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BRIEF COMMUNICATION

Anti-inflammatory phomalichenones from an endolichenic fungus *Phoma* sp.

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Abstract

Four new compounds, phomalichenones A–D (**1**–**4**), and seven known compounds (**5**–**11**) were isolated from the cultures of an endolichenic fungus *Phoma* sp. EL002650. Their structures were determined by the analysis of their spectroscopic data (NMR and MS). Compounds **1** and **6** inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In addition, compound **1** diminished the protein expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and decreased the mRNA expression levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin(IL)-1β, and IL-6.

Lichens are composite organisms consisting of a fungal organism (mycobiont) and a photosynthesizing organism (photobiont), such as algae or cyanobacteria [1, 2]. Endolichenic fungi are found living with lichen-forming fungi similarly to endophytic fungi living symbiotically with the healthy tissues of plants [2–4]. Since metabolites from endolichenic fungi were first reported 10 years ago, research on endolichenic fungal secondary metabolites has increased, and endolichenic fungi have become a proven source of bioactive secondary metabolites [2], including alkaloids [5, 6], quinones [7–10], peptides [11], chromones [12], and terpenes [13–16]. These metabolites have shown antiviral, antibacterial, antifungal, and anti-Alzheimer's disease activities. We investigated new bioactive compounds from the endolichenic fungus *Phoma* sp. EL002650. The fungal strain *Phoma* sp. was cultured in potato dextrose broth (PDB, 5 l) for 7 days at 25 °C, and the broth and mycelia extracts were partitioned with EtOAc/H_2O. Four new phomalone derivatives phomalichenones A–D (**1**–**4**) and seven known (**5**–**11**) compounds were separated from the EtOAc extract. Herein, we describe their isolation, structure elucidation, and biological activities.

Compound **1** was obtained as a yellow amorphous powder. The molecular formula of **1** was deduced as $C_{13}H_{16}O_4$ based on the analysis of the HRESIMS and NMR data. The ^{1}H, ^{13}C, and DEPT data in conjunction with the HSQC-DEPT spectrum of **1** suggested the presence of 13 carbons, containing one carbonyl carbon (δ_C 191.8), five nonprotonated carbons (δ_C 164.6, 162.9, 160.4, 109.4, and 103.8), three olefinic methine carbons (δ_C 142.1, 131.7, and 90.9), one methoxy carbon (δ_C 55.5), one methylene carbon (δ_C 15.1), and two methyl carbons (δ_C 18.3, and 13.5). The ^{1}H NMR data of **1** indicated the presence of two coupled olefinic protons (δ_H 7.23 and 6.94), one singlet olefinic proton (δ_H 6.06), one methoxy proton (δ_H 3.79), one methylene proton (δ_H 2.45), one doublet methyl proton (δ_H

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Table 1 NMR spectroscopic data for **1**, **2**, **3**, and **4** in DMSO-d_6 at 700 MHz for ^{1}H and 175 MHz for ^{13}C

	1		**2**		**3**		**4**	
Position	δ_C, type	δ_H (mult, *J* in Hz)	δ_C, type	δ_H (mult, *J* in Hz)	δ_C, type	δ_H (mult, *J* in Hz)	δ_C, type	δ_H (mult, *J* in Hz)
1	191.8, C		204.1, C		181.4, C		175.9, C	
2	131.7, CH	7.23 (d, 14.7)	39.6, CH_2	2.93 (t, 7.4)	107.6, CH	6.10 (s)	110.6, CH	5.88 (s)
3	142.1, CH	6.94 (dq, 14.7, 7.0)	27.9, CH_2	1.71 (m)	166.8, C		162.7, C	
4	18.3, CH_3	1.92 (d, 7.0)	60.4, CH_2	3.43 (t, 6.4)	19.8, CH_3	2.32 (s)	19.1, CH_3	2.26 (s)
5	103.8, C		103.4, C		102.4, C		106.9, C	
6	160.4, C		161.1, C		155.9, C		157.9, C	
7	90.9, CH	6.06 (s)	91.2, CH	5.98 (s)	93.2, CH	6.31 (s)	95.8, CH	6.42 (s)
8	162.9, C		165.1, C		158.9, C		159.5, C	
9	109.4, C		104.7, C		108.6, C		109.1, C	
10	164.6, C		164.3, C		164.4, C		156.7, C	
1´	15.1, CH_2	2.45 (q, 7.4)	26.1, CH_2	2.63 (t, 7.7)	26.1, CH_2	2.71 (t, 7.0)	15.6, CH_2	2.64 (q, 7.4)
2´	13.5, CH_3	0.98 (t, 7.4)	60.1, CH_2	3.38 (t, 7.7)	59.8, CH_2	3.44 (t, 7.0)	13.8, CH_3	1.07 (t, 7.4)
1-OCH_3	55.5, CH_3	3.79 (s)	55.4, CH_3	3.77 (s)			55.5, CH_3	3.72 (s)

