Novel Cytotoxic Phenanthrenequinone from *Odontioda* Marie Noel 'Velano'

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A new phenanthrenequinone, 5-hydroxy-2,3-dimethoxy-1,4-phenanthrenequinone (1), was isolated along with a known 9,10-dihydrophenanthrenequinone, ephemeranthoquinone B (2) from an MeOH extract of *Odontioda* Marie Noel 'Velano' through bioassay-guided fractionation. Their structures were elucidated by spectroscopic analysis, and the compounds were tested for *in vitro* cytotoxic activity. The compounds showed slightly higher cytotoxicity in human oral squamous cell carcinoma and leukemic cell lines as compared with human oral normal cells. The results suggest that apoptosis may not be involved in the cytotoxicity induction.

Key words *Odontioda* Marie Noel 'Velano'; phenanthrenequinone; cytotoxicity

The Orchidaceae family is widely considered to be the largest plant family in the world with over 800 genera and at least 24000 species.[1)] Various pharmacologically active components have been isolated from some orchid species. During our cytotoxicity screening of extracts from some orchid plants, we found that an EtOAc soluble fraction of a methanolic extract of *Odontioda* Marie Noel 'Velano' demonstrated significant cytotoxicity in some human oral cavity cancer cell lines as compared with human normal oral cells.[2,3)] *Odontioda* is an intergeneric hybrid of *Odontoglossum* and *Cochilida*.[4)] *O.* Marie Noel 'Velano' is mainly cultivated as an ornamental plant. No phytochemical study of this plant has been reported to date. The present report describes the isolation, structure elucidation and evaluation of the cytotoxic potential of a new phenanthrenequinone (**1**) and a known 9,10-dihydrophenanthrenequinone, ephemeranthoquinone B[5)] (**2**) isolated from the dried bulb of *O.* Marie Noel 'Velano.'

Air-dried bulbs of *O.* Marie Noel 'Velano' were extracted with MeOH. The MeOH extract was evaporated and resolved in aqueous methanol and then partitioned with *n*-hexane, EtOAc, and *n*-BuOH to give the respective fractions as described previously.[2)] The cytotoxicity of each soluble fraction was evaluated in a human oral squamous cell carcinoma cell line.

The EtOAc soluble cytotoxic fraction was further fractionated on a silica gel column and the elution with $CHCl_3$–MeOH provided nineteen fractions (Frs. 1–19). Further chromatographic fractionation of Frs. 5 and 6 allowed the isolation of a new phenanthrenequinone (**1**) and ephemeranthoquinone B (**2**), respectively (Fig. 1). Their structures were elucidated by extensive analysis of ^{1}H-, ^{13}C-NMR and two dimensional (2D)-NMR spectra including correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond coherence (HMBC) experiments. The known compound (**2**) was identified by the comparison of the spectral data with those reported.[5)]

Compound **1** was obtained as a red solid and high resolution (HR)-electro ionization (EI)-MS (*m/z* 284.0687) established the molecular formula as $C_{16}H_{12}O_5$. The UV absorption maxima at 219, 302 and 503 nm indicated the presence of a typical phenanthrene derivative.[6,7)] Sixteen carbon signals, including two methoxy, five methine and nine quarternary carbons, were observed in the ^{13}C-NMR spectrum of **1**. Among the nine quarternary carbons, two were identified as carbonyl carbons on the basis of chemical shifts at δ 181.9 and δ 188.4. Therefore, **1** was postulated to be a phenanthrenequinone. In the ^{1}H-NMR spectrum, the signals due to three adjacent aromatic protons at δ 7.25 (overlapped with solvent signal), δ 7.56 (1H, t, J=7.8 Hz) and δ 7.42 (1H, dd, J=1.5, 7.8 Hz) were observed and attributed to H-6, H-7 and H-8, respectively. A pair of *ortho*-coupled aromatic protons at δ 8.12 (1H, d, J=8.5 Hz) and δ 8.15 (1H, d, J=8.5 Hz) were assigned to H-9 and H-10. Furthermore, the ^{1}H-NMR spectrum showed signals for two methoxyl functions (δ 4.10 and δ 4.20) and a hydroxyl group (δ 11.30). Chelation of a hydroxyl proton with a carbonyl function through a hydrogen bond might have caused the appearance of a hydroxyl proton signal in the lower field (δ 11.30). Therefore, the hydroxyl group should be located at the C-5 position. In the HMBC spectrum of **1**, methoxy protons at δ 4.10 (3H, s) and δ 4.20 (3H, s) showed interactions with carbons at δ 145.5 and δ 147.1, respectively (Fig. 2). Thus, these methoxy groups were connected to the C-2 and C-3 positions. The ^{13}C resonances at C-2 and C-3 could not be distinguished unambiguously due to the heavily substituted nature of the aromatic ring and the absence of long range correlations. Therefore, these oxygenated quarternary carbons were assigned by comparison with previously reported data[8)] shown in Table 1. The new compound **1** was therefore elucidated as 5-hydroxy-2,3-dimethoxy-1,4-phenanthrenequinone.

