# Chemical Constituents of the Endophytic Fungus *Hypoxylon* sp. 12F 0687 Isolated from Taiwanese *Ilex formosana*

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Bioassay-guided fractionation of an AcOEt-soluble fraction of the liquid fermentation of an endophytic fungus *Hypoxylon* sp. BCRC 12F 0687 associated with the root of Taiwanese *Ilex formosana* (Aquifoliaceae) resulted in the isolation of two new compounds, *i.e.*, one benzenoid, hypoxyphenone (**1**), and one azaphilone derivative, hypoillexidiol (**2**), two metabolites isolated for the first time from natural source, (−)-(3*S*)-3-hydroxy-3-methyloxindole (**3**) and (+)-vermelone (**4**), along with twelve previously identified compounds, **5**–**16**. Their structures were determined through in-depth spectroscopic and mass-spectrometric analyses. The effects of some isolates on the inhibition of NO and IL-6 production in lipopolysaccharide-activated RAW 264.7 murine macrophages were evaluated. Of the isolates, **2** and **3** exhibited potent anti-NO production activity, with $IC_{50}$ values of $17.5 \pm 1.8$ and $24.7 \pm 1.6$ μM, respectively, compared to that of quercetin, an iNOS inhibitor with an $IC_{50}$ value of $35.9 \pm 1.7$ μM. Compounds **2**, **4**, **5**, and **12** also showed moderate inhibition of IL-6 production, with $IC_{50}$ values ranging from $27.2 \pm 1.8$ to $35.3 \pm 5.8$ μM. This is the first report on an oxindole metabolite from the genus *Hypoxylon*.

**Introduction.** – Secondary metabolites obtained from plant-derived fungi which possess unique structures and interesting biological properties have attracted considerable attention by chemists in recent years [1–3]. Endophytic fungi are microorganisms that reside in the internal tissues of living plants without causing any apparent symptoms or immediate overt negative effects in the host plant [1]. The research on endophytes showed that they are potential sources of diverse and bioactive secondary metabolites for exploitation in medicine, agriculture, and industry [1–5]. Fungi of the genus *Hypoxylon*, a class of widely distributed endophytic fungi, were reported as plentiful producer of secondary metabolites, including antimicrobial carneic acids A and B [6], cytotoxic daldinones C and D [7], and active against *Gram*-positive bacteria-phomadecalins A–D [8]. However, many species of this genus still remain chemically and biologically unexplored. In our effort to search for structurally interesting and bioactive natural products from endophytic fungi, we have isolated and identified over 100 endophytic fungal strains from Formosan plants, and the crude AcOEt and BuOH extracts from these endophytic strains were screened for their inhibitory activity on lipopolysaccharide (LPS)-induced nitric oxide (NO) release and

interleukin-6 (IL-6) production in RAW 264.7 murine macrophages. Our previous work revealed three new and seven known compounds isolated from the active BuOH extract of *H. investiens* BCRC 10F 0115, an endophytic fungus isolated from a Lauraceous plant, *Litsea akoensis* var. *chitouchiaoensis* [9].

In our continual effort, a fungal strain, *Hypoxylon* sp. 12F 0687 from a Taiwanese *Ilex formosana* (Aquifoliaceae) was proven to exhibit inhibitory activity on LPS-induced nitric oxide (NO) release and interleukin-6 (IL-6) production, as determined by our primary screening (approximately 98% inhibition at a concentration of 10 μg/ml). To efficiently utilize the plant fungal sources and increase the number of novel bioactive metabolites available from a single fungal strain, we have attempted to explore the chemical diversity of plant-derived fungi by changing the cultivation parameters (see *Exper. Part*). In a preliminary HPLC screening, the AcOEt extract of *Hypoxylon* sp. 12F 0687 was found to be able to produce diverse secondary metabolites in different culture media. This strain was fermented using MEA (malt extract agar) medium in a large scale, its bioactive AcOEt extract was undergoing chromatography over a silica gel column and by HPLC to give one benzenoid, hypoxyphenone (**1**), one azaphilone derivative, hypoillexidiol (**2**), and two metabolites isolated for the first time from natural source, (−)-(3*S*)-3-hydroxy-3-methyloxindole (**3**) and (+)-vermelone (**4**), and as well as twelve known constituents (*Fig. 1*). Herein, we describe the structure identification of the new compounds and the inhibitory activity of the main isolates on NO and IL-6 productions in macrophage RAW 264.7 cells.

