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Phenanthrenes from *Dendrobium nobile* and their inhibition of the LPS-induced production of nitric oxide in macrophage RAW 264.7 cells

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ABSTRACT

Bioactivity-guided isolation of the methanol extract of the stems of *Dendrobium nobile* yielded a new phenanthrene together with nine known phenanthrenes and three known bibenzyls. Their structures were elucidated by analysis of the spectroscopic data including 2D-NMR. All of the isolates were evaluated for their potential to inhibit the LPS-induced production of nitric oxide in murine macrophage RAW 264.7 cells. Compounds **1**–**4**, **7**–**13** inhibited nitric oxide production with the IC_{50} values ranging from 9.6 μM to 35.7 μM.

Dendrobium nobile (Orchidaceae) is one of the most popular *Dendrobium* plants and has been used as traditional folk remedies for the treatment of various disease, such as chronic atrophic gastritis, diabetes, and cardiovascular diseases.[1,2] *Dendrobium* species have been demonstrated to produce a wide variety of potentially useful constituents that include alkaloids,[3,4] bibenzyls,[5,6] fluorenones,[7] phenanthrenes,[8,9] and sesquiterpenoids.[10–12] Recent pharmacological studies have demonstrated that some species displayed antitumor,[13] anti-angiogenic,[14] anti-platelet aggregation,[15] anti-inflammtatory,[16] and immuno-regulatory activities.[17,18]

As part of our ongoing research program for the discovery of plant-derived inhibitors of nitric oxide (NO) production, we found that the methanolic extract of the stems of *D. nobile* inhibited the production of NO in RAW 264.7 cells. A new phenanthrene along with nine known phenanthrenes and three known bibenzyls was isolated from the stems of *D. nobile*. Their structures were characterized on the basis of spectroscopic methods including extensive 2D-NMR and MS spectrometry. Furthermore, their inhibition of LPS-induced NO production in RAW 264.7 cells was also evaluated. Here, we report the isolation and structure determination in addition to the inhibitory activity of NO production of these compounds.

The air-dried *D. nobile* (2.7 kg) were extracted three times with MeOH. The combined extracts were concentrated under vacuum. The residue was suspended in 80% MeOH and then partitioned with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH, successively. The bioassay indicated that the CH_2Cl_2 extract inhibited 79.7% inhibitory effect on the production of NO at the concentration of 100 μg/mL. Accordingly, the CH_2Cl_2 extract was further separated using vacuum liquid chromatography on RP-18 (15 × 15 cm) and eluted with MeCN–H_2O (20%, 40%, 60%, 80% and 100% MeCN), to give five fractions (C1–C5). Fraction C2 (5.9 g) was subjected to silica gel (4 × 25 cm) column chromatography eluting with CH_2Cl_2:MeOH step gradient systems to obtain six fractions (C2A–C2F). Fraction C2C (0.6 g) exhibiting 92.5% inhibitory effect on the production of NO at the concentration of 100 μg/mL was rechromatographed on silica gel (3 × 20 cm) using *n*-hexane–EtOAc as a gradient elution to give eight fractions (C2B1–C2B8). The third fraction (C2B3, 20 mg) was purified by employing a silica gel column (1 × 15 cm) to obtain a new compound (**1**, 5 mg).[19] Further purification by semi-preparative HPLC (Waters system, YMC ODS H-80, 150 × 20 mm i.d., MeCN–H_2O = 40:60, flow rate 6.5 mL/min) to yield hircinol (**2**, 13 mg),[20] erianthridin (**3**, 3 mg)[21] and ephemeranthol A (**4**, 9 mg)[22] from C2B4 (110 mg), 3,4′-dihydroxy-5,5′-dimethoxydihydrostilbene (**5**, 9 mg)[21] and 3-*O*-methylgigantol (**6**, 11 mg)[22] from C2B6 (140 mg) and 5,7-dimethoxyphenanthrene-2,6-diol (**7**, 5 mg)[23] and moscatilin (**8**, 4 mg)[24] from C2B8 (35 mg). Furthermore, fraction C2E (1.1 g) exhibiting 89.8%