Oral and oropharyngeal squamous cell carcinoma represents a large, worldwide health burden with approximately 350000 cases diagnosed annually.[9)] Most oral cancers are diagnosed at an advanced stage with already being spread to the lymph nodes located under the jaw and in the neck. Due to the late

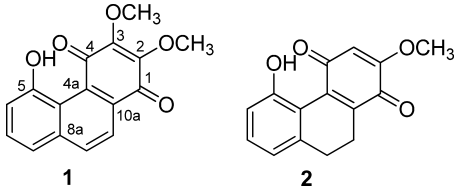

Fig. 1. Structures of Compounds **1** and **2** from *Odontioda* Marie Noel 'Velano'

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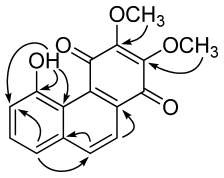

Fig. 2. Selected HMBC Correlations of Compound **1**

diagnosis of this disease, the 5-year survival rate of the patients has remained at approximately 50% during the past 30 years because of the weak activity of recently used anticancer drugs. For squamous cell carcinoma and most other types of oral cancer, surgery and radiation remain as the sole therapy.[10] Moreover, surgery for oral cancers can be disfiguring and psychologically traumatic. Therefore, the development of new treatment strategies and the early diagnosis of oral cancer are of great importance.[11] Therefore, we have been searching for new lead compounds derived from natural sources to treat oral cancer.

The extracts were subjected to cytotoxicity tests using the following cell lines; human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4), human promyelocytic leukemia HL-60 cells and normal human oral cells (gingival fibroblast (HGF), pulp cells (HPC), and periodontal ligament fibroblast (HPLF)).

The cytotoxicity in both human normal and tumor cells increased during the purification step (Table 2). Both compounds **1** and **2** showed approximately one order higher cytotoxicity as compared with the original MeOH extract and EtOAc extract. It should be noted that the tumor-specificity of compound **1** (TS=2.3) was higher than that of the MeOH extract (TS=1.3) and EtOAc extract (TS=2.1) (Table 2).

HL-60 cells were found to be the most sensitive to **2** (50% cytotoxic concentration (CC_{50})=3.0 μM), followed by HSC-2 (CC_{50}=15.4 μM), HSC-3 (CC_{50}=23.1 μM), and HSC-4 cells (CC_{50}=26.2 μM) (Table 2). Human normal oral cells (HGF, HPC, HPLF) showed relatively lower sensitivity (CC_{50}=23.0, 38.3, 39.6 μM, respectively), yielding the TS value of 2.0.

HL-60 cells again showed the highest sensitivity against **1** (CC_{50}=4.7 μM), followed by HSC-3 (CC_{50}=25.8 μM), HSC-2 (CC_{50}=31.9 μM), and HSC-4 cells (CC_{50}=34.8 μM) (Table 2). Normal oral cells (HGF, HPC, HPLF) were less sensitive (CC_{50}=36.3, 72.5, 59.4 μM, respectively), yielding the TS value of 2.3. **2** was more cytotoxic than **1** in all the cells investigated (Table 2). The present study demonstrated that the cytotoxicity of **1** and **2** in HL-60 cells (CC_{50}=4.7, 3.0 μM) was comparable or higher than that of flavonoids (5–31 μM), stilben (6–810 μM),[12] and phenanthrenes (17–55.8 μM).[13,14]

So far the mechanism of cytotoxicity induced by **1** and **2** remains unknown. A DNA fragmentation assay was performed at the initial stage to determine whether the test compounds may induce apoptotic cell death (Fig. 3). Both compounds failed to induce internucleosomal DNA fragmentation, a biochemical marker of apoptosis in HSC-2 cells, regardless of the concentration (6.5–25 μM) and incubation time (24 or 48 h). These compounds occasionally induced a smear pattern of DNA fragmentation, a marker of necrosis (Fig. 3, upper panel). Similarly, **2** (4–16 μM) did not induce DNA fragmentation even in HL-60 cells, which are known to readily undergo commitment to apoptosis by many inducers,[15] regardless of incubation time (24 or 48 h). On the other hand, **1** induced marked production of large DNA fragments with only slight amounts of oligomeric DNA fragmentation patterns, especially at higher concentrations (8, 16 μM) at 24 h, and which

