A New Sesquiterpene Ester from *Celastrus orbiculatus* Reversing Multidrug Resistance in Cancer Cells

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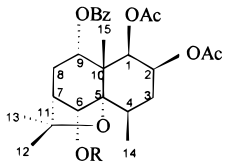
In a search for revertants of multidrug-resistance in cancer cells, a novel (**1**) and two known (**2**, **3**) sesquiterpene esters were isolated from the root of *Celastrus orbiculatus*. The structure of **1** was elucidated as 1β,2β-diacetoxy-6α,9α-bis(benzoyloxy)dihydro-β-agarofuran. Compounds **1**−**3** partially or completely reversed resistance to adriamycin, vinblastine, and paclitaxel of multidrug-resistant KB-V1 and MCF7/ADR cells.

One of the major problems of cancer chemotherapy is intrinsic or acquired multidrug resistance (MDR). A primary mechanism of MDR is attributed to the overexpression of P-glycoprotein (P-gp) in the plasma membrane of resistant cells where the P-gp acts as an energy-dependent efflux pump, reducing intracellular accumulation of anticancer drugs.[1] A number of drugs, such as, calcium channel blockers, calmodulin inhibitors, and indole alkaloids, are known to reverse MDR by competing with anticancer drugs for binding to P-gp.[2] However, they have not been proven clinically useful yet. For example, verapamil, the most extensively studied MDR reversing agent, induces cardiovascular toxicity at the concentration that it reverses MDR.[2] Thus, there remains a need to develop new classes of MDR reversing agents with less toxicity to the host.

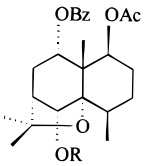
Celastrus orbiculatus Thunb. (Celastraceae) has been used as a treatment for rheumatoid arthritis and bacterial infection in folk medicine.[3] Several sesquiterpene esters were reported as chemical constituents of *C. orbiculatus* seed oil.[4,5] The family of Celastraceae has been known to produce various dihydro-β-agarofuran derivatives,[6] some of which exhibited insecticidal or insect antifeedant activities and antitumor activities.[7,8] Recently, the antitumor-promoting activity of dihydro-β-agarofuran compounds has also been reported.[9]

In our search for MDR reversing agents from natural product, the MeOH extract of the roots of *C. orbiculatus* was found to strongly potentiate the activities of anticancer drugs in multidrug-resistant KB-V1 cells at nontoxic concentrations. Bioactivity-guided fractionation of the MeOH extract of the plant, followed by repeated column chromatography, led to isolation of a novel sesquiterpene ester **1**, and two known compounds, celafolin A-1 (**2**) and celorbicol ester (**3**). Compounds **1**, **2**, and **3** reversed resistance to adriamycin (ADR), vinblastine (VLB), and paclitaxel (TX) of human multidrug-resistant cell lines, KB-V1 and MCF7/ADR, partially or completely. The structure elucidation of a novel sesquiterpene ester **1** along with the effect of the isolates on the MDR is described.



1 R= Bz

4 R= Nic



2 R= Cin

3 R= Bz

The IR spectrum of **1** showed a carbonyl absorption at 1716 cm^{-1}, and the UV spectrum showed the presence of an aromatic moiety (231 and 275 nm). The ^{13}C NMR spectrum revealed four methyls, two methylenes, six methines, three quaternary carbons, and four ester carbonyl carbons. The ^{1}H NMR spectrum revealed the presence of two acetyl groups (δ 1.62 and 2.02), two benzoyl esters [δ 8.06 (4H, d, J = 7.5 Hz), 7.49 (2H, t, J = 7.5 Hz), 7.44 (2H, t, J = 7.5 Hz), 7.62 (1H, t, J = 7.5 Hz), 7.56 (1H, t, J = 7.5 Hz)], three tertiary methyl groups (δ 1.53, 1.44, 1.45), and one secondary methyl group (δ 1.25). The signals observed at δ 5.62 (2H, br s), 5.66 (1H, s), and 5.02 (1H, d, J = 6.8 Hz) were assigned to the four protons attached to the carbons bearing secondary esters. These facts agreed well with the molecular formula, $C_{33}H_{38}O_9$, which was supported by HRMS data. The NMR spectra of **1** were almost identical with those of **4**, triptogelin C-2.[10] Thus, compound **1** was deduced as a 1,2,6,9-tetraesterified dihydro-β-agarofuran compound with the same stereochemistry as that of **4**. The assignments of the proton and carbon signals of **1** as shown in Table 1 were confirmed by the ^{1}H−^{1}H and ^{1}H−^{13}C COSY spectra. Generally, in this class of compounds H-6 has an axial configuration and appears as a singlet.[6] The ^{1}H NMR signals of H-1 and H-2 of **1** were not resolved when measured in $CDCl_3$ but were separated in pyridine-d_5