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inhibitory effect on the production of NO at the concentration of 100 μg/mL was applied to a silica gel column (3 × 20 cm) and eluted with a CH_2Cl_2–EtOAc gradient to yield five fractions (C2E1–C2E5). Fraction C2E4 (71 mg) was purified further by semi-preparative HPLC (Waters system, YMC ODS H-80, 150 × 20 mm i.d., MeCN–H_2O = 40:60, flow rate 6.5 mL/min) to yield coelonin (**9**, 10 mg),[23,25] flavanthridin (**10**, 8 mg)[26] and epheneranthol C (**11**, 12 mg).[27] Fraction C2E5 (75 mg) was also purified by semi-preparative HPLC (Waters system, YMC ODS H-80, 150 × 20 mm i.d., MeCN–H_2O = 40:60, flow rate 6.5 mL/min) to afford lusianthridin (**12**, 10 mg)[26,28] and fimbriol-B (**13**, 6 mg).[27]

Compound **1** was isolated as a brownish amorphous powder and gave a molecular ion peak $[M-H]^-$ at *m/z* 299.0929 (calcd for $C_{17}H_{15}O_5$, 299.0919) in the HR-ESIMS. The ^1H NMR spectrum of **1** showed two *ortho*-coupled AB systems at δ_H 7.18 and 7.01 (each 1H, d, *J* = 8.5 Hz, H-6 and H-7, respectively) and δ_H 8.15 and 7.49 (each 1H, d, *J* = 9.1 Hz, H-9 and H-10, respectively) and an isolated aromatic proton at δ_H 7.27 (1H, s, H-1). In addition, the signals for two hydroxyl groups at δ_H 6.00 (1H, br s, OH-2) and 9.69 (1H, s, OH-5) and three aromatic methoxy groups at δ_H 4.19 (3H, s, OCH_3-3), 3.99 (3H, s, OCH_3-8) and 3.79 (3H, s, OCH_3-4); δ_C 62.24 (OCH_3-3), 56.42 (OCH_3-8) and 62.60 (OCH_3-4), were observed in the ^1H and ^{13}C NMR spectra of **1** as shown Table 1. A complete assignment of protons and carbons was assisted by HMQC and HMBC experiments. HMBC correlations (Fig. 2) were observed: H-10/C-1, 4a, 8a, 10a, H-9/C-4b, 8, 10a, H-7/C-5, 8a, H-6/C-4b, 8, H-1/C-3, 4a, 10, OH-5/C-5, 6, OH-2/C-1, 4, 10a, OCH_3-3/C-3, OCH_3-4/C-4 and OCH_3-8/C-8. On the basis of the data obtained, the structure of **1** was determined as 3,4,8-trimethoxyphenanthrene-2,5-diol.

In addition to the above a new compound described, nine known phenanthrenes and three known bibenzyls were isolated. Their structures were elucidated by spectral methods, especially MS, 1D- and 2D-NMR techniques.

The NO radical, synthesized by the oxidation of L-arginine catalyzed by nitric oxide synthase, is involved in a number of physiological and pathological processes in mammals. However, excessive production of NO by iNOS in macrophages is involved in various acute and chronic inflammatory diseases.[29] Therefore, inhibitors of NO production in macrophages are an important target in the treatment of certain inflammatory diseases.

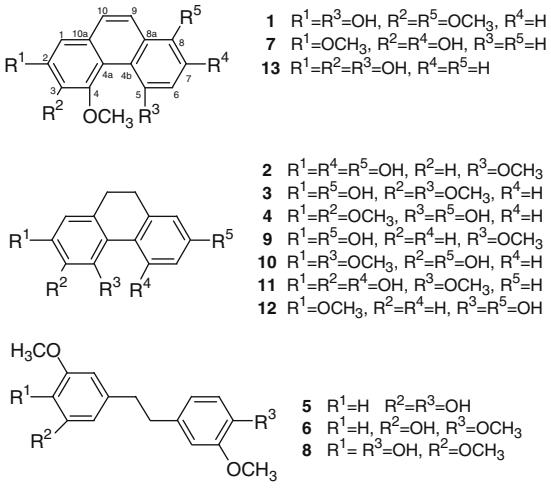

Figure 1. Structures of compounds **1–13**.