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) Spectral Data for Compound **1** (in $CDCl_3$)

Position	δ_C	δ_H (J in Hz)
1	181.9 (s)	
2	145.5 (s)*	
3	147.1 (s)*	
4	188.4 (s)	
4a	128.6 (s)	
4b	121.1 (s)	
5	155.0 (s)	
6	117.8 (d)	7.25[a)]
7	130.6 (d)	7.56 (1H, t, J=7.8)
8	121.5 (d)	7.42 (1H, dd, J=1.5, 7.8)
8a	138.7 (d)	
9	137.7 (d)	8.12 (1H, d, J=8.5)
10	121.7 (d)	8.15 (1H, d, J=8.5)
10a	132.4 (s)	
2-OMe	61.8 (q)**	4.10 (3H, s)***
3-OMe	61.3 (q)**	4.20 (3H, s)***
5-OH		11.30 (1H, s)

a) Overlapped with solvent signal. *,**,*** Maybe interchanged.

Table 2. Cytotoxicity of **1** and **2** Isolated from *Odontioda* Marie Novel 'Velano'

	CC_{50} (μM)							TS
	Tumor cell lines				Normal cell lines			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	
1	31.9±1.1	25.8±5.2	34.8±3.7	4.7±0.5	36.3±1.9	72.5±1.9	59.4±11.3	2.3
2	15.4±2.3	23.1±1.3	26.2±1.2	3.0±1.0	23.0±1.4	38.3±0.5	39.6±1.1	2.0
	CC_{50} (μg/mL)							
MeOH extract	>246±7	>221±32	188±9	106±29	>243±13	>239±13	>243±12	1.3
EtOAc fraction	106±39	95.1±39.9	45.1±8.9	35.0±20.8	142±84	133±80	166±59	2.1
1	9.1±0.3	7.3±1.5	9.9±1.1	1.3±0.1	10.3±0.5	21.1±0.5	16.9±3.2	2.3
2	4.0±0.6	6.0±0.3	6.8±0.3	0.8±0	5.9±0.4	9.9±0.1	10.2±0.3	2.0

CC_{50}, 50% cytotoxic concentration; TS, tumor-specificity; HGF, human gingival fibroblast; HPC, human pulp cells; HPLF, human periodontal ligament fibroblast.

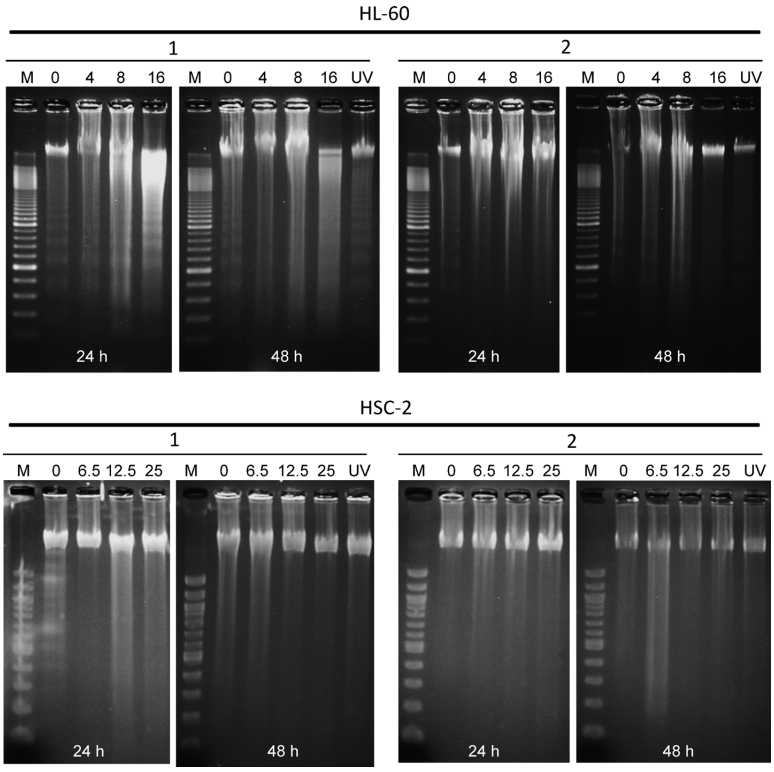

Fig. 3. Induction of DNA Fragmentation by **1** and **2** in HL-60 and HSC-2 Cells

changed into a smear pattern at 48 h (Fig. 3, lower panel). These results suggest that apoptosis may not be involved in the cytotoxicity induced by **1** and **2**. An inability to induce apoptosis may be related to the structure. We have previously reported that compounds with the backbone structures of α,β-unsaturated ketones[16] and α-hydroxy ketones[17] induced autophagic cell death. It remains to be determined whether **1** and **2**, which have one and two ketone groups, respectively, induce autophagic cell death.

