Bioactive Bibenzyl Derivatives and Fluorenones from *Dendrobium nobile*

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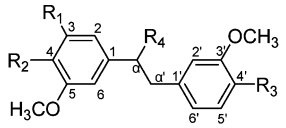
Bioassay-guided fractionation of the 60% ethanol extract of the stems of *Dendrobium nobile* using the DPPH assay led to the isolation of two new bibenzyl derivatives, nobilin D (**1**) and nobilin E (**2**), and a new fluorenone, nobilone (**3**), together with seven known compounds (**4**−**10**). Their structures were determined on the basis of spectroscopic analyses. Compounds **1**, **2**, **4**, **7**, **8**, and **10** exhibited significant antioxidant activity higher than or equivalent to vitamin C in the DPPH assay, and compounds **1**, **3**, **4**, and **7**−**10** displayed higher antioxidant activity than vitamin C in the ORAC assay. Compounds **1**, **2**, and **10** also exhibited stronger inhibitory effects on NO production than resveratrol.

Recently, much attention has been paid to reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, hydrogen peroxide, and peroxide radical, which are the intermediates of regular aerobic metabolism.[1] Excessive concentrations of ROS in the human body lead to peroxidation of membrane lipids and cellular damage of proteins and DNA and can be involved in a number of pathological events.[2,3] More than 60 kinds of diseases have been reported to be associated with ROS.[4]

The traditional Chinese medicine "Shi Hu", derived from the dried or fresh stems of several *Dendrobium* species (Orchidaceae), is widely used as both traditional Chinese and folk remedies for treatment of various diseases, such as chronic atrophic gastritis, diabetes, skin aging, and cardiovascular disease, which to a great extent are believed to be closely associated with the metabolic disorders of ROS in the human body.[5,6] *Dendrobium nobile* Lindl. is one of the most popular *Dendrobium* plants and has been recorded in the Chinese Pharmacopoeia (2005 Edition) as one of the original materials of "Shi Hu".

In our preliminary study, the EtOAc-soluble fraction of a 60% EtOH extract of the stems of *D. nobile* was found to exhibit significant antioxidant activity in the 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging assay (IC_{50} = 32 μg/mL). Bioassay-guided fractionation of the active fraction led to the isolation of two new bibenzyl derivatives, nobilin D (**1**) and nobilin E (**2**), and a new fluorenone, nobilone (**3**), along with seven known compounds (**4**−**10**). The oxygen radical absorbance capacity (ORAC) assay was also used to evaluate the antioxidant activity of the 10 compounds. In addition, all the above compounds except **6** were measured for their inhibitory effects on NO production in murine macrophages (RAW 264.7) activated by lipopolysaccharide (LPS) and interferon (IFN)-γ. No previous investigations have been reported on the antioxidant activity using the ORAC method and NO production inhibitory effects of the components obtained from the genus *Dendrobium*. In this paper, we describe the isolation and structure elucidation of three new compounds (**1**−**3**) and the biological activities of compounds **1**−**10**.



1 R_1 = OCH_3 R_2 = R_3 = R_4 = OH
4 R_1 = R_2 = OCH_3 R_3 = OH R_4 = H
5 R_1 = R_2 = R_3 = OCH_3 R_4 = H
6 R_1 = R_2 = OH R_3 = R_4 = H
7 R_1 = R_3 = OCH_3 R_2 = OH R_4 = H
8 R_1 = OCH_3 R_2 = R_3 = OH R_4 = H
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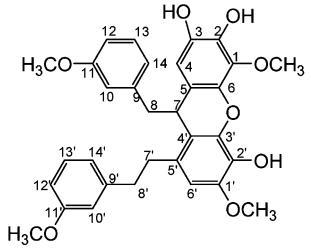

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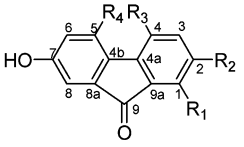

3 R_1 = R_4 = H R_2 = OH R_3 = OCH_3
10 R_1 = R_3 = OH R_2 = H R_4 = OCH_3

Results and Discussion

The 60% EtOH extract of the stems of *D. nobile* was suspended in H_2O and partitioned with EtOAc and *n*-BuOH successively. The EtOAc-soluble fraction, which showed significant antioxidant activity in the DPPH assay, was subjected to bioassay-guided fractionation with repeated column chromatography on silica gel, Sephadex LH-20, and ODS and further purified by reversed-phase HPLC to afford 10 compounds.

