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Polar cardenolide monoglycosides from stems and twigs of *Nerium oleander* and their biological activities

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Abstract Twelve polar cardenolide monoglycosides, **1**, **2**, **4**–**13**, and oleagenin (**3**) were isolated from the methanol extract of stems and twigs of *Nerium oleander*. Among these, oleagenin (**3**) and cardenolide monoglycosides named cardenolide B-1 (**1**) and cardenolide B-2 (**2**) were isolated from natural sources for the first time. 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 **4**–**7** were active at an IC_{50} value of less than 0.4 μM. The cytotoxic activity of compounds **1**–**13** was evaluated against three human cell lines: normal human fibroblast cells (WI-38), malignant tumor cells derived from WI-38 (VA-13), and human liver tumor cells (HepG2). Compounds **4**, **6**, and **7** were active toward these three cell lines at IC_{50} values of less than 0.7 μM, and compounds **5** and **8** were active toward the cell lines at IC_{50} values of less than 1.5 μM. The multidrug resistance (MDR) cancer-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. Compound **1** and **12** showed significant effects on calcein accumulation.

Key words Bioactive cardenolide monoglycoside · *Nerium oleander* · Anti-inflammatory agent · Cytotoxic activity · MDR cancer-reversal agent

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Introduction

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, subtropical 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 interest 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 atrioventricular contraction. Anticancer utilization of digitoxin, digoxin, and related cardenolides has also been investigated.[15,16] We recently reinvestigated the cardenolide monoglycosides from *N. oleander* and isolated thirteen compounds, four of which were new compounds.[17] As a part of ongoing study of new types of anti-inflammatory agents, anticancer agents, and multidrug-resistant (MDR) cancer-reversal agents among the cardenolides, we are conducting further investigation on more polar cardenolide monoglycosides from the methanol extract of stems and twigs of *N. oleander*.

Experimental

General

Melting points are uncorrected. Optical rotation values were measured using a Horiba Sepa-200 polarimeter. IR spectra were recorded on a Shimadzu FTIR-4200 infrared spectrometer and UV spectra were recorded on a JASCO V-550 UV/vis spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (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 correlation spectroscopy (COSY) experiments. ^{13}C NMR assignments were determined using distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) experiments. High resolution fast atom bombardment (HRFABMS) were recorded on a JEOL JMS-HX110. Silica gel (70–230 mesh) was employed for column chromatography and silica gel (230–400 mesh) for flash column chromatography. High-performance liquid 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

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 × 1000 ml). Water (1.3 l) was added to the MeOH layer, extracted with EtOAc (3 × 3000 ml), dried (Na_2SO_4), and concentrated to give an oily material (96.5 g). The water layer was further extracted with *n*-BuOH (3 × 500 ml), dried (Na_2SO_4), and concentrated to give an oily residue (53.76 g).

The EtOAc extract (96.5 g) was separated by column chromatography [silica gel (1.1 kg), gradient of hexane, EtOAc, and MeOH] into five fractions, A–E. On drying, fraction B [hexane–EtOAc (1:1), EtOAc], fraction C (EtOAc), and fraction D [EtOAc–MeOH (1:1)] gave viscous oils weighing 29.58, 23.33, and 32.15 g, respectively. Fraction B was dissolved in EtOAc (200 ml), stirred for 1 h, filtered, and concentrated to a give a viscous oil (19.86 g), which was further separated by column chromatography [silica gel (1 kg), a gradient of hexane, EtOAc, and MeOH] into nine fractions, B1–B9. On drying, fractions B7 [EtOAc (100%)] and B8 [EtOAc(100%)] gave viscous oils [B7 (1.76 g), B8 (0.84 g)]. Fraction B7 was subjected to column chromatography [silica gel (300 g), gradient of hexane, EtOAc, and MeOH] to give five fractions, B71–B75. B73 (1.31 g) afforded compound **6** [53.51 mg (0.00027%)] by separation using HPLC [octadecyl silane (ODS), MeOH–MeCN–H_2O(1:1:2)]. B8 was subjected to column chromatography [silica gel (80 g), gradient of hexane, EtOAc, and MeOH] to give five fractions, B81–B85. B83 (296.0 mg) afforded compound **8** [9.7 mg (0.00005%)] by separation using HPLC [ODS, MeOH–MeCN–H_2O (1:3:5)]. Fraction C was subjected to flash column chromatography [silica gel (1 kg), hexane–EtOAc (1:59)] to give six fractions, C1–C6. Fraction C3 (8.65 g) was further separated by flash column chromatography [silica gel (800 g), hexane–EtOAc (3:7)] into four fractions, C31–C34. Fraction C33 (3.8 g) afforded compounds **3** [10.2 mg (0.000052%)] and **12** [132.3 mg (0.00068 %)] by successive separation using HPLC [ODS, MeOH–MeCN–H_2O (1:6:9)], [ODS, MeOH–MeCN–H_2O (4:4:9)], and [ODS, MeOH–MeCN–H_2O (3:4:10)]. Fraction C34 (1.134 g) was divided into $CHCl_3$-soluble (C341) and $CHCl_3$-insoluble (C342) fractions. C342 (0.80 g) afforded compound **2** [13.9 mg (0.000071%)] by separation using HPLC [ODS, MeOH–MeCN–H_2O (4:4:10)]. Fraction C4 (0.96 g) was separated by flash column chromatography [silica gel (100 g), hexane–EtOAc (2:8)] into eight fractions, C41–C48. C47 was compound **4** [81.7 mg (0.00042%)]. Compound **11** [93.7 mg (0.00048%)] was obtained by crystallization of C43 from EtOAc. Fraction C5 (9.06 g) was separated by flash column chromatography [silica gel (900 g), hexane–EtOAc (1:10)] into three fractions, C51–C53. C51 was compound **4** [575.1 mg (0.00295%)]. Additional compound **4** [165.7 mg (0.00085%)] was obtained from C52 by crystallization from MeOH. Fraction C53 (2.16 g) was separated by HPLC [ODS, MeOH–MeCN–H_2O (4:4:10)] to give compounds **4** [704.0 mg (0.00362%)] and **5** [451.5 mg (0.00232%)]. Fraction C6 (851 mg) was separated by flash column chromatography [silica gel (90 g), EtOAc] into four fractions, C61–C64. Fraction C62 was crystallized from EtOAc to give compound **9** [107.2 mg (0.00055%)]. Fraction D was dissolved in EtOAc (200 ml), stirred for 1 h, filtered, and concentrated to give a viscous oil (17.059 g), which was separated by column chromatography [silica gel (620 g), gradient of $CHCl_3$ and MeOH] into 12 fractions, D1–D12. Fraction D4 [$CHCl_3$–MeOH (98:2), 1.56 g] was further separated by flash column chromatography [silica gel (160 g), EtOAc] into six fractions, D41–D46. D42 (178 mg) was separated by silica gel HPLC [silica gel (20 g), EtOAc], followed by HPLC [ODS, MeOH–H_2O (55:45)] to give compound **13** [18.6 mg (0.00095%)]. The soluble portion of D43 (0.385 g) in EtOAc (D431, 0.314 g) was separated by HPLC [ODS, MeOH–H_2O (55:45)] to give compounds **4** [40.2 mg (0.00021%)], **5** [56.2 mg (0.00029%)], and **10** [46.7 mg (0.00024 %)]. The insoluble portion of D43 in EtOAc (D432, 68 mg) was subjected to HPLC [ODS, MeOH–H_2O (55:45)] to give D4323 [**1**, 4.2 mg (0.00002%)], D4324, and D4325 [**10**, 9.6 mg (0.000049%)]. Separation of D4324 by HPLC [ODS, MeOH–MeCN–H_2O (1:1:2.5)] gave compounds **1** [5.4 mg (0.000028%)] and **10** [6.8 mg (0.000035%)].