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Table 1. ^1H (300 MHz) and ^{13}C (75 MHz) NMR Data for Orbiculin A (**1**) ($CDCl_3$)

position	δ C	δ H[a]
1	71.17 d	5.62 br s
2	69.96 d	5.62 br s
3	31.06 t	2.52 m
		1.85 d (15.0)
4	34.19 d	2.54 m
5	89.80 s	
6	79.88 d	5.66 s
7	48.95 d	2.41 m
8	31.70 t	2.26 m
		2.23 dd (16.4, 3.0)
9	73.12 d	5.02 d (6.8)
10	50.02 s	
11	82.89 s	
12	26.05 q	1.44 s
13	30.03 q	1.45 s
14	17.59 q	1.25 d (7.5)
15	18.90 q	1.53 s

[a] 2 × Bz: δ 8.06 (4H, d, 7.5), 7.49 (2H, t, 7.5), 7.44 (2H, t, 7.5), 7.62 (1H, t, 7.5), 7.56 (1H, t, 7.5). 2 × Ac: δ 1.62 (3H, s), 2.02 (3H, s).

Table 2. Effects of **1**−**3** on the Growth of the Various Human Cancer Cell Lines

cell line[b]	anticacer drug	IC_{50}[a] (μM)		
		1	**2**	**3**
KB-3-1		49.02 ± 2.83	8.64 ± 0.74	9.26 ± 0.55
KB-V1		64.90 ± 4.54	14.67 ± 1.29	12.95 ± 0.95
KB-V1	VLB (0.1 μM)	0.80 ± 0.13	0.77 ± 0.08	1.36 ± 0.11
MCF7		53.16 ± 9.61	11.18 ± 0.49	10.80 ± 2.02
MCF7/ADR		61.91 ± 4.09	14.00 ± 1.56	14.36 ± 2.48
MCF7/ADR	ADR (10 μM)	1.15 ± 0.05	1.45 ± 0.23	1.39 ± 0.18
SNB-19		40.78 ± 2.85	17.56 ± 1.04	18.26 ± 3.34
SK-OV-3		48.42 ± 3.12	15.36 ± 2.02	19.27 ± 0.85
NCI-H23		41.94 ± 3.76	15.73 ± 1.97	16.82 ± 1.90
UACC-62		38.77 ± 5.56	14.86 ± 1.88	12.94 ± 2.12
KM-12		38.56 ± 3.38	9.93 ± 0.84	14.88 ± 1.88
MOLT		7.33 ± 2.15	1.51 ± 0.25	3.05 ± 0.56

[a] Data are mean ± SD from two or three separate experiments. [b] Cell lines: KB-3-1 (human oral epidermal cancer), KB-V1 (drug-resistant KB-3-1), MCF7 (human breast cancer), MCF7/ADR (drug-resistant MCF7), SNB-19 (human CNS cancer), SK-OV-3 (human ovarian cancer), NCI-H23 (human lung cancer), UACC-62 (human melanoma), KM-12 (human colon cancer), MOLT (human leukemia).