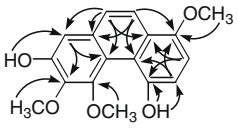

Figure 2. Selected HMBC correlations of compound **1**.

We examined the inhibitory effects of all compounds (**1–13**, Fig. 1) isolated from *D. nobile* on LPS-induced NO generation in RAW 264.7 cells.[30] The concentration required to inhibit the production of NO by 50% (IC_{50} value) was calculated on the basis of concentrations of nitrite released into the culture media by Griess method. Eleven compounds **1–4**, **7–13** inhibited NO production in LPS-stimulated RAW 264.7 cells with the IC_{50} values that ranged between 9.6 μM and 35.7 μM (Table 2). The cell viability measured by CCK-assay[31] indicated that all the compounds lacked significant cytotoxic effects. Although further investigation are needed to clarify the detailed structure–activity relationship, these results indicate that 9,10-dihydrophenanthrenes such as compounds **3**, **4**, **9**, **11**, and **12** showed relatively stronger inhibitory activity (IC_{50} values: 9.6–19.5 μM) than phenanthrenes and bibenzyls such as compounds **1**, **5–8**, and **13** (IC_{50} values: 20.4 to >50 μM).

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data of **1** in $CDCl_3$[a]

Position	δ_H multiplicity (*J* in Hz)	δ_C	HMBC
1	7.27[b] s	109.39	C-3, 4a, 10
2		148.10	
3		140.63	
4		147.53	
4a		116.84	
4b		119.12	
5		147.40	
6	7.18 d (*J* = 8.5 Hz)	115.41	C-4b, 8
7	7.02 d (*J* = 8.5 Hz)	108.11	C-5, 8a
8		149.14	
8a		124.19	
9	8.15 d (*J* = 9.1 Hz)	121.21	C-4b, 10a, 8
10	7.49 d (*J* = 9.1 Hz)	125.46	C-1, 4a, 8a, 10a
10a		131.24	
3-OCH_3	4.19 s	62.24	C-3
8-OCH_3	3.99 s	56.42	C-8
4-OCH_3	3.79 s	62.60	C-4
2-OH	6.00 br s		C-1, 4, 10a
5-OH	9.69 s		C-5, 6

[a] All assignments were made by extensive analyses of 1D and 2D-NMR (COSY, DEPT, HMQC, and HMBC).
[b] Overlapped with solvent signals.

Table 2
Inhibitory effects of the isolated compounds **1–13** on the LPS-induced NO production in RAW 264.7 cells

Compound	IC_{50} (μM)
1	20.4 ± 0.8 [a]
2	26.4 ± 0.2
3	19.5 ± 0.4
4	12.0 ± 0.3
5	>50
6	>50
7	35.7 ± 0.6
8	27.6 ± 0.5
9	10.2 ± 0.2
10	34.1 ± 0.9
11	17.6 ± 0.4
12	9.6 ± 0.3
13	28.9 ± 0.6
Aminoguanidine [b]	17.5 ± 0.3

[a] The experiments were repeated in triplicate, and the values were expressed as mean ± standard deviation.
[b] Aminoguanidine was used as a positive control.

Recently, bibenzyl derivatives and fluorenones from *D. nobile* exhibited significant inhibitory effects of NO production in macrophage cells.[7] Furthermore, phenanthrene derivatives such as phenanthraquinone and a phenanthrene derivative with a spirolactone ring from *D. nobile* have been reported to inhibit the NO production.[16,32]

According to these results and previously reported data, phenanthrene and bibenzyl derivatives isolated from *D. nobile* may have benefit in the prevention of inflammatory diseases associated with the increase of NO production. However, further studies are needed to elucidate how these active compounds inhibit NO production.

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19. *3,4,8-Trimethoxyphenanthrene-2,5-diol* (**1**): Brownish amorphous powder; mp 106.9 °C; UV (MeOH) λ_{max} (log ε) 219 (2.98), 254 (3.11) nm; IR ν_{max} 3364, 3220, 2926, 2850, 1606, 1502, 1460, 858 cm^{-1}; ^1H NMR and ^{13}C NMR data see Table 1; HR-ESIMS *m/z* 299.0929 (calcd for $C_{17}H_{15}O_5$, 299.0919).
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