Compound **7** (17.4 mg, 0.000089%) was obtained from the *n*-BuOH extract (53.76 g) by separation using column chromatography [silica gel, a gradient of $CHCl_3$ and MeOH], followed by HPLC [ODS, MeOH–MeCN–H_2O (1:2:7)].

Identification of isolated compounds

Cardenolide monoglycosides named cardenolide B-1 (**1**), cardenolide B-2 (**2**), and oleagenin (**3**) (Fig. 1) were isolated from natural sources for the first time in this study. Their physical constants and infrared (IR), ultraviolet (UV), and high-resolution fast atom bombardment mass spectrometric (HR FAB-MS) data are given below. Their ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) data are shown in Tables 1 and 2, respectively. The detailed discussion of the structure determination of **1**, **2**, and **3** will appear in separate report.[18]

Cardenolide B-1 [3β-O-(β-D-digitalosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide]. **1** was obtained as colorless microcrystals; mp 203°–206°C (acetone–hexane); $[\alpha]^{20}_D$ + 28.57° (*c* 0.392, $CHCl_3$). ^{1}H NMR: see Table 1. ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3539, 2936, 1786, 1751, 1631. UV (MeOH): λ_{max} nm (log ε) 222 (4.05). HR FAB-MS *m/z*: 533.3104 [calculated for $C_{30}H_{45}O_8$ (M + H)$^+$, 533.3115].

Cardenolide B-2 [3β-O-(β-D-diginosyl)-7β,8-epoxy-14-hydroxy-5β,14β-card-20(22)-enolide]. **2** was obtained as colorless microcrystals; mp 167°–171°C (acetone–hexane); $[\alpha]^{20}_D$ –6.06° (*c* 0.330, $CHCl_3$). ^{1}H NMR: see Table 1. ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3537, 3010, 2932, 1765, 1746. UV (MeOH): λ_{max} nm (log ε) 218 (4.20). HR FAB-MS *m/z*: 533.3104 [calculated for $C_{30}H_{45}O_8$ (M + H)$^+$, 533.3115].

Oleagenin [(8R)-3β-hydroxy-14-oxo-15(14→8)abeo-5β-card-20(22)-enolide]. **3** was obtained as colorless prisms; mp 278°–285°C (MeOH); $[\alpha]^{20}_D$ + 49.60° (*c* 0.254, MeOH). ^{1}H NMR: see Table 1. ^{13}C NMR: see Table 2. IR (KBr): ν_{max} cm^{-1} 3399, 2937, 1748, 1692. UV (MeOH): λ_{max} nm (log ε) 207 (4.32). HR FAB-MS *m/z*: 373.2376 [calculated for $C_{23}H_{33}O_4$ (M + H)$^+$, 373.2379].

The structures of the known compounds **4**–**13** (Fig. 1) were confirmed by the analyses of their NMR, IR, UV, and HRFABMS spectrometric data and by comparison of their physical constants indicated here with those in the literature. ^{13}C NMR data of **4**–**13** are shown in Table 2 for the identification of compounds.

Odoroside H [3β-O-(β-D-digitalosyl)-14-hydroxy-5β,14β-card-20(22)-enolide].[19] **4** was obtained as colorless microcrystals; mp 231°–234°C (MeOH); $[\alpha]^{20}_D$ + 5.57° (*c* 0.556, MeOH). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3539, 3462, 2880, 1780, 1728, 1620. UV (MeOH): λ_{max} nm (log ε) 218 (4.08). HR FAB-MS *m/z*: 535.3271 [calculated for $C_{30}H_{47}O_8$ (M + H)$^+$, 535.3271].

