

## TWO NEW ANTIBACTERIAL NAPHTHOQUINONES FROM A CIGAR TOBACCO-DERIVED ENDOPHYTIC *Fusarium solani*

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In this study, two new (**1** and **2**), together with five known (**3–7**) naphthoquinones were isolated from the cigar tobacco-derived endophytic *Fusarium solani*. Their structures were determined by means of HR-ESI-MS and extensive 1D and 2D NMR spectroscopic studies. The new compounds were evaluated for antibacterial activities against *Pseudomonas syringae* (the main pathogenic sources of tobacco angular leaf spot disease). Interestingly, compounds **1** and **2** exhibited inhibitory effects with MIC<sub>50</sub> values of 6.8 and 5.9 µg/mL, respectively. These rates are lower than that of the positive control (agricultural streptomycin, with MIC<sub>50</sub> of 2.2 µg/mL).

**Keywords:** naphthoquinones, cigar tobacco, fungi *Fusarium solani*, antibacterial activities.

Endophytes are microorganisms that inhabit the interior of plants (especially the leaves, stems, and roots) and do no apparent harm to their host [1]. Recently, endophytes have been viewed as an outstanding source of secondary metabolites, and many scientists have shown interest in studying new biologically natural products from endophytes [2, 3]. Among the numerous existing natural products, naphthoquinones are considered privileged structures in medicinal and pesticide chemistry owing to their structural properties [4–6]. They are present in various families of plants and microorganisms, and serve as vital links in the electron transport chains in the metabolic pathway, participating in multiple biological oxidative processes [6, 7]. In addition, the interest of many investigators in this class of compounds is due to their broad-range biological action, such as phytotoxic [8, 9], insecticidal [10–12], antibacterial [13–15], and fungicidal [16–18].

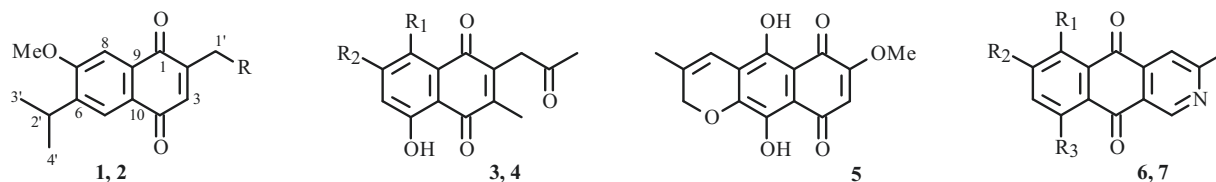
With their ability to colonize a large variety of plants species, *Fusarium* represents a large cosmopolitan genus comprising more than 70 species capable of producing a wide array of active metabolites [19, 20]. Among the numerous existing endophytic fungi in *Fusarium* genus, *Fusarium solani* constitutes one of the most prolific sources of natural products, and which had also been proven to be an important source of naphthoquinones [20–22]. In a continuation of our search for bioactive secondary metabolites from endophytic fungi, a *Fusarium solani* strain isolated from cigar tobacco was studied. The chemical investigation of the ethyl acetate extract of its fermentation products led to the isolation of two new (**1** and **2**) along with five known (**3–7**) naphthoquinones. We report herein the isolation and structural elucidation of the above metabolites, and the antibacterial activities against *Pseudomonas syringae* (the main pathogenic sources of tobacco angular leaf spot disease) of the new naphthoquinones.

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TABLE 1.  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  (125 MHz) NMR Data of Compounds **1** and **2** ( $\text{CDCl}_3$ ,  $\delta$ , ppm, J/Hz)

C atom	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	–	183.5 (C)	–	184.9 (C)
2	–	146.7 (C)	–	149.3 (C)
3	7.47 (s)	138.9 (CH)	7.37 (s)	135.9 (CH)
4	–	185.6 (C)	–	183.2 (C)
5	7.65 (s)	128.4 (CH)	7.67 (s)	128.0 (CH)
6	–	142.4 (C)	–	142.6 (C)
7	–	163.0 (C)	–	163.2 (C)
8	8.40 (s)	108.3 (CH)	8.38 (s)	108.5 (CH)
9	–	132.1 (C)	–	132.0 (C)
10	–	125.7 (C)	–	125.9 (C)
1'	2.25 (s)	16.3 ( $\text{CH}_3$ )	4.33 (s)	61.8 ( $\text{CH}_2$ )
2'	3.14 (m)	28.6 (CH)	3.17 (m)	28.4 (CH)
3', 4'	1.23 (d, $J = 6.8$ )	23.9 ( $\text{CH}_3$ )	1.24 (d, $J = 6.8$ )	23.7 ( $\text{CH}_3$ )
7-OMe	3.74 (s)	56.1 ( $\text{CH}_3$ )	3.75 (s)	56.1 ( $\text{CH}_3$ )