**Results and Discussion.** – *Structure Elucidation*. The AcOEt-soluble fraction produced by the endophytic fungus *Hypoxylon* sp. BCRC 12F 0687 was fractionated by a combination of sillica gel, *RP-18* columns, and preparative TLC to furnish 16 compounds, **1**–**16**, including one benzenoid (**1**), one azaphilone derivative (**2**), one alkaloid (**3**), and one $\alpha$-tetralone derivative (**4**), the structures of which were elucidated by 1D- and 2D-NMR spectra and comparison with literature data. Compound **1** was obtained as yellowish needles with a m.p. of 149–150°, and its molecular formula was deduced as $C_{10}H_{10}O_5$ from the HR-ESI-MS data (*m*/*z* 233.0425 ($[M+Na]^+$, $C_{10}H_{10}NaO_5^+$; calc. 233.0426)), implying six degrees of unsaturation. The UV absorption bands at $\lambda_{max}$ 208, 228, 266, and 375 nm and a bathochromic shift upon addition of KOH suggested the presence of a phenolic skeleton [10]. The IR spectrum showed bands corresponding to the absorptions of OH and CHO groups and aromatic-ring moieties at 3244, 1670, 1601, and 1479 $cm^{-1}$, respectively. The $^1$H-NMR spectrum of **1** exhibited resonances for an aromatic H-atom at $\delta$(H) 7.08 (*s*, H–C(5)) corroborated by a $^{13}$C-NMR signal at $\delta$(C) 118.1 (C(5)). Two H-bonded OH groups ($\delta$(H) 9.86 (*s*, HO–C(6))) and ($\delta$(H) 10.63 (*s*, HO–C(3))), a Me group ($\delta$(H) 2.46 (*s*, Me(8))), an aldehyde moiety ($\delta$(H) 9.89 (*s*, CHO)), and a COOMe group ($\delta$(H) 4.02 (*s*, COOMe)) located at the aromatic ring were determined by $^{13}$C-NMR signals at C(6) ($\delta$(C) 152.9), C(3) ($\delta$(C) 151.8), C(2) ($\delta$(C) 129.2), C(4) ($\delta$(C) 122.7), and C(1) ($\delta$(C) 120.7) and the HMBCs of COOMe/C(7), Me(8)/C(1) and C(2), HO–C(6)/C(1), C(5), and C(6), HO–C(3)/C(2) and C(4), CHO/C(4), C(3), and C(5) (*Fig. 2*). The correlations of Me(8)/COOMe, and HO–C(3), HO–C(3)/CHO, and CHO/H–C(5) were also observed in the NOESY experiment (*Fig. 3*) and further supported the position of each aromatic substitution. The $^1$H- and $^{13}$C-NMR, COSY (*Fig. 2*), NOESY

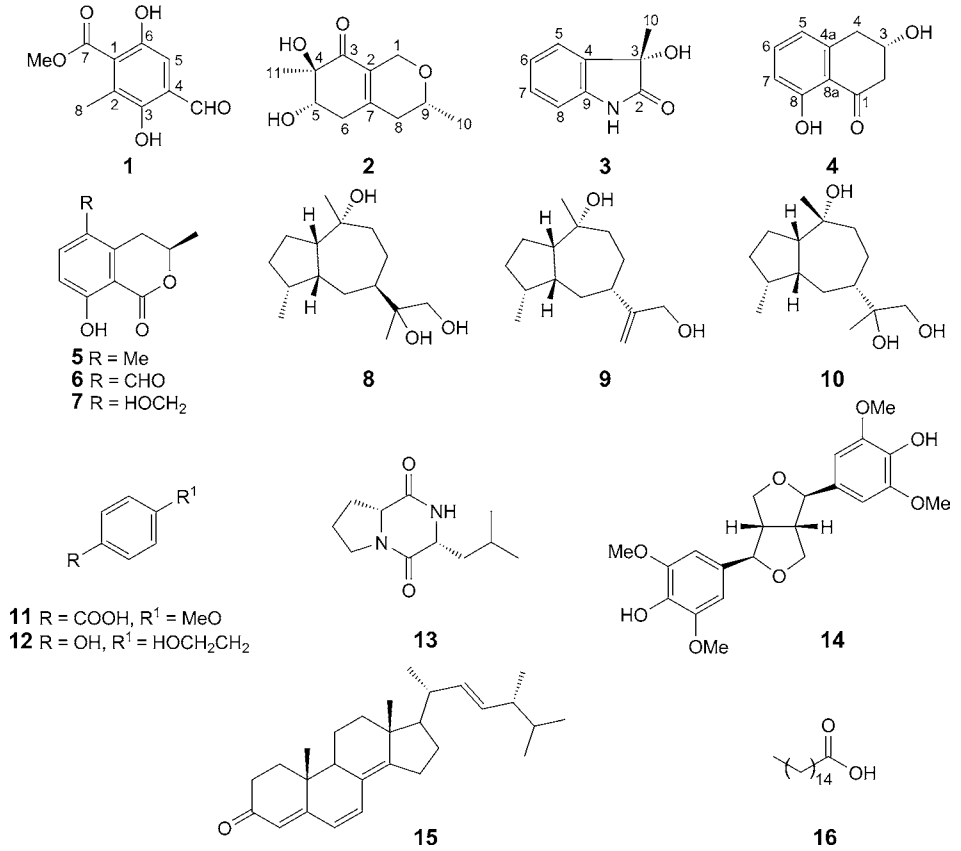


Fig. 1. *Structures of compounds* **1**–**16**

(*Fig. 3*), HSQC and HMBC (*Fig. 2*) experiments confirmed the structure as methyl 4-formyl-3,6-dihydroxy-2-methylbenzoate, and designated hypoxyphenone.