Neritaloside [3β-O-(β-D-digitalosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide].[19,20] **5** was obtained as colorless microcrystals; mp 143°–146°C (acetone–hexane); $[\alpha]^{20}_D$ + 6.78° (*c* 1.046, $CHCl_3$). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3516, 3456, 3013, 2939, 1743. UV (MeOH): λ_{max} nm (log ε) 217 (4.04). HR FAB-MS *m/z*: 593.3326 [calculated for $C_{32}H_{49}O_{10}$ (M + H)$^+$, 593.3326].

Oleandrin [3β-O-(α-L-oleandrosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide].[4,5] **6** was obtained as colorless

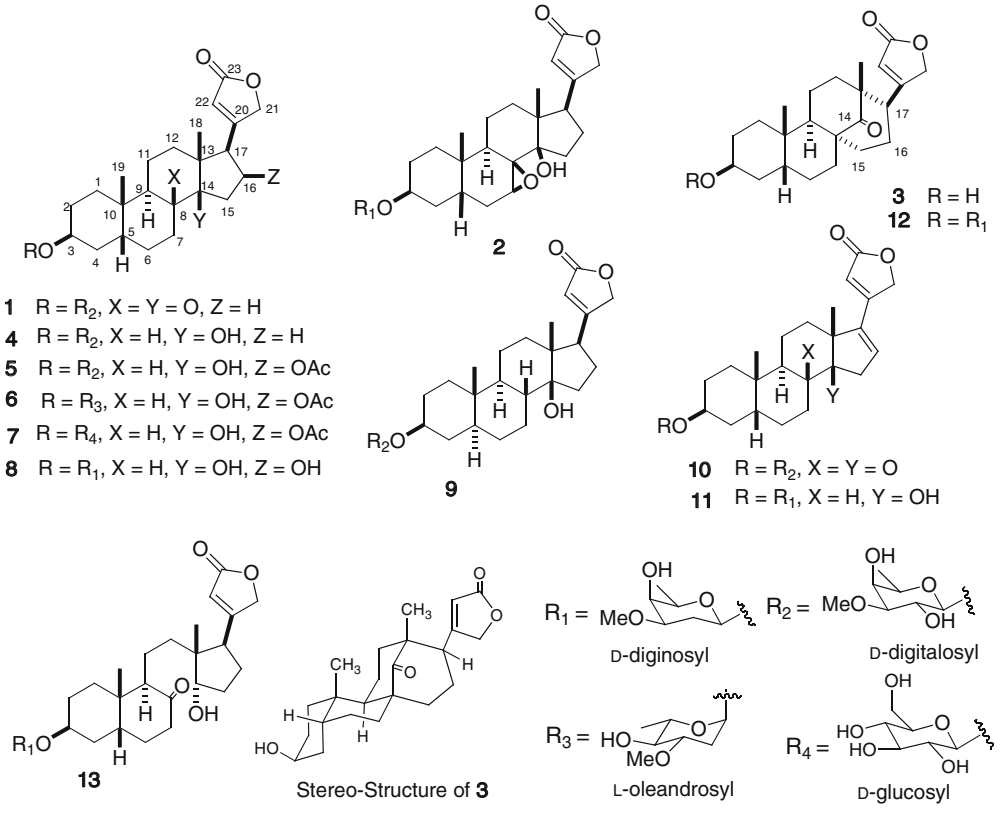

Fig. 1. Polar cardenolide monoglycosides from *Nerium oleander*

Table 1. ^{1}H nuclear magnetic resonance (NMR) data of **1**–**3** (500 MHz)

Position	**1**		**2**		**3**			
	δ	(J in Hz) in $CDCl_3$	δ	(J in Hz) in $CDCl_3$	δ	(J in Hz) in C_6D_5N	δ	(J in Hz) in $CDCl_3$
1	1.5	(1H, m)	1.4	(1H, m)	1.8	(1H, m)	1.45	(1H, m)
	1.5	(1H, m)	1.1	(1H, m)	1.6	(1H, m)	1.58	(1H, m)
2	1.5	(1H, m)	1.6	(1H, m)	1.6	(1H, m)	1.54	(1H, m)
	1.8	(1H, m)	1.80	(1H, m)	1.70	(1H, m)	1.72	(1H, m)
3	4.1	(1H, br s, $W_{h/2}$ = 7.5)	4	(1H, br s, $W_{h/2}$ = 7.5)	4.3	(1H, br s, $W_{h/2}$ = 8.0)	4.11	(1H, br s, $W_{h/2}$ = 7.5)
4	1.80	(1H, m)	1.35	(1H, m)	1.85	(1H, m)	1.84	(1H, m)
	1.60	(1H, m)	1.48	(1H, m)	1.52	(1H, br dd, 14.2, 3.2)	1.35	(1H, m)
5	1.8	(1H, m)	1.6	(1H, m)	2.1	(1H, br, d, 13.2)	1.75	(1H, m)
6	1.30	(1H, m)	1.47	(1H, m)	1.12	(1H, m)	1.14	(1H, m)
	2.2	(1H, m)	2.30	(1H, m)	2.35	(1H, m)	2.19	(1H, m)
7	1.8	(1H, m)	3.2	(1H, d, 5.9)	1.1	(1H, ddd, 13.9, 13.9, 4.6)	1.07	(1H, m)
	1.1	(1H, m)			2	(1H, m)	1.96	(1H, m)
8								
9	1.90	(1H, dd, 11.0, 4.6)	2.23	(1H, m)	2.51	(1H, br d, 8.3)	2.48	(1H, d, 8.6)
10								
11	1.2	(1H, m)	1.4	(1H, m)	2.3	(1H, m)	2.38	(1H, m)
	1.3	(1H, m)	1.6	(1H, m)	1.7	(1H, m)	1.81	(1H, m)
12	1.2	(1H, m)	1.5	(1H, m)	2	(2H, m)	2.06	(1H, m)
	1.6	(1H, m)	1.8	(1H, m)			2.09	(1H, m)
13								
14			2.37	(14-OH)				
15	2.00	(1H, m)	2.2	(1H, m)	1.9	(1H, dd, 14.4, 6.1)	2.04	(1H, m)
	1.7	(1H, m)	1.8	(1H, m)	1.7	(1H, ddd, 14.4, 14.4, 6.1)	1.77	(1H, m)
16	1.9	(1H, m)	2.3	(1H, m)	2.7	(1H, dddd,15.1, 14.4, 7.1, 6.8)	2.85	(2H, m)
	2	(1H, m)	2	(1H, m)	1.4	(1H, br dd,15.1, 6.8)		
17	2.6	(1H, dd, 11.2, 6.6)	2.8	(1H, dd, 8.3, 5.7)	3	(1H, br d, 7.1)	3.08	(1H, d, 7.1)
18	0.9	(3H, s)	0.90	(3H, s)	0.91	(3H, s)	0.94	(3H, s)
19	1	(3H, s)	1	(3H, s)	0.8	(3H, s)	0.80	(3H, s)
20								
21	4.7	(1H, dd, 17.4, 1.0)	4.8	(1H, dd, 18.1, 1.2)	4.80	(1H, dd, 17.6, 1.7)	4.56	(1H, dd, 17.6, 1.5)
	4.8	(1H, dd, 17.5, 1.7)	4.9	(1H, dd, 18.1, 1.2)	4.7	(1H, dd, 18.1, 1.2)	4.68	(1H, dd, 17.6, 1.5)
22	5.9	(1H, br s)	5.9	(1H, br s)	5.9	(1H, br s)	5.69	(1H, br s)
23								
1′	4.3	(1H, d, 7.8)	4.4	(1H, dd, 9.8, 1.7)				
2′	3.7	(1H, dd, 9.5, 7.8)	1.9	(1H, m)				
			1.7	(1H, m)				
3′	3.2	(1H, dd,(1H, dd, 9.5, 3.4)	3.3	(1H, ddd, 12.1, 4.8, 3.2)				
4′	3.9	(1H, br s)	3.70	(1H, br s)				
5′	3.6	(1H, br q, 6.3)	3.4	(1H, br q, 6.6)				
6′	1.4	(3H, d, 6.3)	1.3	(3H, d, 6.6)				
OMe	3.5	(3H, s)	3.40	(3H, s)				