and appeared at δ 5.98 (d, J = 3.3 Hz) and 6.01 (m). The coupling constant ($J_{1,2}$ = 3.3 Hz) between H-1 and H-2 indicated their *cis*-relationship. In the NOESY spectrum, the signal at δ 5.98 (H-1) was correlated with that of H-3_{ax} and the signal at δ 5.02 (H-9) was correlated with that of H-15. The signal at δ 5.66 (H-6) was correlated with those of H-14/H-15. Therefore, H-1, H-2, and H-9 were assigned as α-axial, α-equatorial, and β-equatorial, respectively. The HMBC spectrum demonstrated that two acetates were respectively bound at C-1 and C-2 and two benzoates at C-6 and C-9, respectively. From these facts, the structure of **1** was elucidated as 1β,2β-diacetoxy-6α,9α-bis(benzoyloxy)dihydro-β-agarofuran and was given the trivial name of orbiculin A. Compounds **2** and **3** were identified as celafolin A-1 and celorbicol ester, repectively, by comparison of their physical and spectroscopic data with literature report.[11]

The cytotoxicities of **1**−**3** were measured in both drug-sensitive KB-3-1 and MCF7 cells and multidrug-resistant KB-V1 and MCF7/ADR cells. As shown in Table 2, all the compounds exhibited weak cytotoxic activity. They showed no discernible difference in the cytotoxic activity between sensitive and resistant cells. However, in the presence of 100 nM of VLB or 10 μM of ADR, IC_{50} values of **1**, **2** and **3** for KB-V1 and MCF7/ADR cells were remarkably reduced to the extents of 10−100 fold. The concentrations of VLB and ADR added were lethal to drug sensitive KB-3-1 and MCF7 cells, but had no effect on the growth of drug-resistant KB-V1 and MCF7/ADR cells. These results clearly demonstrated that **1**, **2**, and **3** reversed MDR in KB-V1 and MCF7/ADR cells to the level of sensitive cells. The compounds were also tested for cytotoxic activity against various human cancer cells including the SNB-19, SK-OV-3, NCI-H23, UACC-62, KM-12, and MOLT cell lines. The IC_{50} values were found to be in the range of those for KB and MCF7 cells except for MOLT cells, which showed 5−8-fold more sensitivity than the others.

To further test the MDR reversing activity of compound **1**, both drug-resistant and the parent cells were treated with various concentrations of ADR, VLB, and TX in the presence of 0, 1, 3, and 10 μM of **1**. As shown in Figure 1, compound **1** did not influence the sensitivity of KB-3-1 and MCF7 cells to ADR, VLB, and TX, even at the concentration of 10 μM. On the other hand, KB-V1 and MCF7/ADR cells became sensitive to ADR, VLB, and TX in the presence of **1** in dose-dependent manners. Compared to KB-3-1 cells, KB-V1 cells were 648-fold more resistant to ADR, 1017-fold to VLB, and 2217-fold to TX. When KB-V1 cells were treated with various concentrations of ADR, VLB, or TX in the presence of 10 μM of **1**, the sensitivities of KB-V1 cells to each drug were completely restored to the level of drug-sensitive KB-3-1 cells. The relative resistance (RR) of KB-V1 cells to KB-3-1 was only 1.11 for ADR, 1.60 for VLB, and 0.95 for TX in the presence of 10 μM of **1**. Similar results were also obtained in experiments with MCF7 and MCF7/ADR cells.

The MDR reversing effects of compounds **2** and **3** on the resistant cells were also determined by a similar method (data not shown). The relative resistance of KB-V1 to KB-3-1 was reduced to 15.59-fold for ADR, 20.99 for VLB, and 10.66 for TX in the presence of 3 μM **2** and 98.78 for ADR, 63.92 for VLB, and 37.15 for TX in the presence of 3 μM **3**. In a parallel experiment with 3 μM verapamil, the level of relative resistance were 58.92-fold for ADR, 81.0 for VLB, and 82.25 for TX. These results demonstrated that compounds **1**, **2**, and **3** had more potent MDR reversing activity than verapamil.

With regard to the structures of sesquiterpene esters, the tetraesterified compound **1** was less toxic and showed more potent MDR reversing activity than the triesterified compounds **2** and **3**. It would be of interest to compare the MDR reversing activity of various dihydro-β-agarofuran sesquiterpene esters according to the number of esterification, sites of esters, and different ester moieties.