Compound **1** was obtained as a colorless oil, $[\alpha]^{27}_D$ +1.5 (*c* 0.6, MeOH). The HRTOFMS (*m*/*z* 321.1356, $[M + H]^+$) and NMR analyses revealed the molecular formula as $C_{17}H_{20}O_6$. The presence of phenolic group(s) in the structure was indicated by its characteristic color reaction with $FeCl_3$ (violet). The UV absorption λ_{max} (MeOH) at 281 nm (log ϵ 2.62) was indicative of a bibenzyl. The ^{1}H NMR spectrum of **1** showed resonances for two phenolic

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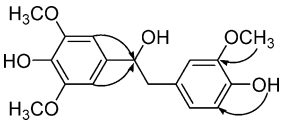

Figure 1. HMBC correlations of compound **1**.

hydroxyl protons at δ 5.47 and 5.51 (disappearing on deuterium exchange), three *O*-methyl groups at δ 3.84 (3H, s) and 3.88 (6H, s), one methine proton at δ 4.77 (1H, dd, J = 8.0, 5.5 Hz), two methylene protons at δ 2.91 (1H, dd, J = 13.8, 8.0 Hz) and 2.93 (1H, dd, J = 13.8, 5.5 Hz), and five aromatic protons, appearing as a two-proton singlet at δ 6.56 (2H, s) and an ABX system at δ 6.62 (1H, d, J = 1.7 Hz), 6.70 (1H, dd, J = 8.0, 1.7 Hz), and 6.85 (1H, d, J = 8.0 Hz). Seventeen carbon signals due to three methyl (oxygenated), one methylene, one methine (oxygenated), five aromatic methine, and seven aromatic quaternary carbons (five oxygenated) were observed in the ^{13}C NMR and DEPT spectra. According to the ^{1}H and ^{13}C NMR data and molecular formula, a bibenzyl skeleton with three hydroxyl and three *O*-methyl groups was deduced for the structure of **1**. The ^{1}H and ^{13}C NMR spectra of **1** suggested a symmetrical aromatic ring by the signals found at δ 6.56 (2H, s, H-2 and H-6), 3.88 (6H, s, 3-*O*CH_3 and 5-*O*CH_3), and δ 102.7 (C-2 and C-6), 147.0 (C-3 and C-5), and 56.4 (3-*O*CH_3 and 5-*O*CH_3). The ABX system revealed that the other benzene ring was 1,3,4-substituted. One hydroxyl and one *O*-methyl group were assigned to this benzene ring according to the ^{13}C−^{1}H long-range correlations (Figure 1) found at δ 5.51 (4′-OH)/δ 114.4 (C-5′) and δ 3.84 (3′-OCH_3)/δ 146.4 (C-3′) in the HMBC spectrum. A significant difference between **1** and some other bibenzyls[7−9] was observed for the benzylic protons. The four equivalent benzylic proton signals in the upfield region in other known bibenzyls were replaced by the signals for one oxygenated methine and two methylene protons in **1**. Thus, one benzylic proton should be substituted by the remaining hydroxyl group in the structure of **1**. In the HMBC spectrum of **1**, ^{13}C−^{1}H long-range correlations (Figure 1) were found from H-2 and H-6 to the oxygenated methine, suggesting the remaining hydroxyl group was located at C-α. On the basis of the above evidence and results of HSQC and HMBC spectra, the structure of **1** was established as 4,4′,α-trihydroxy-3,3′,5-trimethoxybibenzyl. Due to the small amount of **1**, which was mainly used for evaluating its biological activities, we were unable to determine the absolute configuration at the C-α position. Compound **1** is a new bibenzyl derivative, designated as nobilin D.

