Two Phenanthraquinones from *Dendrobium moniliforme*

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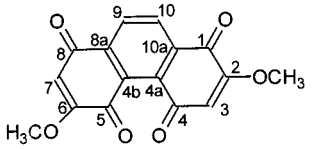
Two phenanthraquinones were isolated from the stems of *Dendrobium moniliforme*. They were identified by interpretation of spectral data as 2,6-dimethoxy-1,4,5,8-phenanthradiquinone and 5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone, named moniliformin (**1**) and denbinobin (**2**), respectively. This is the first report of compound **1**, which possesses a novel 1,4,5,8-diquinone moiety in the phenanthraquinone skeleton. Compound **2** showed potent antiinflammatory effects in vitro.

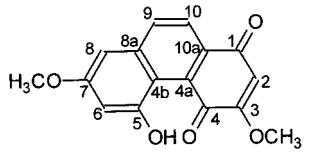
The genus *Dendrobium* (Orchidaceae) includes about 1600 species in the world,[1] 15 of which are found in Taiwan.[2] Its major natural distribution regions extend from Japan, Korea, China, and Taiwan through the Indo-Malayan region and Indonesia to New Guinea and Australia. *Dendrobium* species are important for their wide variety of medicinal properties.[3,4]

In the course of our studies on the Taiwanese Ochidaceae plants, we earlier reported that the *n*-hexane extract of the stems of *Dendrobium moniliforme* (L.) Sw. contained 13 compounds, including denbinobin (**2**), a number of aromatics, long chain fatty acids and esters, and phytosterols.[5]



1

Herein, we describe the isolation and structural determination of a new phenanthradiquinone, moniliformin (**1**), as well as the in vitro antiinflammatory activity of denbinobin (**2**).



2

Compound **1**, obtained as yellow gum, was determined to have the molecular formula $C_{16}H_{10}O_6$ by HREIMS. IR absorptions at 1694 and 1653 supported the presence of a chelated quinone, and absorptions at 1598, 1537, and 1469 cm^{-1} as well as UV absorptions at 273, 329, and 445 nm indicated an aromatic system. ^{1}H NMR data (Table 1) indicated the presence of two methoxyl groups at δ 3.91 and 3.94, two aromatic protons at δ 8.37 (1H, d, J = 8.4 Hz) and 8.41 (1H, d, J = 8.4 Hz) for H-9 and H-10, and two olefinic protons at δ 6.16 (1H, s) and 6.32 (1H, s) for H-7 and H-3, respectively. Signals for two methoxyl groups (56.6, 56.8 ppm), four methines (108.2, 111.8, 129.7, and 130.7 ppm), and 10 quaternary carbons (133.2, 134.5, 135.0, 137.2, 158.9, 163.0, 178.6, 179.3, 182.5, and 182.5 ppm) were evident in the ^{13}C NMR and DEPT spectra (Table 1).

Locations of the methoxyl groups were established by difference NOE experiments. Irradiation of the methine protons at δ 6.16 (H-7) and 6.32 (H-3) gave an NOE of the methoxyl groups at δ 3.94 (6-OCH_3) and 3.91 (2-OCH_3), respectively. Detailed analyses of ^{1}H and ^{13}C NMR spectra with HMQC and HMBC spectra clarified the connectivities of the structure. As shown in Table 1, the two olefinic protons at δ 6.32 (H-3) and 6.16 (H-7) showed long-range correlations with the carbons at δ 134.5 (C-4a), 158.9 (C-2), 178.6 (C-1), 182.5 (C-4) and δ 137.2 (C-8a), 163.0 (C-6), 179.3 (C-5), 182.5 (C-8). Two aromatic protons at δ 8.37 (H-9) and 8.41 (H-10) showed long-range correlations with the carbons at δ 133.2 (C-4b), 135.0 (C-10a), 182.5 (C-8) and δ 134.5 (C-4a), 137.2 (C-8a), 178.6 (C-1). Two methoxyl protons at δ 3.91 (2-OCH_3) and 3.94 (6-OCH_3) showed long-range correlations with the carbons at δ 158.9 (C-2) and 163.0 (C-6). From the above data, the structure of the novel natural product **1**, named moniliformin, was elucidated as 2,6-dimethoxy-1,4,5,8-phenanthradiquinone.