Most MDR inhibitors are known to interact with P-gp, thereby inhibiting the efflux of anticancer drugs. They share some physicochemical characteristics such as hydrophobicity, a conjugated planar ring, and a substituted tertiary amino group.[2] Recently, a new class of compounds, scytophycins, which do not share common features such as a conjugated planar ring and a tertiary amino group with MDR revertants, have also been

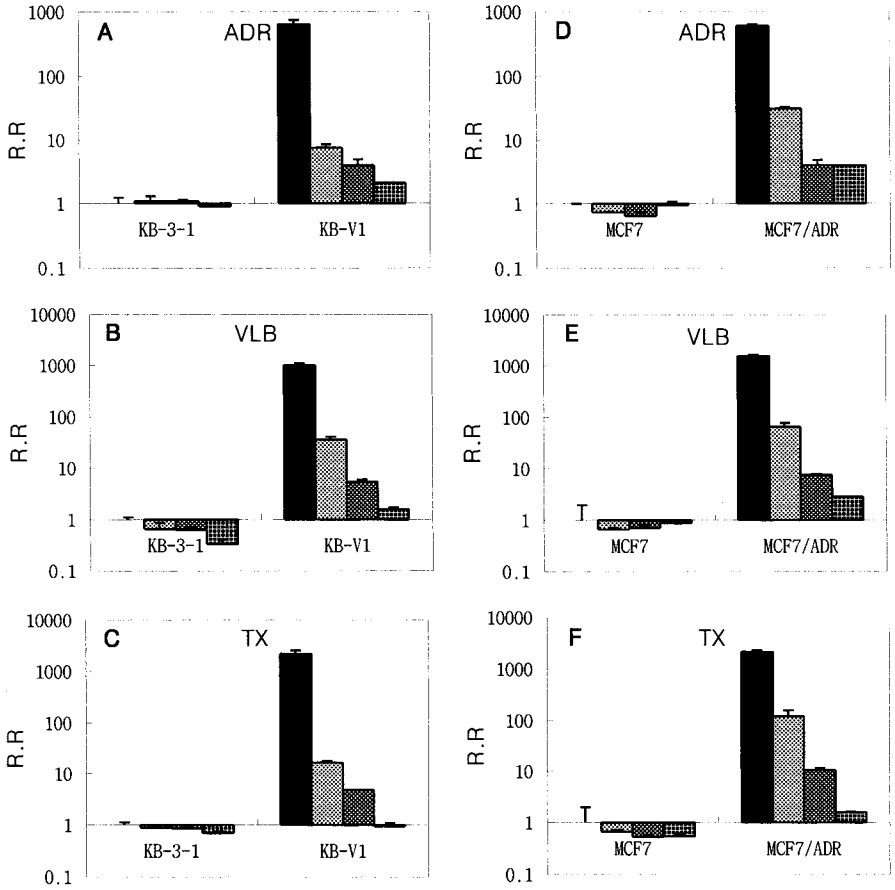

Figure 1. Effects of compound **1** on the multidrug resistance. Drug-sensitive and -resistant cells were treated with various concentrations of ADR, VLB, and TX in the presence of 0 (solid), 1 (dotted), 3 (cross-hatched), and 10 (grid) μM of compound **1**. Cell growth was measured, and RR was calculated as described in the Experimental Section. A−C: KB cells. D−F: MCF cells. Bars: mean ± SD of triplicate assays.

reported as MDR revertants.[12] Dihydro-β-agarofuran derivatives do not have the common structural elements but share hydrophobicity. However, considering the potent effect of **1** on reversing MDR in different cell types and to various anticancer drugs, the dihydro-β-agarofuran sesquiterpene esters appear to be promising leads to the development of MDR reversing agents.