Compound **2** was obtained as a reddish-yellow oil, $[\alpha]_D^{27}$ +0.6 (*c* 1.0, MeOH). The molecular formula of $C_{32}H_{32}O_8$ was determined by HREIMS (*m*/*z* 544.2092, $[M]^+$), which was compatible with the results of ESIMS (*m*/*z* 567, $[M + Na]^+$; *m*/*z* 543, $[M - H]^-$) and NMR analyses. A characteristic color reaction with $FeCl_3$ revealed the phenolic nature of the compound. A UV absorption maxima at 280 nm (log ϵ 3.26) was similar to other bibenzyls. In combination with the HSQC spectrum, the ^{1}H NMR spectrum displayed resonances for 10 aromatic protons, one methine proton, three pairs of methylene protons, and four *O*-methyl groups. In the ^{13}C NMR and DEPT spectra of **2**, 32 carbon signals belonging to four aromatic *O*-methyl, three methylene, one methine, 10 aromatic methine, and 14 aromatic quaternary carbons were observed. On the basis of the ^{1}H and ^{13}C NMR data and molecular formula, the skeleton of **2** was identified as a bisbibenzyl derivative with three hydroxyl and four *O*-methyl groups.

According to the ^{1}H−^{1}H COSY correlations and splitting patterns of protons at δ 7.02 (1H, t, J = 8.0 Hz) and 7.19 (1H, t, J = 7.8 Hz), the aromatic protons at δ 7.02, 6.69, 6.28 and δ 7.19, 6.73, 6.75 were assigned to H-13, H-12, H-14 and H-13′, H-12′, H-14′, respectively. In the NOESY spectrum, correlations were found between δ 3.61 and 6.69 (H-12), 6.16 and between δ 3.75 and 6.73 (H-12′), 6.67, indicating the *O*-methyl groups at δ 3.61 and 3.75 were located at C-11 and C-11′, and aromatic protons at δ 6.16 and 6.67 were assigned to H-10 and H-10′. ^{13}C−^{1}H long-range correlations (Figure 2) were observed for H-8′/C-5′,7′,9′,10′,14′ and H-6′/C-2′,4′,7′ in the HMBC spectrum. In combination with the NOE correlation between H-6′ and 1′-OCH_3 in the NOESY spectrum, one bibenzyl skeleton was determined. In the HMBC spectrum, ^{13}C−^{1}H long-range correlations (Figure 2) were found at H-8/C-5,9,10,14; H-7/C-4,5,6; and H-4/C-2,6, which resulted in the assignment of most proton and carbon signals of the second bibenzyl skeleton. One of the linkage sites for the two bibenzyl skeletons was determined at C-7 and C-4′ on the basis of HMBC correlations (Figure 2) between H-7 and C-3′,4′,5′. According to the molecular formula and unsaturation degree, C-6 and C-3′ were linked with an oxygen atom, which formed a hexacyclic ring between two bibenzyl skeletons. The *O*-methyl-substituted aromatic quaternary carbon at δ 135.1, which was confirmed by a HMBC cross-peak from *O*-methyl protons at δ 4.00 to δ 135.1, was assigned to C-1 on the basis of its chemical shift. Both the *ortho*-positions of C-1 were substituted by oxygenated groups, but only one position of C-3 was oxygenated, which suggested that the chemical shift at C-1 should be relatively upfield. The fact that no correlation was found between H-4 and the protons at δ 4.00 in the NOESY spectrum also supported this conclusion. The three hydroxyl groups were located at C-2, C-3, and C-2′. The specific rotation for **2** was +0.6, suggesting it was likely racemic. Compound **2** is a new bisbibenzyl derivative, named nobilin E.

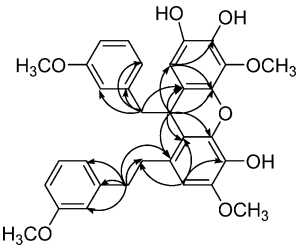

Figure 2. HMBC correlations of compound **2**.