**1:** R = H; **2:** R = OH; **3:** R<sub>1</sub> = OH, R<sub>2</sub> = OMe; **4:** R<sub>1</sub> = H, R<sub>2</sub> = OH  
**6:** R<sub>1</sub> = OH, R<sub>2</sub> = OMe, R<sub>3</sub> = H; **7:** R<sub>1</sub> = H, R<sub>2</sub> = OH, R<sub>3</sub> = OMe

The whole culture broth of *F. solani* was extracted with EtOAc, and then the extract was partitioned between EtOAc and  $\text{H}_2\text{O}$ . The EtOAc-soluble layer was subjected repeatedly to column chromatography on silica gel, MCI, RP-18, and preparative HPLC to afford compounds **1–7**, including two new naphthoquinones, 6-isopropyl-7-methoxy-2-methyl-1,4-naphthoquinone (**1**) and 2-(hydroxymethyl)-6-isopropyl-7-methoxy-1,4-naphthoquinone (**2**), along with five known ones (**3–7**). The NMR data of **1** and **2** were listed in Table 1. The new compounds were confirmed by a search of the newly updated SciFinder database (an electronic database for chemical structure published by the American Chemical Society). The known compounds, javanicin (**3**) [23], 5,7-dihydroxy-3-methyl-2-(2-oxopropyl)naphthalene-1,4-dione (**4**) [24], anhydrofusarubin (**5**) [23], bostrycoidin (**6**) [25], and 7-desmethylscorpinone (**7**) [26], were identified by comparison of their spectroscopic data with the literatures.

Compound **1** was obtained as a yellow powder, and its molecular formula was determined as  $\text{C}_{15}\text{H}_{16}\text{O}_3$  according to the HR-ESI-MS peak at  $m/z$  267.0987 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (calcd 267.0992) and NMR data (Table 1), requiring 8 degrees of unsaturation. The IR spectrum displayed the absorption bands of carbonyl (at  $1668\text{ cm}^{-1}$ ) and aromatic group ( $1632$ ,  $1457$ , and  $1346\text{ cm}^{-1}$ ). The UV spectrum showed absorption maxima at 262 and 328 nm, also suggesting the presence of an aromatic ring in the molecule.

The  $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT NMR data of **1** displayed resonances for 15 carbons and 16 hydrogen atoms, which were ascribed to a 1,2,4,5-tetrasubstituted benzene ring (C-5–C-10, H-5, and H-8), two carbonyls (C-1 and C-4), a pair of double bonds (C-2, C-3, and H-3), a methyl group (C-1',  $\text{H}_3$ -1'), an isopropyl group ( $-(\text{CH})(\text{CH}_3)_2$ , C-2'–C-4', H-2', and  $\text{H}_6$ -3', 4') [27], and a methoxy group ( $\delta_{\text{C}}$  56.1,  $\delta_{\text{H}}$  3.74 s). By further analysis of the above NMR data, the double bonds and two carbonyls should be incorporated with the benzene ring to form a 1,4-naphthoquinone to support the existence of 8 degrees of unsaturation. In addition, the existence of 1,4-naphthoquinone moiety can also further be confirmed by the HMBC correlations (Fig. 1) from H-3 to C-1/C-4/C-10, from H-5 to C-4/C-9/C-10, from H-8 to C-1/C-9/C-10.

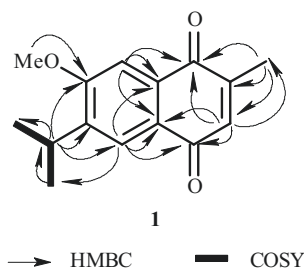


Fig. 1. Key HMBC correlations and  $^1\text{H}$ - $^1\text{H}$  COSY of **1**.