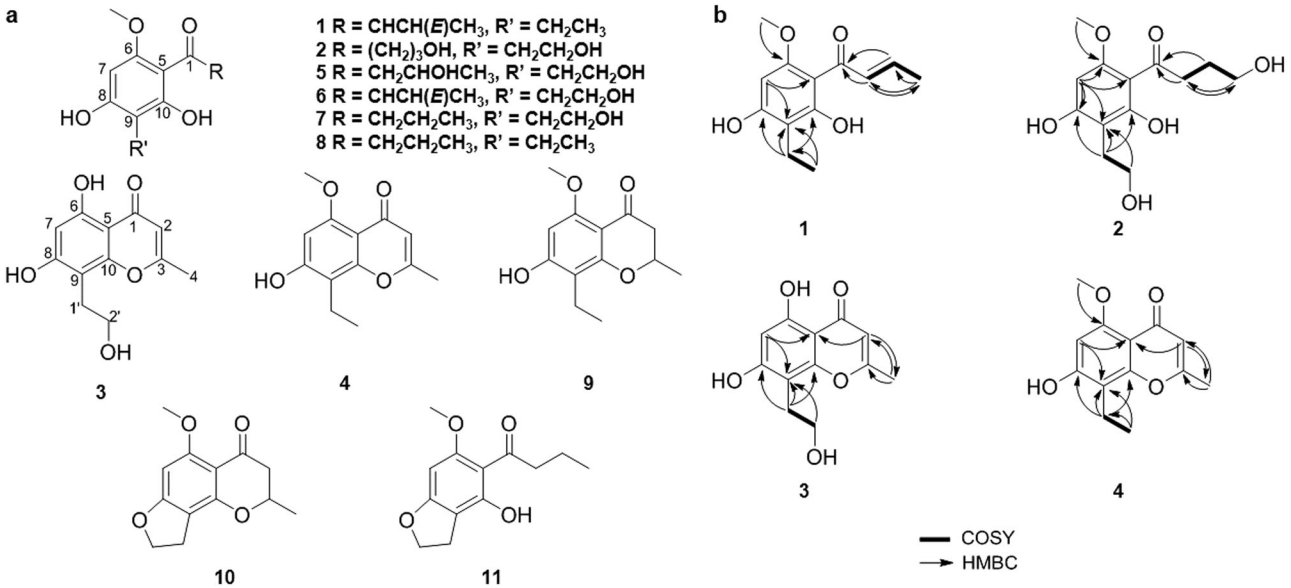

Fig. 1 (**a**) Structures of **1**–**11**, (**b**) Key 2D correlations of compounds **1**–**4**

1.92), and one triplet methyl proton (δ_H 0.98) (Table 1). The interpretation of the 2D NMR data, including the COSY, HSQC-DEPT, and HMBC spectra, led to the construction of the planar structure of **1** (Fig. 1b). The COSY correlations of H_2-1´/H_3-2´ and the HMBC correlations of H_3-2´ to C-1´ and C-9 and H_2-1´ to C-8, C-9, and C-10 indicated the presence of an ethyl side chain that was connected at C-9. Another side chain was established by the COSY correlations of H-2/H-3/H_3-4 and the HMBC correlations of H-2 to C-1 and C-4, H-3 to C-1, and H_3-4 to C-2. Thus, the planar structure of phomalichenone A (**1**) was assigned as shown in Fig. 1. The large coupling constants ($J_{H2–H3} = 14.7$ Hz) revealed that the H-2 and H-3 double bond had a *trans* configuration.