Compound **3** was obtained as a red, amorphous powder. Its molecular formula was established as $C_{14}H_{10}O_4$ by HRTOFMS, giving a quasimolecular ion $[M + H]^+$ at *m*/*z* 243.0637, which was consistent with the results of ESIMS (*m*/*z* 265, $[M + Na]^+$; *m*/*z* 241, $[M - H]^-$) and NMR analyses. The phenolic nature of the compound was indicated by its characteristic color reaction with $FeCl_3$ (violet) and its IR spectrum exhibiting absorptions at 3309 (OH), 1697 (carbonyl), and 1612 and 1454 cm^{-1} (aromatic rings). Compound **3** showed typical fluorenone UV absorptions λ_{max} (MeOH) at 334, 319, 274, and 265 nm (log ϵ 2.13, 2.12, 3.17, and 3.14). The ^{1}H NMR spectrum of **3** exhibited resonances for one *O*-methyl group at δ 4.13 (3H, s) and five aromatic protons, appearing as a pair of *meta*-coupled doublets at δ 6.80 (1H, d, J = 2.0 Hz) and 6.82 (1H, d, J = 2.0 Hz) and an ABX system at δ 6.94 (1H, dd, J = 7.3, 1.9 Hz), 7.11 (1H, d, J = 1.9 Hz), and 7.12 (1H, d, J = 7.2 Hz). The ^{13}C NMR and DEPT spectra of **3** showed the presence of one *O*-methyl, five aromatic methine, seven aromatic quaternary carbons (three oxygenated), and one carbonyl carbon. These indicated a fluorenone skeleton with one *O*-methyl and two hydroxyl groups in compound **3**. In the HMBC spectrum, both aromatic protons at δ 6.82 and 7.11 showed ^{13}C−^{1}H long-range correlations with the carbonyl carbon signal at δ 193.4, implying these two protons were located at C-1 and C-8. Therefore, the aromatic protons at δ 6.80, 7.12, and 6.94 were assigned to H-3, H-5, and H-6. The *O*-methyl protons at δ 4.13 showing an NOE correlation with H-3 but not with H-1 in the NOESY spectrum indicated the location of the *O*-methyl group at C-4. Thus, the two hydroxyl groups were assigned to C-2 and C-7. On the basis of the above evidence, compound **3** was deduced to be 2,7-dihydroxy-4-methoxy-9-fluorenone, which is a new fluorenone named nobilone.

Table 1. Antioxidant Activity[a] of Compounds **1**−**10** from *Dendrobium nobile*

compound	DPPH IC_{50} (μM)[b]	ORAC (μM Trolox equivalent μM^{-1})[c]
1	19.9 ± 0.8	0.274 ± 0.006
2	21.0 ± 0.4	0.031 ± 0.001
3	>200	0.432 ± 0.005
4	21.8 ± 0.4	0.299 ± 0.008
5	>200	0
6	40.3 ± 0.1	0.090 ± 0.001
7	14.0 ± 0.1	0.280 ± 0.005
8	14.5 ± 0.3	0.625 ± 0.013
9	56.4 ± 0.9	0.234 ± 0.005
10	16.2 ± 0.2	0.596 ± 0.003
vitamin C	18.0 ± 0.2	0.172 ± 0.004
BHT	90.9 ± 2.4	NT[d]

[a] Data were expressed as mean ± SD. [b]Concentration of sample required to scavenge 50% DPPH free radicals. [c]Micromoles of Trolox equivalents per micromole of sample. [d]Not tested.

Table 2. Inhibitory Effects on NO Production[a] of Compounds **1**−**5** and **7**−**10** from *Dendrobium nobile*

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	15.3	**7**	cytotoxicity[b]
2	19.2	**8**	36.8
3	38.1	**9**	32.9
4	cytotoxicity[b]	**10**	13.4
5	48.2	resveratrol	23.5

[a] Data were expressed as mean for duplicate independent experiments. [b] IC_{50} value of compound lies within the cytotoxic concentration range.

In addition, seven known compounds were isolated from the active fraction and identified as crepidatin (**4**),[10] chrysotobibenzyl (**5**),[11] dendrobin A (**6**),[7] chrysotoxine (**7**),[12] moscatilin (**8**),[13] gigantol (**9**),[14] and dendroflorin (**10**)[15] by spectroscopic analyses and by comparison of data with those reported. Compounds **4**, **5**, **7**, and **10** are reported from this plant for the first time.