Since the 1,4-naphthoquinone skeleton of **1** was determined unambiguously, the remaining signals (methyl, isopropyl, and methoxy groups) can be considered as substituents, and the existence of an isopropyl group was also supported by the comparison of typical proton signals ( $\delta_{\text{C}}$  28.6 d and 23.9 q;  $\delta_{\text{H}}$  3.14 m (CH), and 1.23 (d,  $J = 6.8$  Hz,  $(\text{CH}_3)_2$ ) with known compounds [27], the HMBC correlations from  $\text{H}_6\text{-3'}$ ,  $4'$  to C-2', from H-2' to C-3', C-4', and  $^1\text{H}$ - $^1\text{H}$  COSY correlations of  $\text{H}_3\text{-3'}$ /H-2'/ $\text{H}_3\text{-4'}$  (Fig. 1). Moreover, the isopropyl linked to C-6 was established by the HMBC correlations from H-5 to C-2', from H-2' to C-5/C-6/C-7, and from  $\text{H}_6\text{-3'}$ ,  $4'$  to C-6. Moreover, the methyl located at C-2 was established by the HMBC correlations from  $\text{H}_3\text{-1'}$  to C-1/C-2/C-3, from H-3 to C-1'. Finally, the position of the methoxy group at C-7 was supported by an HMBC correlation from methoxy protons ( $\delta_{\text{H}}$  3.74 s) to C-7. Hence, the structure of **1** was fully assigned, and given the systematic name of 6-isopropyl-7-methoxy-2-methyl-1,4-naphthoquinone.

2-(Hydroxymethyl)-6-isopropyl-7-methoxy-1,4-naphthoquinone (**2**), was also obtained as a yellow powder, and its molecular formula was determined to be  $\text{C}_{15}\text{H}_{16}\text{NaO}_4$  by HR-EI-MS ( $m/z$  283.0939 [ $\text{M} + \text{Na}$ ] $^+$ ). The  $^1\text{H}$  and  $^{13}\text{C}$  spectral data of **2** depict a similar structure to compound **1**. The obvious chemical shift differences resulted from the disappearance of a methyl resonance ( $\delta_{\text{C}}$  16.3 q and  $\delta_{\text{H}}$  2.25 s), and the appearance of a hydroxymethyl signal ( $\delta_{\text{C}}$  61.8 t,  $\delta_{\text{H}}$  4.33 s). These changes led us to speculate that the methyl at C-6 in **1** is converted into a hydroxymethyl group [23] in **2**. The position of the hydroxymethyl at C-2 was determined by the HMBC correlations from  $\text{H}_2\text{-1'}$  to C-1/C-2/C-3, from H-3 to C-1'. In addition, the position of the isopropyl group at C-6 and the methoxy group at C-7 can also be determined by further analysis of its HMBC correlations. Therefore, the structure of **2** was determined.

Since certain of the naphthoquinones exhibit potential antibacterial activities [13–15], and the bacteria, *Pseudomonas syringae* pv. *angulata* is the main cause of tobacco angular leaf spot disease [28]. Compounds **1** and **2** were tested for their anti-*Pseudomonas syringae* activity. The compounds were assayed for their antimicrobial activities in 96-well plates according to the literatures [29, 30]. Compounds **1** and **2** showed activities with  $\text{MIC}_{50}$  values of 6.8 and 5.9  $\mu\text{g/mL}$ , respectively, whereas the  $\text{MIC}_{50}$  value of the positive control (agricultural streptomycin) is 2.2  $\mu\text{g/mL}$ .

## EXPERIMENTAL

**General.** UV and IR (KBr) spectra were obtained on an UV-1900 spectrophotometer (Shimadzu, Kyoto, Japan) and a FTS185 spectrophotometer (Bio-Rad, Hercules, CA, USA). NMR experiments were carried out on Bruker DRX-500 NMR spectrometer (Bruker, Karlsruhe, Germany) with TMS as the internal standard. ESI-MS and HR-ESI-MS analyses were performed on a 6540 Q-TOF mass spectrometer equipped with an Agilent 1290 UPLC (Agilent Technologies, Wilmington, DE, USA). 80–100 mesh or 200–300 mesh silica gel (Qingdao Marine Chemical, Qingdao, China) and 75–150  $\mu\text{m}$  MCI CHP20P gel (Mitsubishi Chemical Corporation, Tokyo, Japan) was used for normal column chromatography. The fractions were monitored by thin-layer chromatography (Qingdao Marine Chemical, Inc., Qingdao, China), and the spots were visualized by heating silica gel plates (approximately 120°C) sprayed with 5%  $\text{H}_2\text{SO}_4$  in ethanol. Semipreparative HPLC was performed with an Agilent 1260 preparative liquid chromatography (Agilent Technologies, Wilmington, DE, USA) with a Venusil MP  $\text{C}_{18}$  column (5  $\mu\text{m}$ , 2.0 cm  $\times$  25 cm, Bonna-Agela, Tianjin, China) or a Zorbax PrepHT GF  $\text{C}_{18}$  column (5  $\mu\text{m}$ , 2.12 cm  $\times$  25 cm; Agilent, Palo Alto, USA).