Compound **2** was obtained as optically active oil with $[\alpha]_D^{25} = +231$ ($c = 0.09$, MeOH). The ESI-MS and HR-ESI-MS were used to establish the molecular formula of **2** as $C_{11}H_{16}O_4$, which implies four degrees of unsaturation. The UV spectrum absorption $\lambda_{max}$ (MeOH) at 240 nm, and a strong IR absorption at 1667 $cm^{-1}$, as well as the observation of the C-atom resonances ($\delta$(C) 199.2 (C(3)), 152.8 (C(7)), and 128.3 (C(2))) in the $^{13}$C-NMR spectrum, revealed the presence of an $\alpha,\beta$-unsaturated C=O functionality in **2**. Its residual IR spectrum showed absorption bands for OH groups (3412 $cm^{-1}$) and one vinyl (1648 $cm^{-1}$) functionality. The $^1$H- and $^{13}$C-NMR spectra displayed one Me group at $\delta$(H) 1.26 (*s*, Me(11)); $\delta$(C) 17.8 (C(11)), one *sec*-Me group ($\delta$(H) 1.29 (*d*, $J = 6.1$, Me(10)); $\delta$(C) 21.1 (C(10))), an O-bearing CH group ($\delta$(H) 4.00 (*dd*, $J = 10.4$, 5.2, $H_{ax}$–C(5)); $\delta$(C) 72.3 (C(5))), a CH group ($\delta$(H) 3.63 (*dq*, $J = 12.8$, 6.1, $H_{ax}$–C(9); $\delta$(C) 69.3 (C(9))), signals for three non-equivalent $CH_2$ groups ($\delta$(H) 2.16–2.18 (*m*, $H_{eq}$–C(8)), 2.19–2.21 (*m*, $H_{ax}$–C(8)), 2.35 (br. *dd*, $J = 18.4$, 10.4, $H_{ax}$–C(6)), 2.60 (*dd*, $J = 18.4$, 5.2, $H_{eq}$–C(6)), 4.14 (br. *d*, $J = 15.8$, $H_{ax}$–C(1)), and 4.62

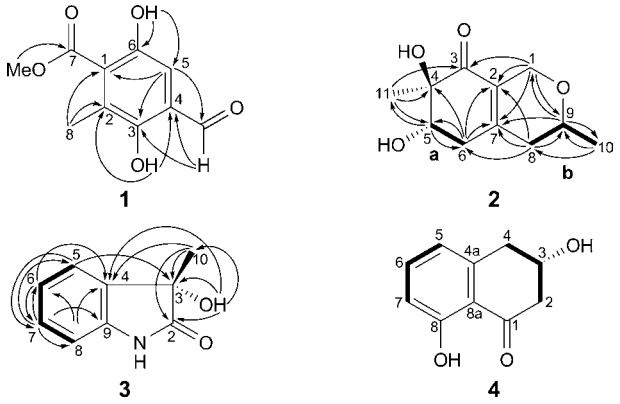


Fig. 2. *Key* $^{1}H,^{1}H$*-COSY* (—) *correlations of compounds* **2**–**4**, *and HMBCs* (H → C) *of compounds* **1**–**3**

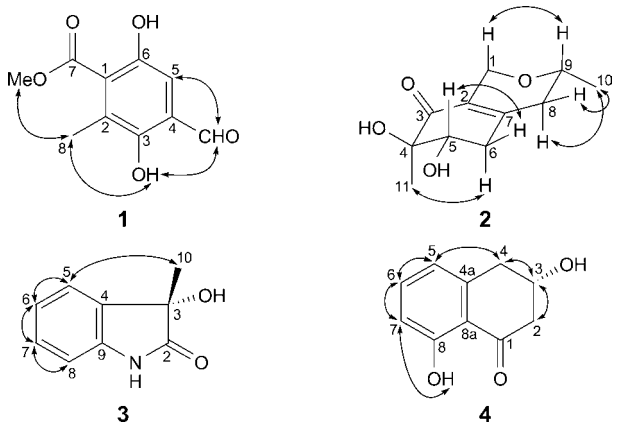


Fig. 3. *NOESY* (H ↔ H) *correlations of compounds* **1**–**4**

($d$, $J = 15.8$, $H_{eq}$–C(1$\alpha$)); $\delta$(C) 36.1 (C(6)), 37.7 (C(8)), and 63.5 (C(1))), and two $D_2O$-exchangeable H-atoms due to OH groups ($\delta$(H) 2.40 (br. *s*, HO–C(5)), 3.71 (br. *s*, HO–C(4))). The $^{13}$C-NMR, DEPT, and HSQC spectra of **2** showed eleven C-atom signals including those of two Me, three $CH_2$, two CH groups, and four quaternary C-atoms, of which one $CH_2$ group ($\delta$(C) 63.5 (C(1))), one CH group ($\delta$(C) 72.3 (C(5))), and one quaternary C-atom ($\delta$(C) 77.3 (C(4))) were O-bearing, and three quaternary C-atoms ($\delta$(C) 128.3 (C(2)), $\delta$(C) 152.8 (C(7)), and $\delta$(C) 199.2 (C(3))) were olefinic C-atoms. Since two out of four unsaturation equivalents were accounted for by the above-mentioned $^{13}$C-NMR data, **2** was inferred to have two rings. In addition, the two rings were further determined as a cyclohex-2-enone skeleton combined with one dihydropyran ring (azaphilone analog) by the following HMBC and COSY analyses (*Fig. 2*).

The above observation followed by the COSY (*Fig. 2*) and HSQC spectra of **2** established the presence of the two partial fragments: **a** (C(5)–C(6)) and **b**

(C(8)–C(9)–C(10)), for the skeleton of **2**. The entire skeleton of **2** was constructed by the aid of the HMBC spectrum (*Fig. 2*).

The COSY correlations, together with the HMBCs Me(11)/C(3), C(4), and C(5), $H_{ax}$–C(5)/C(4), C(6), and C(11), $H_{eq}$–C(6)/C(2), C(4), C(5), C(7), and C(8), $H_{eq}$–C(1)/C(9), and $H_{ax}$–C(9)/C(1) and C(10), established the presence of cyclohexenone (from C(2) to C(7)) and dihydropyran moiety (from $CH_2$(1) to H–C(9)), respectively.