The antioxidant activity of compounds was evaluated by DPPH free radical scavenging and ORAC assays (Table 1). The well-known antioxidants vitamin C and butylated hydroxytoluene (BHT) were used as positive controls. In the DPPH assay, compounds **1**, **2**, **4**, **7**, **8**, and **10** exhibited significant scavenging activity higher than or equivalent to vitamin C. Although less active than vitamin C, compounds **6** and **9** showed higher activity than BHT. In the ORAC assay, compounds **1**, **3**, **4**, and **7**−**10** displayed potent peroxyl radical scavenging activity higher than vitamin C, and compound **6** showed weak activity. Some differences between the results of these two methods were possibly due to their different reaction mechanisms and measurements.[16] Compound **5**, without any phenolic hydroxyl group, was inactive both in the DPPH and ORAC assays, which was in accordance with the previous reports that the phenolic hydroxyl group played an important role in antioxidant activity.[17−19] Compound **1** was less active than compound **8** in both the DPPH and ORAC assays, suggesting that antioxidant activity decreases with the presence of a hydroxyl group at C-α in bibenzyl derivatives.

All isolated compounds except for compound **6**, for which insufficient material was available, were also measured for their inhibitory effects on NO production in murine macrophages (RAW 264.7) activated by LPS and interferon (IFN)-γ (Table 2). Macrophages play major roles in inflammation and host defense mechanisms against bacterial and viral infections.[20] The inorganic free radical nitric oxide (NO), produced by the oxidation of L-arginine catalyzed by NO synthase (NOS), has been implicated in many physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation.[21,22] Therefore, inhibition of NO production may be of therapeutic benefit in various diseases induced by pathological concentrations of NO. In this assay, resveratrol, which has been reported to have an inhibitory effect on NO production in LPS-activated RAW 264.7 macrophages by down-regulation of the inducible NOS and mRNA, was used as a positive control.[23,24] Cell viability in the present experiment was determined by the MTT method to exclude the bioactivity resulting from the cytotoxicity of tested compounds (data not shown). Compounds **1**−**3**, **5**, and **8**−**10** showed inhibitory effects on NO production without cytotoxicity. Compounds **1**, **2**, and **10** exhibited stronger activity than resveratrol, while significant cytotoxicity was found for compounds **4** and **7**. In contrast with antioxidant activity, compound **1** displayed a more potent inhibitory effect on NO production than compound **8**, suggesting that the presence of a hydroxyl group at C-α in bibenzyl derivatives was beneficial to the inhibition of NO production.

Several bibenzyls and fluorenones have been identified from this plant before. Our study indicated these two types of components possessing one or more hydroxyl groups in TCM "Shi Hu" could contribute to its prophylactic and therapeutic effects on many diseases through scavenging of the redundant radicals in the body and inhibiting excessive NO production.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Jasco P-1020 polarimeter. UV spectra were obtained with a Shimadzu UV2401PC UV−vis recording spectrophotometer in MeOH. IR spectra were recorded on a Shimadzu FTIR 8400 spectrophotometer as KBr disks. NMR spectra were run on a Bruker AVANCE 400 NMR spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) with TMS as internal standard. ESIMS spectra were performed on a Bruker Esquire 2000 mass spectrometer. HRTOFMS spectra were obtained on a Finnigan MAT95 mass spectrometer, and HREIMS spectra were measured on a Micromass mass spectrometer. The analytical and preparative HPLC were performed on a Shimadzu Pak with RI detector using a Shim-pack VP-ODS column (4.6 × 250 mm) and a Shim-pack PREP-ODS column (10 × 250 mm), respectively. Column chromatography was carried out on silica gel H60 (Qingdao Haiyang Chemical Group Corp., Qingdao, China), Sephadex LH-20 (Amersham Biosciences AB), and ODS (60−80 μm, Merck) as packing materials. Silica gel G was used for analytical TLC.

Plant Material. The fresh stems of *D. nobile* were collected in Yunnan Province in 2004 and identified by Ms. Li-Ping Xiao of Hongkong Kadoorie Farm and Botanic Garden. A voucher specimen (YZXDN-2004) is deposited at the Key Laboratory for New Drugs Research of Traditional Chinese Medicine in Shenzhen, China.