Denbinobin (**2**) was obtained as a dark green amorphous powder. The ^{1}H and ^{13}C NMR data (Table 1) of **2** agree with those in the literature.[5−8] This compound has also been previously isolated from *D. nobile*,[6] *Ephemerantha lonchophylla*,[7] and *E. fimbriata*[8] and has been reported to exhibit cytotoxicity against human lung carcinoma, ovary adenocarcinoma, and promyelocytic leukemia cell lines.[9] We examined the in vitro antiinflammatory effects of denbinobin. At 1 μM, **2** significantly inhibited the formation of tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) (about 62 and 43% inhibition, respectively) in RAW 264.7 cells (mouse macrophage-like cell line) stimulated with 1 μg/mL of lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4). However, it did not affect the nitrite formation at similar concentrations in RAW 264.7 cells. In N9 cells (murine microglial cell line) stimulated with 10 ng/mL of LPS plus 10 unit/mL of interferon-γ (IFN-γ), **2** reduced TNF-α and nitrite formation (about 70 and 44% inhibition, respectively) (Table 2). The cytotoxicity was less than 10% (as measured the lactate dehydrogenase release) under the tested conditions. In addition, at 30 μM, **2** significantly attenuated the degranulation of rat peritoneal mast cells

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Table 1. NMR Data for Compound **1** (600 MHz) and **2** (300 MHz) in $CDCl_3$ [a]

	1					**2**				
				HMBC					H MBC	
position	C atom	δ^1_H (J in Hz)	δ^{13}_C	^2J	^3J	C atom	δ^1_H (J in Hz)	δ^{13}_C	^2J	^3J
1	C		178.6			C		184.3		
2	C		158.9			CH	6.16 (s)	107.3	C-1, C-3	C-4, C-10a
3	CH	6.32 (s)	111.8	C-2, C-4	C-1, C-4a	C		161.2		
4	C		182.5			C		186.5		
4a	C		134.5			C		139.9		
4b	C		133.2			C		117.2		
5	C		179.3			C		156.3		
6	C		163.0			CH	6.94 (d, 2.7)	108.6	C-5, C-7	C-4b
7	CH	6.16 (s)	108.2	C-6, C-8	C-5, C-8a	C		160.8		
8	C		182.5			CH	6.83 (d, 2.7)	101.8	C-7	C-4b, C-6, C-9
8a	C		137.2			C		128.6		
9	CH	8.37 (d, 8.4)	129.7		C-4b, C-8, C-10a	CH	8.07 (d, 8.6)	137.4		C-4b, C-8, C-10a
10	CH	8.41 (d, 8.4)	130.7	C-10a	C-1, C-4a, C-8a	CH	8.14 (d, 8.6)	122.6		C-1, C-4a, C-8a
10a	C		135.0			C		132.4		
2-OCH_3	CH_3	3.91 (s)	56.6		C-2	CH_3				
3-OCH_3	CH_3					CH_3	3.98 (s)	56.9		C-3
6-OCH_3	CH_3	3.94 (s)	56.8		C-6	CH_3				
7-OCH_3	CH_3					CH_3	3.94 (s)	55.5		C-7
5-OH							10.99 (s)		C-5	C-4b, C-6

[a] Assignments confirmed by $^1H-^1H$ COSY, HMQC, and HMBC experiments.

Table 2. Effect of Denbinobin (**2**) on the Formation of Nitrite, TNF-α, and PGE_2 in RAW 264.7 and N9 Cell Lines[a]

	RAW 264.7		N9	
	control	denbinobin (**2**)	control	denbinobin (**2**)
Nitrite (μM)	46.6 ± 0.4	40.4 ± 1.0	52.1 ± 1.4	28.8 ± 1.0[b]
TNF-α (ng/mL)	146.9 ± 8.6	56.4 ± 12.1[b]	3.5 ± 0.1	1.0 ± 0.1[b]
PGE_2 (ng/mL)	26.8 ± 3.2	15.1 ± 1.3[b]	ND	ND

[a] Cells were pretreated with DMSO (as control) or **2** (1 μM for RAW 264.7 and 3 μM for N9 cells) at 37 °C for 1 h before stimulation with 1 μg/mL of LPS or 10 ng/mL of LPS plus 10 unit/mL of IFN-γ for RAW 264.7 and N9 cells, respectively, for 24 h. Values are expressed as means ± SEM (N = 3−4). ND: not determined. [b] $P < 0.01$ as compared with the corresponding control values.