Experimental

General Experimental Procedures Silica gel column chromatography was performed on silica gel 60 (Merck, Germany). UV spectra were measured using a Molecular Devices SpectraMax M5^e. IR spectra were recorded on a Shimadzu FTIR-8200PC. NMR spectra were obtained on a JEOL AL400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. EI-MS was recorded on a JEOL JMN700 spectrometer.

Plant Material Bulbs of *O.* Marie Noel 'Velano' (Orchidaceae) were supplied by the Orchid Garden Co., Ltd., Nagano Prefecture, Japan, in April 2009. This specimen was verified and identified by Mr. H. Sumiyoshi (Orchid Garden Co., Ltd.) and a voucher specimen (#20090617) was deposited in the Medicinal Plant Garden of Josai University.

Extraction and Isolation The air-dried bulbs (553 g) of *O.* Marie Noel 'Velano' were extracted with MeOH three times under reflux for 3 h. The methanolic extract (48.0 g) was fractioned by sequential organic solvent extraction as described previously.[2] The EtOAc soluble portion (6.3 g) was chromatographed on a silica gel column by gradient elution with a chloroform–methanol mixture to afford 19 fractions (Frs. 1–19). Fraction 5 (25.8 mg) was rechromatographed on silica gel and eluted with a benzene-acetone mixture to yield **1** (4.8 mg). Fraction 6 (698.4 mg) was fractionated with silica gel open column chromatography eluting with a hexane–EtOAc mixture to give **2** (6.8 mg).

Compound **1**: Amorphous solid; UV λ_{max} (MeOH) nm (logε): 219 (3.93), 302 (3.74), 503 (2.08). IR (KBr) cm^{-1}: 3417, 2916, 2850, 1667, 1645, 1583, 1513, 1462, 1427, 1260. ^{1}H- and ^{13}C-NMR ($CDCl_3$): Table 1. EI-MS (70 eV) *m/z*: 284 $[M]^+$ (100), 239 (16), 213 (25), 197 (14), 170 (15), 142 (12), 114 (17). HR-EI-MS (70 eV) *m/z*: 284.0687 $[M]^+$ (Calcd for $C_{16}H_{12}O_5$: 284.0685).

Assay for Cytotoxic Activity The cells (3×10^3 cells/well, 0.1 mL/well) were seeded into 96-microwell plates (Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) and incubated for 48 h to allow cell attachment. Near-confluent cells were treated for 48 h with different concentrations of the test compounds in fresh medium. The relative viable cell number of adherent cells (except for HL-60 cells) was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[2] In brief, control and sample-treated cells were incubated for 4 h with 0.2 mg/mL of MTT in the culture medium. After removing the medium, the reaction product, formazan, was extracted with dimethyl sulfoxide and the absorbance (the relative viable cell number) was measured at 540 nm by a microplate reader (Multiskan Bichromatic Labsystems, Helsinki, Finland). The viability of the suspended cells, *i.e.* HL-60, was determined by cell counting with a hemocytometer after staining with 0.15% trypan blue. The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve. The tumor-specificity index (TS) was calculated by the following equation: TS=mean CC_{50} (normal cells)/mean CC_{50} (all tumor cell lines).

Assay for DNA Fragmentation The cells were lysed with 50 μL lysate buffer [50 mM Tris–HCl (pH 7.8), 10 mM

ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. The lysate was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1 h at 50°C, and then mixed with 50 μL NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris–HCl, pH 8.0], and 250 μL of ethanol. After centrifugation for 20 min at 15000×$\boldsymbol{g}$, the precipitate was washed with 1 mL of 70% ethanol. After centrifugation for 5 min at 15000×$\boldsymbol{g}$, the precipitate was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 3–5). The samples (10–20 μL) were applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris–HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA molecular marker (Takara Bio Inc., Tokyo, Japan) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min), followed by 3 h incubation, were used for calibration. The DNA fragmentation pattern was examined in a photograph taken under UV illumination.

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