microcrystals; mp 243°–249°C (MeOH); $[\alpha]^{20}_{D}$ – 12.90° (*c* 0.062, MeOH). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3539, 3462, 2944, 1746. HR FAB-MS *m/z*: 577.3377 [calculated for $C_{32}H_{49}O_9$ $(M + H)^+$, 577.3377].

3β-O-(β-D-Glucosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide.[21,22] **7** was obtained as colorless microcrystals; mp 151°–153°C (acetone–hexane); $[\alpha]^{20}_{D}$ – 18.05° (*c* 0.670, MeOH). ^{13}C NMR: see Table 2. IR (KBr): ν_{max} cm^{-1} 3429, 2939, 1738.

3β-O-(β-D-Diginosyl)-14,16β-dihydroxy-5β,14β-card-20(22)-enolide.[11,23] **8** was obtained as an amorphous compound; $[\alpha]^{21}_{D}$ + 5.55° (*c* 0.54, MeOH). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3605, 3499, 3026, 2878, 1782, 1745. HR FAB-MS *m/z* 535.3281 [calculated for $C_{30}H_{47}O_8$ $(M + H)^+$, 535.3271].

3β-O-(β-D-Digitalosyl)-14-hydroxy-5α,14β-card-20(22)-enolide.[9,11] **9** was obtained as colorless microcrystals; mp 230°–234°C (MeOH); $[\alpha]^{20}_{D}$ +0.86° (*c* 1.153, MeOH). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3518, 3011, 2940, 1788, 1746. UV (MeOH): λ_{max} nm (log ε) 218 (3.96).

3β-O-(β-D-Digitalosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide.[11,24] **10** was obtained as colorless microcrystals; mp 217°–220°C (acetone–hexane); $[\alpha]^{20}_{D}$ + 13.36° (*c* 0.546, $CHCl_3$). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3480, 2944, 1782, 1743, 1631. UV (MeOH): λ_{max} nm (log ε) 219 (3.19).

3β-O-(β-D-Diginosyl)-14-hydroxy-5β,14β-card-16,20(22)-dienolide.[23] **11** was obtained as colorless microcrystals; mp 187°–190°C (acetone–hexane); $[\alpha]^{20}_{D}$ + 26.87° (*c* 1.256, $CHCl_3$). ^{13}C NMR: see Table 2. IR ($CHCl_3$): ν_{max} cm^{-1} 3507, 3362, 2943, 1782, 1730, 1697, 1622; UV (MeOH): λ_{max} nm (log ε) 217 (4.12).

Oleaside A [(8R)-3β-O-(β-D-diginosyl)-14-oxo-15(14→8)abeo-5β-card-20(22)-enolide].[2] **12** was obtained as colorless prisms; mp 242°–245°C (MeOH); $[\alpha]^{20}_{D}$ + 27.60° (*c* 0.920, $CHCl_3$). ^{13}C NMR: see Table 2. IR (KBr) ν_{max} cm^{-1} 3420,

Table 2. ^{13}C NMR data of **1–13** (125 MHz, d in ppm *J* in Hz)