Table 3. Effect of Denbinobin (**2**) on the Release of Histamine and β-Glucuronidase in Mast Cells[a]

		release (%)	
		histamine	β-glucuronidase
control		47.6 ± 1.3	29.5 ± 1.1
denbinobin (**2**)	(30 μM)	29.1 ± 0.5[b]	17.6 ± 0.9[b]
mepacrine	(30 μM)	18.4 ± 2.4[b]	8.7 ± 3.0[b]

[a] The cell suspension was preincubated with DMSO (as control) or test drugs at 37 °C for 3 min before stimulation with 10 μg/mL of compound 48/80 for 15 min. Values are expressed as means ± SEM (N = 3−4). [b] $P < 0.01$ as compared with the corresponding control values.

stimulated with a potent secretagogue, compound 48/80 at 10 μg/mL (about 39 and 40% inhibition of the histamine content and the β-glucuronidase activity, respectively), as shown in Table 3. In contrast, **2** had no inhibitory effects on the degranulation and superoxide anion generation of rat neutrophils (data not shown). The dried stem of *D. moniliforme*, known as Shi-Hu in Chinese medicine, has for centuries been used to relieve the common cold, fever, and heat-stroke.[3,4] The present study suggests that denbinobin (**2**) may have benefit in controlling central and peripheral inflammation, but this remains to be investigated.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanaco micromelting point apparatus MP-500D and were uncorrected. IR spectra were obtained on a Nicolet Impact 400 FT-IR spectrometer, and UV spectra were recorded on a Shimadzu UV-160A (UV−visible recording spectrometer). Mass spectra were taken on a JEOL JMS-SX/SX 102A tandem mass spectrometer. HREIMS were measured on a JEOL JMS-HX 110 mass spectrometer. ^1H and ^{13}C NMR spectra were run on either a Varian VXR-300 FT NMR or a Varian INOVA 600 NMR spectrometer. Chemical shifts are given in δ-values, and coupling constants (J) are given in hertz (Hz). Si gel 60 (Merck, 70−230 mesh) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) were used for column chromatography. Precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical TLC, and precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.50 mm) were employed for preparative TLC.

Plant Material. The stems of *D. moniliforme* were collected in Nantou, Taiwan, during July 1997. The plant was identified by Professor Chung-Chuan Chen of the Institute of Chinese Pharmaceutical Sciences, China Medical College, Taichung, Taiwan. A voucher specimen (CMC-1901-DM-1) is deposited in the Herbarium of the Institute of Chinese Pharmaceuitical Sciences.

Extraction and Isolation. The air-dried stems of *D. moniliforme* were chopped into small pieces (0.33 kg) and extracted sequentially with *n*-hexane (3.5 L × 6), ethyl acetate (3.5 L × 7), and methanol (3.5 L) at room temperature. The ethyl acetate extract (7.05 g) was chromatographed on a Si gel 60 (150 g) column and eluted with a step gradient of *n*-hexanes−ethyl acetate (8:2, 7:3, 6:4, 5:5, 3:7, 1:9, and 100% ethyl acetate) to yield 15 fractions. The fractions 6 and 7 (120 mg) obtained with *n*-hexanes−ethyl acetate (6:4) were further separated by Sephadex LH-20 (chloroform−methanol, 1:1) and Si gel column (benzene−ethyl acetate, 4:1) followed by preparative TLC (benzene−ethyl acetate, 4:1) to afford 1.5 mg of **1** (R_f = 0.30, benzene−ethyl acetate, 4:1) and 7.5 mg of **2** (R_f = 0.66, benzene−ethyl acetate, 4:1), respectively.