Further confirmation by the HMBCs (*Fig. 2*) of $CH_2$(8)/C(2), C(6), C(7), and C(9); $H_{ax}$–C(9)/C(7); and $H_{eq}$–C(1)/C(2), C(3), and C(7), verified the junction of the two substitutes **a** to **b** unit at C(2) and C(7). This allowed the skeleton of **2** to be determined as 4,5,8,9-tetrahydro-4,5-dihydroxy-4,9-dimethyl-1*H*-isochromen-3(5*H*)-one.

Unfortunately, the limited amount and the unstable nature of compound **2** prevented us from assigning the absolute configuration at C(5) in **2** using the *Mosher* ester methodology. The relative configuration of **2** was proposed on the basis of the NOESY experiments (*Fig. 3*) and $^1$H,$^1$H coupling constants. The signal of H–C(9) as a *dq* ($J = 12.8$, 6.1) indicated axial-axial coupling between $H_{ax}$–C(9) and $H_{ax}$–C(1), leading to the assignment of H–C(9) as axial, *i.e.*, $\beta$-oriented. This was indeed confirmed by a NOESY correlation (*Fig. 3*). The $\alpha$-orientation of the Me(11) group was deduced from the observation of the *quasi*-1,3-diaxial interactions of $H_\alpha$–C(6) and Me(11). The $H_{ax}$–C(5) was $\beta$-oriented, which was further confirmed by the observation of a NOE between $H_{ax}$–C(5) ($\delta$(H) 4.00) and $H_{eq}$–C(6) ($\delta$(H) 2.60). Thus, the *trans*-configuration for $H_\beta$–C(5) ($\delta$(H) 4.00)/$H_\alpha$–C(6) ($\delta$(H) 2.35) was deduced from the fact, that no NOE correlation between $H_\beta$–C(5)/$H_\alpha$–C(6) was observed, and from the large axial-axial coupling constant ($J = 10.4$ Hz) between $H_\beta$–C(5)/$H_\alpha$–C(6). Based on above spectral evidence, the structure of **2** was elucidated as (4*R**,5*S**,9*R**)-4,5,8,9-tetrahydro-4,5-dihydroxy-4,9-dimethyl-1*H*-isochromen-3(5*H*)-one, named hypoillexidiol.

Compound **3** was obtained as colorless needles with a m.p. of 147 – 149 °. $[\alpha]_D^{25} = -31.9$ ($c = 0.16$, MeOH). The HR-ESI-MS data let us determine the molecular formula to be $C_9H_9NO_2$ (*m*/*z* 186.0522 ($[M + Na]^+$; calc. 186.0525)). UV absorptions ($\lambda_{max}$ bands at 210, 252, and 285 nm) confirmed the presence of an oxindole nucleus [11]. The IR spectrum revealed the presence of NH/OH groups (3259 $cm^{-1}$), and an amidocarbonyl moiety (1717 $cm^{-1}$), respectively.

The $^1$H-NMR spectrum of **3** was similar to that of 2-oxindole [12], except for an OH group ($\delta$(H) 2.85 (*s*, HO–C(3))) and one Me moiety ($\delta$(H) 1.60 (*s*, Me(10))) attached at C(3) in **3**, instead of a $CH_2$ group ($\delta$(H) 3.56 (*s*)) at C(3) in 2-oxindole [12].

The location of the OH and Me groups at C(3) was determined by the HMBC experiment (*Fig. 2*), in which cross-peaks were observed between HO–C(3) ($\delta$(H) 2.85) and C(2) ($\delta$(C) 180.2), C(3) ($\delta$(C) 73.9), C(4) ($\delta$(C) 131.7), and C(10) ($\delta$(C) 24.8); and between Me(10) ($\delta$(H) 1.60) and C(2) ($\delta$(C) 180.2), C(3) ($\delta$(C) 73.9), and C(4) ($\delta$(C) 131.7). The constitution of **3** was elucidated as 3-hydroxy-3-methyloxindole. This substance had been detected from the urine of a schizophrenic patient by *Albrecht et al.* [13], who had not determined the configuration at C(3). The absolute configuration of **3** was assigned as (*S*) from the laevorotatory optical activity ($[\alpha]_D^{25} = -31.9$) by analogy with a previous observation [14]. The $^1$H- and $^{13}$C-NMR

chemical shifts, and key HMBC, COSY, and NOESY correlations are shown in *Figs. 2* and *3*. This is the first isolation of the (*S*) enantiomer of **3** from a natural source, although (−)-(3*S*)-3-hydroxy-3-methyloxindole and (+)-(3*R*)-3-hydroxy-3-methyloxindole have been synthesized by *Monde et al.* [14]

Compound **4** was isolated as optically active colorless oil. $[\alpha]_D^{25} = +37.6$ ($c = 0.011$, EtOH). The UV absorptions at 223, 260, and 315 nm were similar to those of related compounds, (−)-(3*R*)-vermelone [15–18] and (−)-(3*R*)-scytalone [16][18][19], and the bathochromic shift in alkaline solution suggested the presence of a phenolic $\alpha$-tetralone moiety. The IR spectrum showed absorption bands for a OH group at 3423 $cm^{-1}$ and a conjugated C=O group at 1633 $cm^{-1}$. All above data indicated that **4** is an $\alpha$-tetralone derivative (= 3,4-dihydronaphthalen-1(2*H*)-one).