Position	**1**	**2**	**3**		**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
	$CDCl_3$	$CDCl_3$	$CDCl_3$	C_5D_5N	$CDCl_3$	$CDCl_3$	$CDCl_3$	C_5D_5N	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$
1	30.4, CH_2	31.1, CH_2	30.9, CH_2	31.6, CH_2	30.2, CH_2	30.0, CH_2	26.6, CH_2	30.8, CH_2	26.6, CH_2	37.1, CH_2	30.1, CH_2	30.2, CH_2	31.5, CH_2	30.3, CH_2
2	26.6, CH_2	27.1, CH_2	28.1, CH_2	28.9, CH_2	26.5, CH_2	26.4, CH_2	26.5, CH_2	27.1, CH_2	29.9, CH_2	29.2, CH_2	26.6, CH_2	26.5, CH_2	27.0, CH_2	28.3, CH_2
3	73.7, CH	71.9, CH	66.6, CH	65.8, CH	73.9, CH	73.9, CH	71.3, CH	74.2, CH	72.5, CH	77.7, CH	73.8, CH	72.6, CH	72.2, CH	72.6, CH
4	30.0, CH_2	32.7, CH_2	33.5, CH_2	34.5, CH_2	30.0, CH_2	30.0, CH_2	34.5, CH_2	30.4, CH_2	30.1, CH_2	34.2, CH_2	30.0, CH_2	29.9, CH_2	29.9, CH_2	30.8, CH_2
5	36.6, CH	33.6, CH	36.4, CH	37.1, CH	36.5, CH	36.4, CH	36.4, CH	36.7, CH	36.2, CH	44.2, CH	36.5, CH	36.5, CH	36.8, CH	36.1, CH
6	24.5, CH_2	27.9, CH_2	23.9, CH_2	24.8, CH_2	26.6, CH_2	26.4, CH_2	30.4, CH_2	27.1, CH_2	26.6, CH_2	28.5, CH_2	24.6, CH_2	26.6, CH_2	24.2, CH_2	30.9, CH_2
7	26.7, CH_2	51.2, CH	29.0, CH_2	29.5, CH_2	21.2, CH_2	20.7, CH_2	21.0, CH_2	21.7, CH_2	21.8, CH_2	27.4, CH_2	27.0, CH_2	21.2, CH_2	29.1, CH_2	37.8, CH_2
8	65.3, qC	63.9, qC	48.8, qC	49.1, qC	41.9, CH	41.7, CH	41.8, CH	42.0, CH	42.1, CH	41.6, CH	65.1, qC	41.0, CH	48.8, qC	216.7, qC
9	36.7, CH	31.6, CH	45.7, CH	46.0, CH	35.8, CH	35.6, CH	35.6, CH	35.9, CH	35.7, CH	49.8, CH	36.2, CH	36.3, CH	46.0, CH	50.9, CH
10	36.7, qC	33.6, qC	37.5, qC	37.9, qC	35.2, qC	35.0, qC	35.1, qC	35.4, qC	35.2, qC	35.9, qC	36.7, qC	35.1, qC	37.3, qC	42.5, qC
11	16.1, CH_2	20.3, CH_2	21.3, CH_2	21.4, CH_2	21.4, CH_2	21.0, CH_2	20.8, CH_2	21.2, CH_2	21.0, CH_2	21.1, CH_2	15.6, CH_2	19.8, CH_2	21.4, CH_2	27.2, CH_2
12	37.0, CH_2	41.0, CH_2	42.6, CH_2	42.7, CH_2	40.1, CH_2	39.2 CH_2	39.3 CH_2	39.0, CH_2	41.7 CH_2	39.8, CH_2	33.3, CH_2	38.4, CH_2	42.6, CH_2	34.7, CH_2
13	41.8, qC	52.2, qC	47.3, qC	47.5, qC	49.6, qC	49.9, qC	50.0, qC	50.5, qC	49.6, qC	49.5, qC	44.7, qC	52.2, qC	47.4, qC	51.4, qC
14	70.5, qC	81.0, qC	220.8, qC	221.3, qC	85.5, qC	84.2, qC	84.3, qC	83.5, qC	86.3, qC	85.4, qC	70.1, qC	85.6, qC	220.7, qC	78.9, qC
15	25.7, CH_2	34.4, CH_2	44.1, CH_2	44.1, CH_2	33.2, CH_2	41.2, CH_2	41.3, CH_2	41.3, CH_2	41.9, CH_2	33.0, CH_2	33.0, CH_2	40.4, CH_2	44.1, CH_2	26.8, CH_2
16	27.0, CH_2	28.4, CH_2	26.9, CH_2	26.9, CH_2	27.0, CH_2	74.0, CH	73.9, CH	75.0, CH	73.3, CH	26.8, CH_2	132.2, CH	132.1, CH	26.9, CH_2	17.5, CH_2
17	51.5, CH	50.6, CH	53.3, CH	53.0, CH	50.9, CH	56.1, CH	56.1, CH	56.9, CH	58.1, CH	50.8, CH	143.0, qC	144.0, qC	53.1, CH	45.8, CH
18	16.2, CH_3	17.1, CH_3	23.3, CH_3	23.4, CH_3	15.8, CH_3	15.9, CH_3	15.9, CH_3	16.3, CH_3	16.7, CH_3	15.7, CH_3	19.9, CH_3	16.8, CH_3	23.4, CH_3	17.3, CH_3
19	24.7, CH_3	24.0, CH_3	26.4, CH_3	26.6, CH_3	23.7, CH_3	23.6, CH_3	23.8, CH_3	23.7, CH_3	23.6, CH_3	12.1, CH_3	24.5, CH_3	23.8, CH_3	26.3, CH_3	23.9, CH_3
20	169.5, qC	173.6, qC	170.5, qC	171.9, qC	174.4, qC	170.4, qC	167.6, qC	169.7, qC	168.5, qC	174.5, qC	157.6, qC	158.3, qC	170.4, qC	171.4, qC
21	73.2, CH_2	73.3, CH_2	72.8, CH_2	73.4, CH_2	73.4, CH_2	75.6, CH_2	75.6, CH_2	76.2, CH_2	75.4, CH_2	73.4, CH_2	71.4, CH_2	71.7, CH_2	72.8, CH_2	73.8, CH_2
22	116.9, CH	117.8, CH	116.7, CH	116.4 (d)	117.1, CH	121.3, CH	121.4, CH	121.6, CH	119.8, CH	117.6, CH	112.8, CH	112.4, CH	116.4 (d)	116.7, CH
23	173.6, qC	174.2, qC	173.5, qC	173.8, qC	174.4, qC	174.1, qC	174.0, qC	174.1, qC	174.2, qC	174.5, qC	174.2, qC	174.4, qC	173.5, qC	174.0, qC
16-OAc						21.0, CH_3 167.8, qC	21.04, CH_3 170.4, qC	20.7, CH_3 170.2, qC						
1′	101.3, CH	97.9, CH			101.1, CH	101.3, CH	95.5, CH	103.1, CH	97.8, CH	100.8, CH	100.3, CH	97.8, CH	97.5, CH	98.4, CH
2′	70.8, CH	32.0, CH_2			70.8, CH	70.7, CH	29.8, CH	75.4, CH	32.1, CH_2	70.5, CH	70.7, CH	32.1, CH_2	32.1, CH_2	32.1, CH_2
3′	82.8, CH	78.0, CH			82.8, CH	82.8, CH	78.4, CH	78.8, CH	78.0, CH	82.9, CH	82.8, CH	78.0, CH	77.9, CH	77.9, CH
4′	68.2, CH	67.2, CH			68.2, CH	68.1, CH	67.6, CH	72.0, CH	67.2, CH	67.9, CH	68.1, CH	67.2, CH	67.2, CH	66.9, CH
5′	70.4, CH	70.4, CH			70.3, CH	70.4, CH	76.3, CH	78.4, CH	70.4, CH	70.3, CH	70.3, CH	70.4, CH	70.3, CH	70.4, CH
6′	16.2, CH_3	16.8, CH_3			16.4, CH_3	16.4, CH_3	17.8, CH_3	63.0, CH_3	16.8, CH_3	16.5, CH_3	16.4, CH_3	16.6, CH_3	16.9, CH_3	16.8, CH_3
3′-OMe	57.6, CH_3	55.7, CH_3			57.5, CH_3	57.6, CH_3	56.4, CH_3		55.7, CH_3	57.4, CH_3	57.5, CH_3	55.7, CH_3	55.8, CH_3	55.7, CH_3