Moniliformin (1): yellow gum; UV (CH_3OH) λ_{max} (log ϵ) 273 (3.62), 329 (3.13), 445 (sh) (2.36) nm; IR (KBr) ν_{max} 2922, 2847, 1694, 1653, 1598, 1537, 1469, 1407, 1346, 1237 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$), see Table 1; EIMS m/z 298 $[M]^+$ (76), 283 (34), 255 (28), 240 (17), 214 (23), 171 (27), 128 (25), 100 (39), 87 (57), 74 (51), 69 (100), 53 (44); HREIMS m/z 298.0474 (calcd for $C_{16}H_{10}O_6$, 298.0477).

Denbinobin (2): dark green amorphous powder; UV (CH_3OH) λ_{max} (log ϵ) 237 (4.25), 310 (4.05), 406 (sh) (3.15) nm; IR (KBr) ν_{max} 3400, 2919, 2849, 1650, 1508, 1467, 1432, 1340, 1242, 1207, 1172, 1123, 1087, 863, 709 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$), see Table 1;

EIMS *m/z* 284 $[M]^+$ (100), 269 (4), 255 (2), 241 (5), 227 (9), 213 (61), 198 (3), 185 (44).

Antiinflammatory Activity of Denbinobin. Materials. Denbinobin (**2**) was dissolved in dimethyl sulfoxide (DMSO). The final volume of DMSO in the reaction mixture was ≤0.5%. RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA). N9 cells were provided by Dr. P. Ricciardi-Castagnoli (CNR Cellular and Molecular Pharmacology Center, Italy). All chemicals were obtained from Sigma (St. Louis, MO) except for the following: dextran T-500 and PGE_2 enzyme immunoassay (EIA) kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK); Hank's balanced salt solution, Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle medium, and fetal bovine serum were obtained from Gibco Life Technologies (Gaithersburg, MD); mouse IFN-γ, mouse recombinant TNF-α and capture, and biotinylated antibodies against mouse TNF-α were purchased from R&D Systems (Minneapolis, MN).

Cell Cultures and Drugs Treatment. RAW 264.7 cells were plated in 96-well tissue-culture plates in Dulbecco's modified Eagle medium supplemented with 5% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 2×10^5 cells per 200 μL per well. Cells were allowed to adhere overnight. Murine microglial cell lines N9[10] were plated in 96-well tissue-culture plates in Iscove's modified Dulbecco's medium containing 2% heat-inactivated fetal bovine serum and antibiotics at 8×10^4 cells per 200 μL per well. Cells were pretreated with test drugs at 37 °C for 1 h before stimulation with LPS or LPS/IFN-γ for 24 h, and then the medium was collected and stored at −70 °C until used.

Determination of NO, TNF-α, and PGE_2. The production of NO was determined in cell medium by measuring the content of nitrite based on the Griess reaction.[11] Briefly, 40 μL of 5 mM sulfanilamide, 10 μL of 2 M HCl, and 20 μL of 40 mM naphthylethylenediamine were added sequentially to 150 μL of culture medium. After 10 min of incubation at room temperature, absorbance was measured at 550 nm in a microplate reader. A standard nitrite curve was generated in the same fashion using $NaNO_2$. TNF-α content in the medium was measured using the ELISA kit. PGE_2 content in the medium was measured using the EIA kit.

Mast Cell Degranulation. Peritoneal mast cells were isolated as previously described.[12] Briefly, heparinized Tyrode's solution was injected into the peritoneal cavity of exsanguinated rat (Sprague Dawley). After abdominal massage, the cells in the peritoneal fluid were harvested and then separated through 38% bovine serum albumin (BSA). Cells were washed and suspended in Tyrode's solution with 0.1% BSA at $(1-1.5) \times 10^6$ cells/mL. The cell suspension was preincubated with test drugs at 37 °C for 3 min. Fifteen minutes after the addition of compound 48/80, β-glucuronidase (1 mM phenolphthalein-β-D-glucuronide in 0.1 M acetic acid buffer, pH 4.5, as substrate, absorbance was monitored at 550 nm after alkalization) and histamine (0.2% *o*-phthaldialdehyde condensation in pH 12.5, fluorescence was monitored at 350/450 nm after acidification) in the supernatant were determined.[13,14] The total content was measured after treatment of the cell suspension with Triton X-100. The percentage release was determined.

Statistical Analysis. Data are presented as the means ± SEM of three to four separate experiments. Statistical analyses were performed using the least significant difference test method after analysis of variance. P values < 0.05 were considered to be significant.

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