cells (2×10^4 cell/well) were seeded in a microtiter plate the day before the assay and incubated in the presence or absence of test compounds for 24 h. At the last 4 h of incubation, the cell were pulsed with 500 μg/ml of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for 4 h. MTT formazan was solubilized with 5% sodium dodecyl sulfate (SDS) overnight. Absorbance at 595 nm was measured. Cell viability (%) was calculated as follows:

cell viability (%)
=[(experimental absorbance−background absorbance)/(control absorbance−background absorbance)]×100

Cell Growth Inhibitory Activity to WI-38, VA-13, and HepG2 *in Vitro* Medium (100 μl) containing *ca.* 5000 cells (WI-38, VA-13, HepG2) were incubated at 37 °C in humidified atmosphere of 5% CO_2 for 24 h in microtiter plates. Then test samples dissolved in dimethyl sulfoxide (DMSO) were added to the medium and incubation was continued further for 48 h in the same conditions. Coloration substrate, WST-8 [2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450 nm. Cell viability (%) was calculated as [(experimental absorbance−background absorbance)/(control absorbance−background absorbance)]×100. Cell viability at different concentration of compounds was plotted and 50% inhibition of growth was calculated as IC_{50}.

Cellular Accumulation of Calcein Medium (100 μl) containing *ca.* 1×10^6 cells [adriamycin-resistant human ovarian cancer 2780AD cells (AD10)] was incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Test compounds were dissolved in DMSO and diluted with PBS (−). Test sample (50 μl) were added to the medium and incubated for 15 min. Then, 50 μl of the fluorogenic dye calcein AM [1 μl in PBS (−)] was added to the medium, and incubation was continued for a further 60 min. After removing the supernatant, each microplate was washed with 200 μl of cold PBS (−). The washing step was repeated twice and 200 μl of cold PBS (−) was added. Retention of the resulting calcein was measured as calcein-specific fluorescence. The absorption maximum for calcein is 494 nm, and emission maximum is 517 nm.

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