2961, 1788, 1745; UV (MeOH): λ_{max} nm (log ε) 213 (4.10); HR FAB-MS *m/z*: 517.3165 [calculated for $C_{30}H_{45}O_7$ $(M + H)^+$, 517.3166].

Neriaside [3β-O-(β-D-diginosyl)-8,14-seco-14α-hydroxy-8-oxo-5β-card-20(22)-enolide].[4,5] **13** was obtained as colorless prisms; mp 159°–163°C (MeOH); $[\alpha]^{20}_D$ + 21.42° (*c* 0.462, $CHCl_3$). ^{13}C NMR: see Table 2. IR (KBr) ν_{max} cm^{-1} 3483, 3478, 2959, 1782, 1751, 1693, 1626. HR FAB-MS *m/z*: 535.3274 [calculated for $C_{30}H_{47}O_8$ $(M + H)^+$, 535.3271].

Inhibitory activity on induction of intercellular adhesion molecule-1 (ICAM-1)

Cells. Human lung carcinoma A 549 cells were provided by the Health Science Research Resources Bank (Tokyo, Japan). A 549 cells maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum (JRH Bioscience, Lenexa, KS, USA) and a penicillin–streptomycin–neomycin antibiotic mixture (Invitrogen).

Reagents. Mouse anti-human ICAM-1 antibody (clone 15.2) was purchased from Leinco (St. Louis, MO, USA), and horseradish peroxidase-conjugated goat anti-mouse IgG antibody was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Recombinant human IL-1α and TNF-α were kindly provided by Dainippon Pharmaceutical (Osaka, Japan).

Procedures. 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 compound in 75 µl for 1 h, 25 µl of IL-1α (1 ng/ml) or TNF-α (10 ng/ml) was added to the culture and the cells were further incubated for 6 h. The cells were washed once with phosphate-buffered saline (PBS), 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 for 60 min. After being washed three times with 0.02% Tween 20–PBS, the cells were treated with horseradish peroxidase-linked anti-mouse 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 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

Cell viability. 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. During the last 4 h of induction, the cells 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 of compounds toward WI-38 fibroblast cells, VA-13 malignant cells, and HepG2 human liver cells in vitro. Experimental details were described in a previous article.[25]

Cellular accumulation of calcein

Cells. Adriamycin-resistant human ovarian cancer A2780 cells (AD10) were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Fitron) with 80 µg/ml kanamycin.

Procedures. Medium (100 µl) containing ca. 1×10^6 cells was incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Test compounds were dissolved in dimethylsulfoxide and diluted with PBS (–). Test samples (50 µl) were added to the medium and incubated for 15 min. Then, 50 µl of the fluorogenic dye calcein-acetoxymethyl ester (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 florescence. The absorption maximum for calcein is 494 nm, and the emission maximum is 517 nm.