Experimental Section

General Experimental Procedures. UV spectra were obtained on a Milton Roy 3000 spectronic array. IR spectra were run as KBr disks on a Laser Precision Analytical RFX-65 FT-IR. ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, and ^{1}H−^{13}C COSY spectra were obtained on a Varian Unity 300 MHz (^{1}H NMR) and 75 MHz (^{13}C NMR), using $CDCl_3$ as a solvent. ^{1}H NMR and ^{13}C NMR spectra were also obtained on Bruker 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) spectrometers using pyridine-d_5. HMBC and NOESY spectra were determined on a Bruker at 500 MHz. EIMS were measured on a Hewlett-Packard 5989A, HRFABMS on JEOL HX 110 mass spectrometer. Melting points were measured on an Electrothermal Model 9100 without correction. Optical rotations were determined on a JASCO DIP-181 polarimeter. Si gel 60 (Merck) and C-18 (EM) were used for column chromatography. Preparative HPLC was carried out on a DELTA-PAK C18 (∅ 19 mm × 300 mm, Waters) with detection at 230 nm. Fetal calf serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL (Grand Island, NY). The anticancer drugs adriamycin (ADR), vinblastine (VLB), and paclitaxel (TX) were obtained from the Sigma Chemical Co. (St. Louis, MO).

Extraction and Isolation. Roots of *C. orbiculatus* were collected at Cheongju, Chungbuk province, in Korea and identified by Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen is deposited in our institute. Air-dried chopped roots (4.5 kg) were extracted with MeOH at room temperature. The extract (300 g) was concentrated, diluted in H_2O, and extracted with CH_2Cl_2. The CH_2Cl_2 layer (73 g) was chromatographed on a Si gel column using CH_2Cl_2−MeOH (19:1, 9:1, 4:1, 1:1) as eluent to give four fractions. Fraction 2 (12.5 g), which showed strong MDR reversing effect in the KB-V1 cells was subjected to Si gel [eluent: hexane−EtOAc (5:1)] and RP-18 column chromatography (eluent; 75−80% MeOH), successively. Compound **1** (14 mg) from fraction 11 (400 mg) and compounds **2** (16 mg) and **3** (18 mg) from fraction 9 (700 mg) were obtained by preparative HPLC separation (solvent; 70−75% MeOH).

Compound 1 white amorphous powder; mp 122−125 °C; $[\alpha]^{25}_D$ +9.52 (*c* 0.21, MeOH); UV (MeOH) λ max (log ϵ) 202 (4.25), 231 (4.42), 275 (3.29), 281 (3.22) nm; IR (KBr) ν max 2969, 2929, 1747 (C=O, ester), 1716 (C=O, ester), 1602, 1452, 1274, 1247, 1099, 1022, 713 cm^{-1}; EIMS *m/z* $[M]^+$ 578 (1), 352 $[M + H - C_6H_5CO - C_6H_5CO_2H]^+$(8), 292 $[M + H - C_6H_5CO - C_6H_5CO_2H - C_6H_5$-

$CO_2H]^+$(6), 237 (12), 175 (18), 105 $[C_6H_5CO]^+$(100), 77 $[C_6H_5]^+$(39); HRFABMS *m/z* 578.2532 (calcd for $C_{33}H_{38}O_9$, 578.2515); ^{1}H NMR and ^{13}C NMR ($CDCl_3$, 300 MHz and 75 MHz) see Table 1; ^{1}H NMR (pyridine-d_5, 500 MHz) δ 1.30 (3H, d, J = 7.6 Hz, H-14), 1.54 (3H, s, H-12), 1.57 (3H, s, H-13), 1.61 (3H, s, H-15), 1.74 (3H, s, 1-CH_3CO), 1.99 (3H, s, 2- CH_3CO), 2.29 (1H, dd, J = 16.3, 3.0 Hz, H-8_{eq}), 2.44 (1H, m, H-7), 2.52 (1H, m, H-3_{ax}), 2.61 (1H, m, H-4), 2.65 (1H, m, H-8_{ax}), 5.34 (1H, d, J = 6.8 Hz, H-9), 5.93 (1H, s, H-6), 5.98 (1H, d, J = 3.3 Hz, H-1), 6.01 (1H, m, H-2), 8.34 (4H, m, benzoyl-*o*-H), 7.41−7.66 (6H, m, benzoyl-*m*- and *p*-H); ^{13}C NMR (pyridine-d_5, 125 MHz) δ 71.82 (C-1), 69.93 (C-2), 31.43 (C-3), 34.46 (C-4), 90.35 (C-5), 80.06 (C-6), 49.29 (C-7), 31.90 (C-8), 73.54 (C-9), 50.39 (C-10), 83.32 (C-11), 26.13 (C-12), 30.98 (C-13), 18.75 (C-14), 20.52 (C-15), benzoyl esters [δ 165.77, 165.59, 128.78, 129.37, 129.99, 130.33, 130.47, 130.62, 133.62, 133.96], acetyl esters [δ 169.85, 170.18, 21.02, 20.05]