Compound **2** was isolated as a white amorphous powder. Its molecular formula was established as $C_{13}H_{18}O_6$ by the HRESIMS and NMR data. The ^{1}H and ^{13}C NMR data of **2** were closely similar to those of phomalone (**7**) (Supplementary Figures S8 and S35). The different resonance was an oxymethylene at C-4, which was confirmed by the

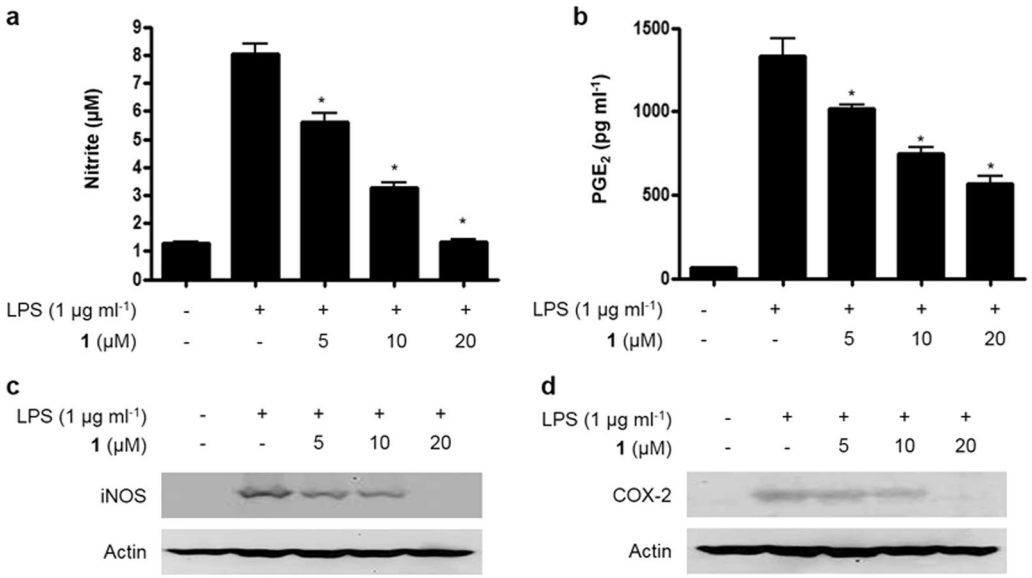

Fig. 2 Effects of **1** on nitrite content (**a**), PGE_2 production (**b**), and protein expression levels of iNOS and COX-2 in RAW264.7 cells stimulated with LPS (**c**, **d**). The cells were pre-treated for 3 h with the indicated concentrations of **1** and stimulated for 24 h with LPS (1 μg ml^{-1}). $^*p < 0.05$ compared with the group treated with LPS

COSY correlation of H_2-2/H_2-3/H_2-4. The hydroxylation of C-4 was supported by the deshielded signals of C-4 (δ_C 60.4) and H_2-4 (δ_H 3.43). Therefore, the structure of **2** was designated as phomalichenone B (**2**).

Compound **3** was isolated as a white amorphous powder. The molecular formula of **3** was determined to be $C_{12}H_{12}O_5$ by the HRESIMS and NMR data. The analysis of the ^{13}C NMR and HSQC-DEPT data suggested the presence of 12 carbons, comprising one carbonyl carbon (δ_C 181.4), six nonprotonated carbons (δ_C 166.8, 164.4, 158.9, 155.9, 108.6, and 102.4), two olefinic carbons (δ_C 107.6 and 93.2), two methylene carbons (δ_C 59.8 and 26.1), and one methyl carbon (δ_C 19.8). The ^1H NMR data indicated two single olefinic protons (δ_H 6.31 and 6.10), one oxymethylene proton (δ_H 3.44), one methylene proton (δ_H 2.71), and one methyl proton (δ_H 2.32) (Table 1). The HMBC correlations of H-2 to C-4 and C-5 and H-7 to C-5 and C-9 established the chromen-4-one structure. The COSY correlations of H_2-1′/H_2-2′ and the HMBC correlations of H_2-2′ to C-1′ and C-9 and H_2-1′ to C-8, C-9, and C-10 indicated the presence of a hydroxyethyl side-chain that was connected at C-9. Thus, the planar structure of **3** was assigned as a new member of the chromone family and designated as phomalichenone C (**3**).