**Fungal Material.** The culture of *Fusarium solani* YNNI-21-48 was isolated from the leaves of cigar tobacco, which was collected from the fermentation plant of Yuanjiang County, Yuxi Prefecture, Yunnan Province, in 2021. The strain was identified by one of authors (Dr. Yin-Ke Li) based on the analysis of the ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28°C. Agar plugs were inoculated into 250 mL Erlenmeyer flasks each containing 100 mL

potato dextrose broth and cultured at 28°C on a rotary shaker at 180 rpm for 5 days. Large-scale fermentation was carried out in 100 Fernbach flasks (1.0 L) each containing of 500 g of rice and 300 mL nutrient solution (glucose 5%; peptone 0.15%; yeast 0.5%; KH<sub>2</sub>PO<sub>4</sub> 0.05%; urea 0.1%; MgSO<sub>4</sub> 0.05% in 1.0 L of deionized water; pH 6.5 before autoclaving). Each flask was inoculated with 5.0 mL of cultured broth and incubated at 27°C for 20 days.

**Extraction and Isolation.** The whole culture broth of *F. solani* was extracted four times with EtOH (4 × 20 L) at room temperature and filtered. The extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated Na<sub>2</sub>CO<sub>3</sub> aq. and extracted with EtOAc again. The crude extract (88.6 g) was applied to silica gel column chromatography, eluting with a CHCl<sub>3</sub>–MeOH gradient system (9:1, 8:2, 7:3, 6:4, 5:5). Five fractions were obtained from the silica gel column and individually decolorized on MCI gel to yield fractions A–E. The further separation of Fr. B (8:2, 7.84 g) by silica gel column chromatography, eluted with CHCl<sub>3</sub>–(Me)<sub>2</sub>CO (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixture subfractions B1–B5. Subfraction B2 (8:2, 1.84 g) was subjected to RP-18 column chromatography (MeOH–H<sub>2</sub>O, 30:70–70:30 gradient) and HPLC to give **1** (18.5 mg) and **2** (22.6 mg). Subfraction B4 (6:4, 2.15 g) was subjected to RP-18 column chromatography (MeOH–H<sub>2</sub>O, 20:80–65:35 gradient) and HPLC to give **3** (18.9 mg), **4** (22.5 mg), **5** (14.8 mg), **6** (26.2 mg), and **7** (19.3 mg).

**Antibacterial Assays.** The strain of bacteria (*Pseudomonas syringae* pv. *angulata*) was obtained from Yunnan Academy of Tobacco Agricultural Sciences. The antibacterial activities were tested by a serial dilution technique using 96-well microtiter plates [29, 30], using agricultural streptomycin (a commercial product for plant bacteria disease in China) as a positive control. The tested compounds and positive control were dissolved in DMSO to give a stock solution.

**6-Isopropyl-7-methoxy-2-methyl-1,4-naphthoquinone (1)**, yellow powder, C<sub>15</sub>H<sub>16</sub>O<sub>3</sub>. UV (MeOH, λ<sub>max</sub>, nm) (log ε): 215 (3.98), 262 (3.64), 328 (3.38). IR (ν<sub>max</sub>, cm<sup>-1</sup>): 2985, 2938, 2872, 1668, 1632, 1457, 1346, 1279, 1182, 810. For <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 267 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* 267.0987 [M + Na]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>16</sub>NaO<sub>3</sub>, 267.0992).

**2-(Hydroxymethyl)-6-isopropyl-7-methoxy-1,4-naphthoquinone (2)**, yellow powder, C<sub>15</sub>H<sub>16</sub>O<sub>4</sub>. UV (MeOH, λ<sub>max</sub>, nm) (log ε): 215 (4.02), 266 (3.68), 333 (3.42). IR (ν<sub>max</sub>, cm<sup>-1</sup>): 3385, 2980, 2931, 2876, 1672, 1638, 1339, 1273, 1179, 836. For <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 283 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* 283.0939 [M + Na]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>16</sub>NaO<sub>4</sub>, 283.0941).

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