The structure was elucidated by analysis of 1D-NMR data, together with the NOESY plot (*Fig. 3*) showing correlations of both H–C(5) and H–C(7) to H–C(6), HO–C(8) to H–C(7), and H–C(3) to both $CH_2$(2) and $CH_2$(4). The absolute configuration at the stereogenic center C(3) in **4** was corroborated by optical activity measurement. Compound **4** was identified by comparison with literature data of (−)-(3*R*)-vermelone [15–17], but showed a dextrorotatory optical activity. With an enantiomeric antipode for comparison, and with the reference to (−)-(3*R*)-vermelone ($[\alpha]_D^{25} = -18$ (EtOH)) [15][16]), (−)-(3*R*)-GTRI-02 ($[\alpha]_D^{20} = -16$ (MeOH) [19]) and (−)-(3*R*)-7-chloroscytalone ($[\alpha]_D^{20} = -3$ (MeOH) [20]), the configuration at C(3) of **4** would appear to be (*S*). Based on spectral evidences, the structure of **4** was elucidated as (+)-(*S*)-3,4-dihydro-3,8-dihydroxynaphthalen-1(2*H*)-one, named as (+)-(3*S*)-vermelone. Although the (−)-(3*R*)-vermelone was reported as a constituent from culture filtrates of the melanin-deficient *brm-l* mutant of *Verticillium dahliaef*, to the best of our knowledge, **4** with (3*S*) configuration was found in nature for the first time.

Additionally, twelve known compounds were assigned as following: three isocoumarins, (−)-(*R*)-5-methylmellein (**5**) [21], (−)-(*R*)-5-formylmellein (**6**) [22][23], (−)-(*R*)-5-hydroxymethylmellein (**7**) [21], three sesquiterpenoids, 2-[(3*R**,3a*R**,5*R**,-8*R**,8a*S**)-8-hydroxy-3,8-dimethyldecahydroazulen-5-yl]propane-1,2-diol (**8**) [9], xylaranol A (**9**) [24], and xylaranol B (**10**) [18], two benzenoids, 4-methoxybenzoic acid (**11**) [25], and tyrosol (**12**) [26], one cyclic peptide, cyclo (L-Leu-L-Pro) (**13**) [27], one lignan, (−)-syringaresinol (**14**) [28], one steroid, ergosta-4,6,8(14),22-tetraen-3-one (**15**) [29], and one fatty acid, palmitic acid (**16**) [30] by comparing their spectroscopic data with published literature values, and compounds **5**, **7**, **8**, **10**, and **15** were previously isolated from genus *Hypoxylon* [9].

*Biological Studies.* The eleven isolates present in sufficient amounts (**1**–**6**, **8**, **11**–**13**, and **15**) were screened by examining their inhibitory effects on LPS-induced inducible nitric oxide synthase (iNOS)-dependent NO and IL-6 production in the murine macrophage cell line RAW 264.7 (*Table*). From the results of our above tests, the following conclusions can be drawn: *a*) As shown in the *Table*, compared to quercetin ($IC_{50}$ value $35.9 \pm 1.7$ μM), which was used as positive control in this study, hypoillexidiol (**2**), and (−)-(3*S*)-3-hydroxy-3-methyloxindole (**3**) displayed NO inhibitory activity with $IC_{50}$ values of $17.5 \pm 1.8$ and $24.7 \pm 1.6$ μM, respectively. *b*) Compounds **2**, **4**, **5**, and **12** moderately inhibited IL-6 production with $IC_{50}$ values of $33.2 \pm 4.2$, $35.3 \pm 5.8$, $27.2 \pm 1.8$, and $33.8 \pm 1.2$ μM, respectively. *c*) Compounds **1** and **4**–**6** were less effective NO inhibitors, with $IC_{50}$ values ranging from $42.3 \pm 4.9$ to

Table. *Inhibitory Effects of the Eleven Isolates* (**1**–**6**, **8**, **11**–**13**, and **15**) *from* Hypoxylon *sp. BCRC 12F0687 on LPS-Activated NO and IL-6 Productions in RAW 264.7 Macrophages*

| Compounds | $IC_{50}$ [μM][a]) | |
|---|---|---|
| | NO | IL-6 |
| Hypoxyphenone (**1**) | 54.2 ± 1.5 | > 100 |
| Hypoillexidiol (**2**) | 17.5 ± 1.8 | 33.2 ± 4.2 |
| (−)-(*S*)-3-Hydroxy-3-methyloxindole (**3**) | 24.7 ± 1.6 | 47.7 ± 3.6 |
| (+)-Vermelone (**4**) | 42.3 ± 4.9 | 35.5 ± 5.8 |
| (−)-(*R*)-5-Methylmellein (**5**) | 64.8 ± 12.9 | 27.2 ± 1.8 |
| (−)-(*R*)-5-Formylmellein (**6**) | 42.8 ± 4.8 | > 100 |
| 2-[(3*R**,3a*R**,5*R**,8*R**,8a*S**)-8-Hydroxy-3,8-dimethyldecahydro-azulen-5-yl]propane-1,2-diol (**8**) | > 100 | > 100 |
| 4-Methoxybenzoic acid (**11**) | > 100 | > 100 |
| Tyrosol (**12**) | > 100 | 33.8 ± 1.2 |
| Cyclo (L-Leu-L-Pro) (**13**) | > 100 | > 100 |
| Ergosta-4,6,8(14),22-tetraen-3-one (**15**) | > 100 | > 100 |
| Quercetin[b]) | 35.9 ± 1.7 | 29.5 ± 1.2 |

[a]) The $IC_{50}$ values are represented as means ± SD based on three independent experiments. [b]) Quercetin was used as a positive control.