Results and discussion

In vitro anti-inflammatory activity

The in vitro anti-inflammatory activity of isolated compounds **1–13** was estimated on the basis of inhibitory activity against the induction of the intercellular adhesion molecule-1 (ICAM-1) in the presence of interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α)[26–29] using human cultured cell line A549 cells. Cell viability was measured by an MTT assay (Table 3). The assay results of **1–13** are summarized as follows: (1) Compounds **4–7**, with a 14-hydroxy-5β,14β-card-20(22)-enolide structure, showed very strong inhibitory activity toward the induction of ICAM-1 at IC_{50} values of less than 0.4 µM. Although the presence or absence of 16β-OAc at C-16 had no influence on the activity, the presence of a more polar hydroxyl group at C-16 reduced the activity, as shown by the activity of **8**. (2) Among compounds **4–7**, cardenolide **7** was the most

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-1α[d]	TNF-α[d]	IC_{50} (μM)
1	220	140	>320
2	6.6	5.7	>330
3	90	54	>320
4	0.20	NT	>1000
5	0.28	0.27	>320
6	0.39	NT	570
7	0.16	0.12	>320
8	5.2	NT	>1000
9	7.5	6.2	>320
10	31	20	>320
11	63	39	>320
12	81	57	>320
13	56	NT	>1000
Odoroside A[e]	0.20	0.48	>316

IL-1α, interleukin-1α; TNF-α, tumor necrosis factor-α; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide; NT, not tested

[a] A549 cells 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 at 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

[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

[d] IC_{50} represent the means of two independent experiments, except for 4, 5, 6, and 7

[e] 3β-*O*-(β-D-Diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide[17]

Table 4. Cell growth inhibitory activities of compounds **1–13** toward WI-38, VA-13, and HepG2 cells

Compound	IC_{50} (μM)[a]		
	WI-38	VA-13	HepG2
1	130	>190	180
2	11	14	6.5
3	180	220	170
4	0.016	0.12	0.41
5	0.013	0.12	1.3
6	0.010	0.014	0.14
7	0.11	0.68	0.14
8	1.50	1.50	1.50
9	18	150	11
10	130	130	74
11	35	80	90
12	1.9	11	18
13	13	9.5	78
Paclitaxel	0.04	0.005	8.1
Adriamycin	0.70	0.40	1.3

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

effective compound and the IC_{50} values were less than 0.2 μM. Since compound **7** showed very weak cytotoxic activity (IC_{50} > 320 μM), it could be a desirable compound as an anti-inflammatory agent. (3) The structural changes in sugar moiety from the 3β-*O*-(D-digitalosyl) group in compound **5** to the 3β-*O*-(L-oleandrosyl) group in compound **6** or the 3β-*O*-(D-glucosyl) group in compound **7** had little influence on the activities, as shown in of the data for **5**, **6**, and **7**, respectively. (4) The change of the 14-hydroxyl group of **4** to an 8β,14β-epoxide ring induced a remarkable decrease of activity, as shown by that of cardenolide B-1 (**1**). (5) Introduction of a double bond at C-16 of the 14-hydroxy-5β,14β-card-20(22)-enolide structure induced a significant decrease of activity, as shown by that of **11**. (6) The change of the 5β,14β-card-20(22)-enolide structure of **4** to the corresponding 5α,14β-card-20(22)-enolide structure of **9** induced a large decrease in activity. (7) The skeletal rearrangement of the 5β,14β-cardenolide structure of **4** to the 15(14→8)abeo-cardenolide derivatives of **3** and **12** and the 8,14-seco-cardenolide derivative of **13** induced a large decrease in activity. (8) Compounds **1–3**, **5**, **7**, and **9–12** showed inhibitory activity on the induction of ICAM-1 induced by IL-1α and TNF-α at nearly the same level. The results suggest that these compounds block the common signaling nuclear factor-κB (NF-κB) activation downstream of inhibitor of NF-κB (IκB) kinase activation. Consistent with this, we have recently shown that odoroside A and ouabain inhibit Na^+/K^+-ATPase and prevent NF-κB-inducible protein expression by blocking Na^+-dependent amino acid transport.[30]

Cytotoxic activity

Cytotoxic activities of compounds **1–13** were evaluated against three cell lines: WI-38 (normal human fibroblast cells), VA-13 (malignant tumor cells derived from WI-38), and HepG2 (human liver tumor cells) (Table 4). The assay results of **1–13** are summarized as follows: (1) the 5β,14β-Card-20(22)-enolide structure is important for cell growth inhibitory activity of cardenolides. Thus, compound **4** with a 5β,14β-card-20(22)-enolide structure showed stronger activity than that of corresponding 5α,14β-card-20(22)-enolide **9**, as shown by the increase of IC_{50} values of **9** in the range from 30 to 1000 times. (2) The skeletal rearrangement of the 3β-*O*-(glycosyl)-5β,14β-cardenolide structure of **4** to the corresponding 3β-*O*-(glycosyl)-15(14→8)abeo-cardenolide **12** and 3β-*O*-(glycosyl)-8,14-seco-cardenolide **13** also induced a decrease in cytotoxic activities of the compounds as shown by the increase of IC_{50} values of **12** and **13** in the range from 40 to 100 times and from 80 to 800 times, respectively. (3) 3β-*O*-(Glycosyl)-16β-acetoxy-14-hydroxy-5β,14β-cardenolides **6** and **7** were the most effective compounds toward HepG2 cells. The change of the 3β-*O*-(glycosyl) moiety of L-oleandrosyl in **6** to D-glucosyl in **7** had no influence on the activity; their IC_{50} values were both 0.14 μM. In contrast, the change of the 3β-*O*-(glycosyl) moiety of L-oleandrosyl in **6** and D-glucosyl in **7** to D-digitalosyl in **5** induced a decrease of activity toward HepG2 with an increase of IC_{50} value of around 10 times. Thus, structural changes involving the sugar moieties of compounds had a big influence on the cytotoxic activities of compounds toward HepG2. Since liver cells transport poisonous substances from the inside to the outside of cells as a mechanism of detoxification, the 3β-*O*-(α-L-oleandrosyl) moiety of **6** and