Cell Lines and Cell Culture. Human oral epidermal cancer cell line KB-3-1 and its VLB selected multidrug-resistant KB-V1 cell line were obtained from M. Gottesman (NCI, MD). KB-3-1 and KB-V1 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 2 mM L-glutamine and 10% heat-inactivated fetal calf serum and split twice a week at 1:16 and 1:8 ratios, respectively. KB-V1 cells were maintained in the presence of 1 μM VLB. Human breast cancer MCF7 and MCF7/ADR cell lines were supplied by D. Newman (NCI, MD). SNB-19, SK-OV-3, NCI-H23, UACC-62, KM-12, and MOLT cell lines were also obtained from D. Newman. They were cultured in RPMI1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine and transferred twice a week by diluting at 1:8 or 1:16 split in fresh media. MCF7/ADR cells maintain resistance to ADR for 20 passages in the absence of ADR. All cells were grown at 37 °C in humidified atmosphere with 5% CO_2.

***In Vitro* Drug Sensitivity.** Cell growth was measured using the SRB method.[13] Cells in exponential growth were trypsinized, dispersed in a single cell suspension, and dispensed in 100 μL volumes into 96-well plates. Correlations between cell growth and spectroscopic absorbance were performed for each cell line, and the initial cell plating density and optimal assay conditions were chosen to ensure a linear relationship between cell number and absorbance. For in vitro assay, 5×10^3 MCF7, 1×10^4 MCF7/ADR, 2.5×10^3 KB-3-1, 5×10^3 KB-V1, 1×10^4 SNB-19, 8×10^3 SK-OV-3, 1×10^4 NCI-H23, 8×10^3 UACC-62, 1×10^4 KM-12, and 1×10^4 MOLT cells/well were inoculated in 100 μL medium containing 5% fetal calf serum and allowed to attach and grow overnight. One hundred microliters of medium containing anticancer drug and/or reversing compound were added and further incubated for 48 h. Drugs were dissolved in small amounts of DMSO or MeOH before dilution with the medium (final concentration of solvent < 0.5%). Controls were exposed to vehicle-containing medium. Cells were fixed by gently layering 50 μL of cold 50% trichloroacetic acid (final concentrations 10%) on the top of the growth medium in each well and incubated at 4 °C for 1 h and then washed five times with tap water. Plates were air-dried, stained with 0.4% (w/v) sulforhodamine B in 1% acetic acid for 15−30 min, and rinsed four times with 1% acetic acid to remove unbound dye. Plates were air-dried, and bound dye was solubilized with 10 mM unbuffered Tris base on a shaker for 5 min. Absorbance was read with a microtiter plate reader sat at 570 nm. IC_{50} was defined as the concentration of each drug that reduced absorbance to 50% of vehicle-treated controls.

MDR Reversing Activity. The effects of compounds on MDR were studied by exposing the cells to a range of concentrations of anticancer drugs in the absence or presence of MDR reversing compounds. The MDR of resistant cells to various anticancer drugs and the MDR reversing effect of each compound were expressed as relative resistance (RR)

$$\mathrm{RR} = \frac{IC_{50} \text{ of drug resistant cells or sensitive cells}}{IC_{50} \text{ of sensitive parent cells}}$$

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