64.8 ± 12.9 μM, whereas compounds, **8**, **11**–**13**, and **15** were inactive (> 100 μM). *d*) Compound **3** (47.7 ± 3.6 μM) displayed weak IL-6 inhibitory activity. *e*) Furthermore, the RT-PCR analysis in the present study indicated that LPS treatment increased the level of iNOS mRNA expression, and that compounds **2** and **3** inhibited this increase in a concentration-dependent manner. At the highest concentration, none of the compounds tested showed any obvious cytotoxicity towards RAW 264.7 cells. *f*) Cytotoxicities were observed in the cells treated with compounds **8**, **11**–**13**, and **15** (cell viability < 70% at 50 μM), whereas the other compounds had no influence on cell viability indicating that the inhibitory activities of LPS-induced NO production by the active compounds were not resulting from their cytotoxicity.

This work is kindly supported by the *Ministry of Economic Affairs*, Taiwan, R. O. C., to *Food Industry Research & Development Institute* (FIRDI). Thanks are also given to the *Fushan Botanical Garden*, Yilan County, Taiwan, for the help of plant collection.

## Experimental Part

*General*. TLC: Silica gel *60 $F_{254}$* precoated plates (*Merck*). Column chromatography (CC): silica gel *60* (70–230 or 230–400 mesh; *Merck*) and *Spherical C18 100A* Reversed-Phase Silica Gel (*RP-18*) (particle size: 20–40 μm; *Silicycle*). HPLC (anal. And prep.): *Shimadzu* model *LCC-6AD* on *YMC-pack*, *R&D ODS* column (250 × 4.6 mm, 250 × 20 mm) and UV detector *Shimadzu SPD-10AVP*. Optical rotation: *Jasco P-2000* polarimeter; in $CHCl_3$. UV Spectra: *Jasco V-530* UV/VIS spectrophotometer; $\lambda_{max}$ (log $\varepsilon$) in nm. IR Spectra: *Jasco FTIR-4200* spectrophotometer; $\tilde{\nu}$ in $cm^{-1}$. $^1H$-, $^{13}C$-, and 2D-NMR spectra: *Varian-VNMRS*-600, and *Varian-Mercury*-400 spectrometers; $\delta$ in ppm rel. to $Me_4Si$ as internal standard, *J* in Hz. EI-MS: *VG-Biotech Quatro-5022* mass spectrometer; in *m*/*z* (rel. %). ESI-MS and HR-ESI-MS: *Bruker APEX-II* mass spectrometer; in *m*/*z*.

*Fungal Strain and Identification.* The fungal strain 12F 0687 was isolated from the root of *Ilex formosana*, collected in Fushan botanical garden, Yilan County, Taiwan, during November of 2012. Fungal identification was performed based on sequencing of the ITS regions. The fungal strain grew slowly on MEA (malt extract agar) medium at 28°. The color of the colony was white for the first 7 d, and then gradually became gray with black areas and eventually turned to black. Spores were generated after more than 14 d, and only a few spores could be observed by naked eye. The fungal strain was identified as *Hypoxylon* sp. (family Xylariaceae) by Dr. *Sung-Yuan Hsieh*, based on cultural and anamorphic data. The identification was further confirmed by sequence analysis of the rDNA-ITS (internal transcribed spacer) region. A nucleotide-to-nucleotide BLAST (nucleotide sequence comparison program) query of the NCBI database yielded *Hypoxylon* sp. with accession No. AB701369.1 as the closest match to the ITS rDNA of 12F 0687 (100%). The strain is preserved with the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI), under the ID No. 12F 0687.

*Culture Media and Method*. Two-week-old colonies of the *Hypoxylon* sp. strain on malt extract agar (MEA) medium in 9-cm *Petri* dish were cut into the bottle and blended for 30 s with 100 ml of dist. $H_2O$ to prepare the fungal inoculum for liquid fermentation. To each 500-ml flask containing 150 ml of liquid cultural media (ingredients: corn starch, 30 g; corn steep liquor, 10 g; yeast extract, 5 g; and sea salt, 2 g in 1 l of dist. $H_2O$, pH 6) were added 10 ml of fungal inocula and incubated at 25° for 2 weeks on a rotary shaker at the speed of 100 circles/min without illumination. A total of 14.0 l of fungal fermentation broth were harvested and then filtered to remove fungal mycelium.