Compound **4** was obtained as a white amorphous powder. The molecular formula of **4** was deduced as $C_{13}H_{14}O_4$ by the HRESIMS and NMR data. The ^1H and ^{13}C NMR spectra of **4** showed similarity to those of **3**. The different resonances were a methoxy group of C-6 and a methyl at C-2′, which were supported by the HMBC correlation of 6-OCH_3 to C-6 and the COSY correlation of H_2-1′/H_3-2′ (Fig. 1b). The structure of **4** was similar to that of **3** and designated as phomalichenone D (**4**).

Compound **5** is known, although the NMR data of **5** was not reported [17]. We report the NMR data of (2,4-dihydroxy-3-(2-hydroxyethyl)-6-methoxyphenyl)-3-hydroxybutan-1-one (**5**) in this study (Supplementary Figures S29–33).

The other compounds were determined to be (*E*)-1-(2,4-dihydroxy-3-(2-hydroxyethyl)-6-methoxyphenyl)but-2-en-1-one (**6**) [18], phomalone (**7**) [18], deoxyphomalone (**8**) [18], 8-ethyl-7-hydroxy-5-methoxy-2-methylchroman-4-one (**9**) [19], LL-D253γ (**10**) [19], and 4-hydroxy-6-methoxy-5-(1'-oxobutyl) benzodihydrofuran (**11**) [18] by comparison of our data with that in the published literature.

To evaluate the anti-inflammatory effect of compounds **1**–**11**, we investigated their inhibitory effects on the nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. Cytotoxic effects of **1**–**11** on RAW264.7 cells were evaluated, cell viability was not altered by the exposure to **1**–**11** at concentrations of 5–40 μM for 24 h; however, cell viability decreased by the exposure to **1** at a concentration of 80 μM for 24 h (Supplementary Figure S40). **1** and **6** significantly inhibited the NO production with IC_{50} values of 9.4 ± 0.5 and 7.4 ± 2.8 μM, respectively, whereas **2**–**5** and **7**–**11** were inactive (Fig. 2a and Supplementary Table S1). This result suggests that the presence of the double bond on the side chain may play an important role for the inhibitory effects of NO production in LPS-stimulated RAW264.7 cells. Under the same conditions, compound **1** decreased PGE_2 production in a dose-dependent manner measured by enzyme

immunoassay with IC_{50} values of 12.7 ± 1.5 μM (Fig. 2b). The overproduction of NO and PGE_2 is associated with the overexpression of inducible nitric oxide synthesis (iNOS) and cyclooxygenase-2 (COX-2) in LPS-induced RAW264.7 cells. In the Western blot analysis, the protein expression levels of iNOS and COX-2 in RAW264.7 cells were significantly up-regulated in response to LPS, while **1** suppressed iNOS and COX-2 protein expression in LPS-treated cells in a dose-dependent manner (Fig. 2c,d). Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, have been reported to be important mediators of inflammation [20]. To further examine the anti-inflammatory effect of **1** in LPS-induced RAW264.7 cells, the mRNA expression levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were estimated by RT-qPCR analysis in the cells stimulated with LPS (1 μg ml^{-1}) for 6 h. The transcript levels of IL-1β, IL-6, and TNF-α were decreased in a dose-dependent manner in LPS-treated RAW264.7 cells (Supplementary Figure S41). The nuclear factor-kappa B (NF-κB) is known to play a key role in the expression of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α and interleukines [21]. Therefore, it is proposed that **1** might suppress the activation of NF-κB in LPS-induced RAW264.7 cells.

In summary, phomalichenones A–D (**1**–**4**), from endolichenic fungus *Phoma* sp. EL002650, are new members of the phomalone derivatives and chromone skeleton. In the evaluation of the anti-inflammatory effects of the isolated compounds, compounds **1** and **6** suppressed the production of NO in LPS-stimulated RAW264.7 cells. Especially, compounds **1** and **6** have a double bond on their side chain. Although the structure–activity relationships of phomalone derivatives with a double bond have not been thoroughly investigated, our results suggest that the presence of a double bond on the side chain may be important for the inhibitory effect against NO production. In addition, the anti-inflammatory effect of **1** was confirmed by observing that **1** inhibited the production of PGE_2 and suppressed the protein levels of iNOS and COX-2. Also, **1** blocked the mRNA transcription of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW264.7 cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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