the 3*β*-*O*-(*β*-D-glucosyl) moiety of **7** may play an important role in disturbing the elimination of compounds from HepG2 cells. (4) Compound **6** showed the strongest activity toward VA-13 cells with an IC_{50} value of 0.014 μM. 3*β*-*O*-(Glycosyl)-14-hydroxy-5*β*,14*β*-cardenolide **4** and its 16-acetoxy derivatives **5** and **7** showed strong activity toward VA-13 with IC_{50} values of less than 1 μM. (5) Thus, 3*β*-*O*-(glycosyl)-14-hydroxy-5*β*,14*β*-card-20(22)-enolide structures with or without an acetoxyl group at C-16 are effective for expression of cytotoxic activity toward VA-13 and HepG2 cells. (6) Introduction of a new epoxide ring at the 7,8-position of **4** induced a decrease in activity as shown by the increase of IC_{50} values of **2** in the range from 20 to 700 times. The change of the functional group of **4** from a 14-hydroxyl group to an 8,14-epoxy ring, such as **1**, induced a further decrease in the activity as shown by the increase of IC_{50} values of **1** in the range from 400 to 8000 times. Introduction of a double bond at C-16 of the 14-hydroxy-5*β*,14*β*-card-20(22)-enolide structure induced a significant decrease in activity as shown in that of **10**. Thus, digitoxigenin (3*β*,14-dihydroxy-5*β*,14*β*-card-20(22)-enolide) and oleandrigenin (16*β*-acetoxy-3*β*,14-dihydroxy-5*β*,14*β*-card-20(22)-enolide) are the essential genin moieties for expression of the strong activity of cardenolide monoglycosides **4**–**7** toward WI-38, VA-13, and HepG2. Actually, compounds **2** with tanghinigenin (7*β*,8-epoxy-3*β*,14-dihyddroxy-5*β*,14*β*-card-20(22)-enolide), **9** with uzarigenin (3*β*,14-dihydroxy-5*α*,14*β*-card-20(22)-enolide), and **11** with Δ^{16}-digitoxigenin (3*β*,14-dihydroxy-5*β*,14*β*-card-16,20(22)-dienolide) as genin moieties showed weaker cytotoxic activities toward WI-38, VA-13, and HepG2 than those of compounds **4**–**7**. (7) Compounds **4**–**6** showed stronger cytotoxic activities toward WI-38 than those of paclitaxel and adriamycin (ADM). (8) Compounds **4**–**6** showed stronger cytotoxic activities toward VA-13 than that of ADM. (9) Compounds **4**, **6**, and **7** showed stronger cytotoxic activity toward HepG2 than those of paclitaxel and ADM. (10) Thus, compounds **4**–**7** with 3*β*-O-(glycosyl)-14-hydroxy-5*β*,14*β*-card-20(22)-enolide or 3*β*-O-(glycosyl)-16-acetoxy-14-hydroxy-5*β*,14*β*-card-20(22)-enolide structures showed strong cytotoxic activity toward WI-38, VA-13, and HepG2 comparable with those of the positive controls, paclitaxel and adriamycin.

Multidrug resistance (MDR) cancer-reversal activity

In cancer chemotherapy, the occurrence of cancer cells with multidrug resistance (MDR) caused by repeated administration of agents is a serious problem. One of the mechanisms of MDR is overexpression of P-glycoprotein (P-gp),[31,32] which boosts the transport of anticancer agents from the inside to the outside of cancer cells. We screened cardenolides **1**–**13** for activity as MDR reversal agents. Fluorogenic dye calcein, which is derived from calcein AM by enzymatic hydrolysis inside the cells, 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 effect of the thirteen cardenolide derivatives **1**–**13** on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells was examined. Compounds **1**, **6**, **12**, and **13** showed MDR reversal activity in comparison with the control (Table 5). Since compound **1** showed very weak cytotoxic activity, it is a potential lead compound as a MDR cancer-reversal agent.

Table 5. Effect of compounds on the accumulation of calcein in multidrug-resistant 2780AD cells

Compound	Calcein accumulation (% of control)[a,b]		
	0.25 μg/ml	2.5 μg/ml	25 μg/ml
1	109[c]	110[c]	130[c]
2	108	81	86
3	91	95	92
4	96	83	91
5	94[c]	85[c]	85[c]
6	97	87	111
7	79	79	75
9	99	84	82
10	101[c]	105[c]	99[c]
11	97	99	98
12	112	126	117
13	108	96	106
Verapamil	103	110	138

[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 each test compound
[b]Values are the relative amount of calcein accumulated in the cell compared with the control experiment and represent the means of triplicate determinations
[c]Values represent the means of duplicate determinations

Conclusions

14-Hydroxy-5*β*,14*β*-card-20(22)-enolide derivatives **4**–**7** were active at an IC_{50} value of less than 0.4 μM in in vitro anti-inflammatory tests of compounds **1**–**13**. The principal structure generating this activity is the 14-hydroxy-5*β*,14*β*-card-20(22)-enolide structure. The most effective compound was 3*β*-O-(D-glucosyl)-16*β*-acetoxy-14-hydroxy-5*β*,14*β*-card-20(22)-enolide (**7**). Since compound **7** showed inhibitory activity on the induction of ICAM-1 induced by IL-1*α* and TNF-*α* at nearly the same level, it is likely that compound **7** blocks the common signaling NF-*κ*B activation downstream of I*κ*B kinase activation in a molecular mechanism similar to that of odoroside A and ouabain. In cytotoxic activities, compounds **4**–**7** showed significant activity. Compound **7**, compounds **4** and **5**, and compounds **6** and **7** were the most active compounds toward WI-38, VA-13, and HepG2, respectively. For these activities also, the principal structure was the 14-hydroxy-5*β*,14*β*-card-20(22)-enolide structure. In terms of multidrug resistance (MDR) cancer-reversal activity, compounds with the 14-hydroxy-5*β*,14*β*-card-20(22)-enolide structure were not effective, but the 8,14-epoxy-5*β*,14*β*-card-20(22)-enolide structure and rearranged cardenolide structures such as **12** or **13** were effective.

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