*Isolation and Characterization of Metabolites.* The liquid fermentation broth ($H_2O$) of *Hypoxylon* sp. 12F 0686 (14 l) was extracted with AcOEt, and the AcOEt-layer (16.0 g) was separated. The $H_2O$-layer was further extracted with BuOH, and the BuOH-layer (7.8 g) was separated. The AcOEt-layer was subjected to CC ($SiO_2$; hexane/acetone gradient) to get obtain eleven fractions (*Frs. 1–11*). There was a large amount of phthalate (11.5 g) remaining before *Fr. 1*, and a large amount of triglycerides (2.3 g) were distributed in *Frs. 1–8*. *Fr. 1* was subjected to MPLC (*RP-18*; MeOH/$H_2O$ 5:1) to produce 17 fractions, *Frs. 1.1–1.17*. *Fr. 1.5* yielded **5** (9.6 mg). *Fr. 1.14* was further purified with prep. TLC (hexane/AcOEt 6:1) to obtain **15** ($R_f$ 0.35; 0.8 mg). *Fr. 3* was subjected to MPLC ($SiO_2$; hexane/AcOEt 6:1) to produce 14 fractions, *Frs. 3.1–3.14*. *Fr. 3.6* was subjected to MPLC ($SiO_2$; hexane/acetone 4:1) to produce four fractions, *Frs. 3.6.1–3.6.4*. *Fr. 3.6.4* was further separated with prep. TLC ($CH_2Cl_2$/AcOEt 10:1) to obtain **6** ($R_f$ 0.83; 0.6 mg), **1** ($R_f$ 0.71; 1.1 mg), **11** ($R_f$ 0.51; 0.4 mg), and **16** ($R_f$ 0.37; 1.7 mg). *Fr. 5* was subjected to MPLC (*RP-18*; acetone/$H_2O$ 1:2) to produce seven fractions, *Frs. 5.1–5.7*. *Fr. 5.1* was subjected to MPLC ($SiO_2$; $CH_2Cl_2$/AcOEt 10:1) to obtain **4** (0.3 mg). *Fr. 6* was subjected to MPLC (*RP-18*; MeOH/$H_2O$ 1:2) to produce eight fractions, *Frs. 6.1–6.8*. *Fr. 6.1* was further purified with prep. TLC (hexane/AcOEt 1:1) to obtain **12** ($R_f$ 0.32; 1.7 mg). *Fr. 6.4* was further purified with prep. TLC (hexane/acetone 2:1) to obtain **7** ($R_f$ 0.40; 1.4 mg). *Fr. 6.7* was subjected to MPLC (*RP-18*; MeOH/$H_2O$ 2:1) to obtain **9** (1.9 mg). *Frs. 7* and *8* were combined after TLC analysis and subjected to MPLC (*RP-18*; MeOH/$H_2O$ 2:1) to produce four fractions, *Frs. 7.1–7.4*. *Fr. 7.2* was subjected to MPLC ($SiO_2$; $CH_2Cl_2$/AcOEt 5:1) to produce 15 fractions, *Fr. 7.2.1–7.2.15*. *Fr. 7.2.4* was further purified with HPLC (*RP-18*; MeOH/$H_2O$ 1:2) to obtain **12** ($t_R$ 12 min; 2 ml/min; 9.7 mg). *Fr. 7.2.10* was subjected to MPLC ($SiO_2$; $CH_2Cl_2$/MeOH 30:1) to obtain **3** (3.1 mg). *Fr. 7.2.12* was subjected to MPLC ($SiO_2$; $CH_2Cl_2$/MeOH 30:1) to obtain **11** (3.2 mg) and **2** (3.6 mg). *Fr. 9* (40 mg) was subjected to $SiO_2$ CC, eluting with $CH_2Cl_2$/AcOEt (15:1), to yield *Frs. 9.1* to *9.3*. *Fr. 9.1* was purified with TLC (*RP-18*; acetone/$H_2O$ 1:1) to obtain **8** ($R_f$ 0.42; 9.9 mg) and **10** ($R_f$ 0.35; 2.4 mg). *Fr. 9.2* was subjected to MPLC ($SiO_2$; $CH_2Cl_2$/MeOH 20:1) to produce ten fractions, *Frs. 9.2.1–9.2.10*. *Fr. 9.2.3* was purified with prep. TLC ($SiO_2$; $CH_2Cl_2$/AcOEt 3:1) to obtain **14** ($R_f$ 0.51; 0.9 mg).

*Hypoxyphenone* (= *Methyl 4-Formyl-3,6-dihydroxy-2-methylbenzoate*; **1**). Yellowish needles. M.p. 149–150°. UV (MeOH): 208 (3.55), 228 (3.61), 266 (3.41), 375 (2.94). UV (MeOH + KOH): 210 (4.27), 243 (3.56), 275 (sh, 3.39), 424 (2.97). IR (KBr): 3244 (OH), 1670 (C=O), 1601, 1479 (aromatic ring). $^1$H-NMR (600 MHz, $CDCl_3$): 10.63 (*s*, HO–C(3)); 9.89 (*s*, CHO); 9.86 (*s*, HO–C(6)); 7.08 (*s*, H–C(5)); 4.02 (*s*, COOMe); 2.46 (*s*, Me(8)). $^{13}$C-NMR ($CDCl_3$, 150 MHz): 196.7 (CHO); 170.7 (C(7)); 152.9 (C(6)); 151.8 (C(3)); 129.2 (C(2)); 122.7 (C(4)); 120.7 (C(1)); 118.1 (C(5)); 52.8 (*Me*O–C(7)); 13.6 (C(8)). ESI-MS: 233 ($[M+Na]^+$). HR-ESI-MS: 233.0425 ($[M+Na]^+$, $C_{10}H_{10}NaO_5^+$; calc. 233.0426).