Extraction and Isolation. The powdered air-dried stems of *D. nobile* (5 kg) were refluxed with 60% EtOH three times. After evaporation of solvent in vacuo, the residue (210 g) was suspended in H_2O and partitioned with EtOAc and *n*-BuOH successively. The EtOAc-soluble fraction exhibited significant antioxidant activity (IC_{50} = 32 μg/mL) in the DPPH assay, while the *n*-BuOH-soluble fraction showed only weak activity (IC_{50} = 105 μg/mL). Then the active EtOAc-soluble fraction (63 g) was first subjected to column chromatography on silica gel (200−300 mesh, 700 g) eluted with $CHCl_3$/MeOH (100:0 → 0:100) to afford 12 fractions. Fractions 5, 6, and 7 were found to be active in the DPPH assay (IC_{50} = 33.6, 29.7, 31.8 μg/mL).

Fraction 5 (11 g) was further chromatographed on silica gel MPLC by gradient elution with cyclohexane/EtOAc (95:5 → 0:100) to give 13 subfractions. Subfraction 7 (1.5 g) was passed over a Sephadex LH-20 column with $CHCl_3$/MeOH (1:1) as eluent and then applied to an ODS column eluted with MeOH/H_2O (4:6 → 8:2). The eluent of 60% MeOH was purified by preparative HPLC (55% MeOH) to yield compounds **4** (181.7 mg) and **5** (208.1 mg). Subfraction 8 (276 mg) was passed over a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and ODS column (MeOH−H_2O, 4:6 → 8:2). Compound **6** (2.4 mg) was finally obtained from the eluent of 60% MeOH by purification with preparative HPLC (55% MeOH). Subfraction 10 (1.4 g) was passed over a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and then applied to an ODS column (MeOH−H_2O, 4:6 → 7:3). The eluent of 50% MeOH was purified by preparative HPLC (50% MeOH) to yield compound **8** (455.4 mg), and the eluent of 60% MeOH was recrystallized in MeOH to afford compound **7** (259.4 mg).

Fraction 6 (6.9 g) was chromatographed on silica gel MPLC by gradient elution with cyclohexane/EtOAc (85:15 → 0:100) to give 10 subfractions. Subfraction 5 (727 mg) was passed over a Sephadex LH-20 column with $CHCl_3$/MeOH (1:1) as eluent and an ODS column eluted with MeOH/H_2O (4:6 → 8:2), then finally purified by preparative HPLC (50% MeOH) to yield compound **9** (10.0 mg). Subfraction 6 (927 mg) was passed over a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and then applied to an ODS column (MeOH−H_2O, 3:7 → 8:2). The eluent of 50% MeOH was recrystallized in MeOH to afford compound **10** (28.2 mg), and the eluent of 60% MeOH was further purified by preparative TLC (cyclohexane/EtOAc, 6:4) to yield compound **3** (48.5 mg). Subfraction 9 (559 mg) was passed over a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and then applied to an ODS column (MeOH−H_2O, 2:8 → 8:2). Compound **1** (2.6 mg) was finally obtained from the eluent of 30% MeOH by purification with preparative HPLC (30% MeOH).

Fraction 7 (6.9 g) was passed over a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and then chromatographed on silica gel MPLC by gradient elution with cyclohexane/EtOAc (8:2 → 0:1) to give nine subfractions. Subfraction 5 (1.0 g) was applied to an ODS column eluted with MeOH/H_2O (3:7 → 8:2). The eluent of 80% MeOH was further purified by preparative HPLC (65% MeOH) to yield compound **2** (15.2 mg).