*Hypoillexidiol* (=*(3*R*,6*S*,7*R*)-1,3,4,5,6,7-Hexahydro-6,7-dihydroxy-3,7-dimethyl-8*H*-2-benzopyran-8-one*; **2**). Colorless oil. $[\alpha]_D^{25} = +231$ ($c = 0.09$, MeOH). UV (MeOH): 240 (3.62). IR (neat): 3412 (OH), 1667 (C=O), 1648 (C=C). $^1$H-NMR (400 MHz, $CDCl_3$): 4.62 (*d*, $J = 15.8$, $H_{eq}$–C(1)); 4.14 (br. *d*, $J = 15.8$, $H_{ax}$–C(1)); 4.00 (*dd*, $J = 10.4$, 5.2, $H_{ax}$–C(5)); 3.71 (br. *s*, HO–C(4)); 3.63 (*dq*, $J = 12.8$, 6.1, $H_{ax}$–C(9)); 2.60 (*dd*, $J = 18.4$, 5.2, $H_{eq}$–C(6)); 2.40 (br. *s*, HO–C(5)); 2.35 (br. *dd*, $J = 18.4$, 10.4, $H_{ax}$–C(6)); 2.19–2.21 (*m*, $H_{ax}$–C(8)); 2.16–2.18 (*m*, $H_{eq}$–C(8)); 1.29 (*d*, $J = 6.1$, Me(10)); 1.26 (*s*, Me(11)). $^{13}$C-NMR ($CDCl_3$, 100 MHz): 199.2 (C(3)); 152.8 (C(7)); 128.3 (C(2)); 77.3 (C(4)); 72.3 (C(5)); 69.3 (C(9)); 63.5 (C(1)); 37.7 (C(8)); 36.1 (C(6)); 21.1 (C(10)); 17.8 (C(11)). ESI-MS: 235 ($[M + Na]^+$). HR-ESI-MS: 235.09406 ($[M + Na]^+$, $C_{11}H_{16}NaO_4^+$; calc. 235.09408).

*(−)-(3*S*)-3-Hydroxy-3-methyloxindole* (=*(3*S*)-1,3-Dihydro-3-hydroxy-3-methyl-2*H*-indol-2-one*; **3**). Colorless needles. $[\alpha]_D^{25} = -31.9$ ($c = 0.16$, MeOH). UV (MeOH): 210 (3.93), 252 (3.38), 285 (2.73). IR (KBr): 3259 (OH, NH), 1717 (C=O), 1623 (amide), 1473 (aromatic ring). $^1$H-NMR ($CDCl_3$, 600 MHz): 7.81 (br. *s*, NH(1)); 7.40 (br. *d*, $J = 7.8$, H–C(5)); 7.26 (*ddd*, $J = 7.8$, 7.8, 1.1, H–C(7)); 7.09 (*ddd*, $J = 7.8$, 7.8, 1.1, H–C(6)); 6.88 (br. *d*, $J = 7.8$, H–C(8)); 2.85 (*s*, HO–C(3)); 1.60 (*s*, Me(10)). $^{13}$C-NMR ($CDCl_3$, 150 MHz): 180.2 (C(2)); 139.7 (C(9)); 131.7 (C(4)); 129.7 (C(7)); 123.9 (C(5)); 123.3 (C(6)); 110.2 (C(8)); 73.9 (C(3)); 24.8 (C(10)). ESI-MS: 164 ($[M + H]^+$). HR-ESI-MS: 186.0522 ($[M + Na]^+$, $C_9H_9NaNO_2^+$; calc. 186.0525).

*(+)-Vermelone* (=*(3*S*)-3,4-Dihydro-3,8-Dihydroxynaphthalen-1(2*H*)-one*; **4**). Brownish powder. $[\alpha]_D^{25} = +37.6$ ($c = 0.011$, EtOH). UV (MeOH): 223 (3.47), 260 (4.48), 315 (3.75). IR (KBr): 3423 (OH), 1633 (C=O), 1578, 1455 (aromatic ring). $^1$H-NMR ($CDCl_3$, 600 MHz): 12.25 (*s*, HO–C(8)); 7.41 (*dd*, $J =$ 7.9, 7.9, H–C(6)); 6.84 (*d*, $J = 7.9$, H–C(7)); 6.75 (*dd*, $J = 7.9$, 0.9, H–C(5)); 4.55 (*m*, H–C(3)); 3.23 (*dd*, $J = 16.5$, 4.2, $H_{eq}$–C(4)); 3.02 (*dd*, $J = 16.5$, 7.8, $H_{ax}$–C(4)); 2.98 (*ddd*, $J = 17.1$, 3.6, 1.2, $H_{eq}$–C(2)); 2.79 (*ddd*, $J = 17.1$, 7.8, 0.6, $H_{ax}$–C(2)). ESI-MS: 179 ($[M + H]^+$). HR-ESI-MS: 201.0530 ($[M + Na]^+$, $C_{10}H_{10}NaO_3^+$; calc. 201.0529).

*Determination of NO and IL-6 Production and Cell Viability Assay.* Mouse macrophage cell line (RAW 264.7) was obtained from Bioresource Collection and Research Center (BCRC 60001) and cultured at 37° in *Dulbecco*'s Modified *Eagle*'s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; *Gibco*), 4.5 g/l glucose, 4 mm glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) in a humidified atmosphere in a 5% $CO_2$ incubator. The cells were treated with 10, 25, and 50 mM natural products in the presence of 1 mg/ml Whitish LPS (*Sigma–Aldrich*) for 20 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by *Griess* reagent assay [31], and cell viabilities were determined using the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay as described in [32]. Levels of IL-6 production were measured in cell culture supernatants with a *Mouse IL-6* ELISA Ready-SET-Go ELISA kit (*eBiosciencee*) according to the manufacturer recommendations and quantified with a microplate reader (*m-Quant*; *Bio-Tek Instruments Inc.*).

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*Received February 24, 2015*