Nobilin D (1): colorless oil: $[\alpha]^{27}_D$ +1.5 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ϵ) 281 (2.62) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.85 (1H, d, J = 8.0 Hz, H-5′), 6.70 (1H, dd, J = 8.0, 1.7 Hz, H-6′), 6.62 (1H, d, J = 1.7 Hz, H-2′), 6.56 (2H, s, H-2,6), 5.51 (1H, s, 4′-OH), 5.47 (1H, s, 4-OH), 4.77 (1H, dd, J = 8.0, 5.5 Hz, H-α), 3.88 (6H, s, 3,5-OCH_3), 3.84 (3H, s, 3′-OCH_3), 2.93 (1H, dd, J = 13.8, 5.5 Hz, H-α′a), 2.91 (1H, dd, J = 13.8, 8.0 Hz, H-α′b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.0 (C × 2, C-3,5), 146.4 (C, C-3′), 144.5 (C, C-4′), 135.1 (C, C-1), 134.1 (C, C-4), 129.7 (C, C-1′), 122.2 (CH, C-6′), 114.4 (CH, C-5′), 112.1 (CH, C-2′), 102.7 (CH × 2, C-2,6), 75.6 (CH, C-α), 56.4 (CH_3 × 2, 3,5-OCH_3), 55.9 (CH_3, 3′-OCH_3), 45.9 (CH_2, C-α′); ESIMS *m*/*z* 343 $[M + Na]^+$; HRTOFMS *m*/*z* 321.1356 $[M + H]^+$ (calcd for $C_{17}H_{21}O_6$, 321.1338).

Nobilin E (2): reddish-yellow oil: $[\alpha]^{27}_D$ +0.6 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 280 (3.26) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.19 (1H, t, J = 7.8 Hz, H-13′), 7.02 (1H, t, J = 8.0 Hz, H-13), 6.75 (1H, m, H-14′), 6.73 (1H, m, H-12′), 6.69 (1H, m, H-12), 6.67 (1H, m, H-10′), 6.49 (1H, s, H-6′), 6.28 (1H, m, H-14), 6.16 (1H, m, H-10), 6.15 (1H, s, H-4), 4.02 (1H, m, H-7), 4.00 (3H, s, 1-OCH_3), 3.86 (3H, s, 1′-OCH_3), 3.75 (3H, s, 11′-OCH_3), 3.61 (3H, s, 11-OCH_3), 2.83 (1H, m, H-7′a), 2.79 (2H, m, H-8′), 2.78 (1H, m, H-8a), 2.74 (1H, m, H-8b), 2.72 (1H, m, H-7′b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.7 (C, C-11′), 159.1 (C, C-11), 145.6 (C, C-1′), 143.1 (C, C-9′), 140.7 (C, C-3′), 139.8 (C, C-9), 139.5 (C, C-3), 138.8 (C, C-6), 135.1 (C, C-1), 134.9 (C, C-2), 132.3 (C, C-2′), 129.4 (CH, C-13′), 129.3 (C, C-5′), 128.8 (CH, C-13), 122.2 (CH, C-14), 120.9 (CH, C-14′), 117.9 (C, C-5), 117.8 (C, C-4′), 114.8 (CH, C-10), 114.4 (CH, C-10′), 112.4 (CH, C-12), 111.4 (CH, C-12′), 109.0 (CH, C-4), 107.3 (CH, C-6′), 61.5 (CH_3, 1-OCH_3), 56.4 (CH_3, 1′-OCH_3), 55.1 (CH_3, 11′-OCH_3), 55.0 (CH_3, 11-OCH_3), 45.6 (CH_2, C-8), 38.8 (CH, C-7), 37.8 (CH_2, C-8′), 33.3 (CH_2, C-7′); ESIMS *m*/*z* 567 $[M + Na]^+$, *m*/*z* 543 $[M - H]^-$; HREIMS *m*/*z* 544.2092 $[M]^+$ (calcd for $C_{32}H_{32}O_8$, 544.2097).

Nobilone (3): red, amorphous powder; UV (MeOH) λ_{max} (log ϵ) 334 (2.13), 319 (2.12), 274 (3.17), 265 (3.14) nm; IR (KBr) ν_{max} 3309, 1697, 1612, 1454 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.12 (1H, d, J = 7.2 Hz, H-5), 7.11 (1H, d, J = 1.9 Hz, H-8), 6.94 (1H, dd, J = 7.3, 1.9 Hz, H-6), 6.82 (1H, d, J = 2.0 Hz, H-1), 6.80 (1H, d, J = 2.0 Hz, H-3), 4.13 (3H, s, 4-OCH_3); ^{13}C NMR (acetone-d_6, 100 MHz) δ 193.4 (CO, C-9), 161.2 (C, C-2), 153.6 (C, C-4), 151.6 (C, C-7), 137.2 (C, C-9a), 135.9 (C, C-8a), 130.2 (CH, C-5), 128.0 (C, C-4b), 125.0 (CH, C-6), 122.5 (C, C-4a), 116.8 (CH, C-8), 106.3 (CH, C-3), 106.1 (CH, C-1), 57.6 (CH_3, 4-OCH_3); ESIMS *m*/*z* 265 $[M + Na]^+$, *m*/*z* 241 $[M - H]^-$; HRTOFMS *m*/*z* 243.0637 $[M + H]^+$ (calcd for $C_{14}H_{11}O_4$, 243.0657).

DPPH Free Radical Scavenging Assay. The DPPH free radical has been widely used to evaluate the antioxidant capacity of fractions and pure compounds, and is stable due to its resonance stabilization and spacial blockade of three benzene rings.[25−27] In its radical form, DPPH has a characteristic absorption at 517 nm in EtOH, which disappears with acceptance of an electron from the antioxidant sample.[28] The method reported[29,30] was adopted with some modifications: 100 μL of the test samples at different concentrations in EtOH and 100 μL of DPPH (Sigma) in EtOH (200 μM) were added in a 96-well microplate. The 96-well microplate was shaken for 1 min in a Spectra Max 340PC microplate spectrophotometer (Molecular Devices Corp.). After 30 min at room temperature in the dark, the absorbance was recorded at 517 nm. The tested samples at different concentrations without DPPH were used as controls to eliminate the influence of the samples' color. Vitamin C and BHT were used as positive controls, and DPPH solution in ethanol served as negative control. All tests were performed in triplicate. The antioxidant activity of the tested samples was compared in terms of IC_{50} (concentration of sample required to scavenge 50% DPPH free radicals).

Oxygen Radical Absorbance Capacity Assay. The ORAC assay is based on free radical damage to a fluorescent probe causing the elimination of its fluorescence characteristic. The presence of the antioxidants can compete with the fluorescent probe and prolong the reduction of its fluorescence.[31,32] The method was used with some modifications.[32] In our assay, sodium fluorescein was used as a fluorescent probe, AAPH as a free radical initiator, and Trolox as a standard. The test samples at different concentrations (20 μL), potassium phosphate buffer (7.5 mM, 20 μL), and sodium fluorescein (63 nM, 20 μL) were added to a 96-well microplate. After incubating at 37 °C for 5 min, AAPH (12.8 mM, 140 μL) was added into the mixture to start the reaction. The fluorescence intensity was measured with Genios multi-detection microplate reader (Tecan) (ex 485 nm, em 538 nm) every 2 min until the fluorescence decay was close to zero. Fluorescence decay of sodium fluorescein without AAPH was used as a control, and fluorescence decay induced by AAPH without tested samples served as a blank. All tests were performed in triplicate. The measurement result was expressed as relative fluorescence intensity, which was obtained by comparison of the fluorescence intensity with the control. The antioxidant capacity was evaluated in terms of ORAC value, which was determined by calculating the net area under the curve (AUC) of the samples and standard. The net AUC was obtained by subtracting the AUC of the blank from that of a sample, and the final ORAC value was expressed as micromoles of Trolox equivalents per micromole of sample.

Inhibitory Effects on NO Production in Activated Murine Macrophage-like Cell Line RAW 264.7.[33] The cells were seeded at 1.2 × 10^6 cells/mL onto 96-well microplates (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Next, the test samples were added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/mL) and recombinant mouse IFN-γ (0.33 ng/mL). The cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. The culture supernatant (100 μL) was placed in duplicate in the wells of 96-well microplates. To quantify nitrite, 50 μL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthylethylenediamide dihydrochloride) was added to each well. After 10 min, the reaction products were colorimetrically quantitated at 570 nm using a model 3550 microplate reader (BIO-RAD). The cell culture without LPS, IFN-γ, and tested samples was used as a blank, the cell culture without tested samples served as a negative control, and resveratrol was used as a positive control. Cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.[34] All tests were performed in duplicate.

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