Original article

2-(2-Phenylethyl)chromones from Endophytic Fungal Strain Botryosphaeria rhodina A13 from Aquilaria sinensis

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ABSTRACT

Objective To study the characteristic 2-(2-phenylethyl)chromone components of endophytic fungal strain of Aquilaria sinensis by solid culture. Methods The compounds were isolated by various chromatographic methods such as silica gel, reverse-phase silica gel, Sephadex-LH20 column chromatography as well as crystallization. Results Seven 2-(2-phenylethyl)chromone analogues were isolated from the solid culture of Botryosphaeria rhodina A13. Their structures were established by spectral data as well as physicochemical properties, and identified as 6-hydroxy-7-methoxy-2-(2-phenylethyl)chromone (1), 6,7-dimethoxy-2-(2-phenylethyl)chromone (2), (5S,6R,7S,8R)-2-(2-phenylethyl)-5,6,7,8-tetrahydrchromone (3), 6-hydroxy-2-(2-phenylethyl)chromone (4), 4′-hydroxy-2-(2-phenylethyl)chromone (5), 6-methoxy-2-phenethyl-4H-chromen-4-one (6), and 6-methoxy-2-(4′-methoxy-phenethyl)-4H-chromen-4-one (7). Conclusion All of the compounds are isolated for the first time from the genus Botryosphaeria. This research opens up a new vista to produce the characteristic components of agarwood by endophytic fungi.

Key words agarwood; Aquilaria sinensis; Botryosphaeria rhodina A13; 2-(2-phenylethyl)chromones; solid culture

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1. Introduction

Agarwood represents the frequently encountered incense which is widely used in many traditionally meaningful usages such as fragrances, medicines, aromatherapy, and religious ceremonies (CITES, 2004; 2005a; 2005b). It was also well

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known as a famous traditional Chinese medicine to be used as clinical sedative, carminative, and antieptic drug (Naef, 2011). The precious, high-priced, and fragrant agarwood is also called Cheixiang in China, Gaharu and Kalambak in Malaysia, Kanankoh and Jinkoh in Japan (Naef, 2011). The natural agarwood is extremely valuable and usually obtained from certain trees in the genus Aquilaria Lam., Thymelaeaceae family (Rogers, 2011). The healthy wood of Aquilaria Lam. trees is white, soft, and without scented resins. It is widely accepted that the dark resinous material of Aquilaria is created as a response to natural injury by lightning strike, animal grazing, insect attack, and microbial invasion (Blanchette and Heuveling, 2009). Agarwood formation occurs slowly and infrequently in the nature and the supply of agarwood from wild sources is far less than market demand. In the recent years, the huge demand for the agarwood has led to Aquilaria spp. being endangered and listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (CITES, 2004). Due to its significant utilities and economic value, considerable efforts aimed at preserving natural Aquilaria Lam. populations were also conducted (Soeharono et al, 2004). Chemical analyses of agarwood extracts and essential oils showed a very complex matrix containing agarofurans, cadinanes, eudesmanes, valencanes, guaianes, prezizanes, vetispiranes, 2-(2-phenylethyl) chromones, tetra-hydro-2-(2-phenylethyl) chromones as well as other volatile aromatic compounds (Naef, 2011). Among which, 2-(2-phenylethyl)chromone was one of the most abundant constitutes (Chen et al, 2012) and considered to be the critical one responsible for the quality and various biological activities of agarwood (Yoon et al, 2006; Liu et al, 2008; Dai et al, 2009; Yang, 1998).

Since 1930, a few endophytic fungi have been isolated from Aquilaria Lam. trees, such as Epicoccum granulatum (Battacharya et al, 1952), Botryosphaeria rhodina (Mohamed et al, 2010), Fusarium sp. (Mohamed et al, 2010), and Trichoderma sp. (Mohamed et al, 2010). In our previous work, an endophytic fungal strain Botryosphaeria rhodina A13 (Figure 1) was successfully isolated from Aquilaria sinensis (Lour.) Gilg (Wang et al, 2009), and it could induce excised twigs of A. sinensis to produce 5,9-dimethyl-2-(1-methylene)deoxy-1-cyclodecanol, a sesquiterpene of agarwood (Tao et al, 2012). Based on the discovery of sesquiterpene from endophytic fungal strain B. rhodina A13, a further extensive chemical constituent investigation in the fungal strain led to the isolation of seven 2-(2-phenylethyl) chromones, including 6-hydroxy-7-methoxy-2-(2-phenylethyl) chromone (1), 6,7-dimethoxy-2-(2-phenylethyl)chromone (2), (5S,6R,7S,8R)-2-(2-phenylethyl)-5,6,7,8-tetrahydrochromone (3), 6-hydroxy-2-(2-phenylethyl)chromone (4), 4’-hydroxy-2-(2-phenylethyl) chromone (5), 6-methoxy-2-phenethyl-4H-chromen-4-one (6), and 6-methoxy-2-(4’-methoxy-phenethyl)-4H-chromen-4-one (7). Hence, we described the isolation and structural elucidation of the 2-(2-phenylethyl)chromones from this endophytic fungal strain of A. sinensis.

![Botryosphaeria rhodina A13](image)

2. Materials and methods

2.1 General procedures

1D and 2D NMR spectra were recorded on a Bruker Avance-500 Spectrometer with TMS as internal standard, δ in ppm, J in Hz (Bruker, Switzerland). EI-MS on a Thermo DSQ El Mass Spectrometer (Thermo Scientific, USA). All solvents were analytical grade (Guangzhou Chemical Plant, Guangzhou, China). Silica gel (200–300 mesh) was used for column chromatography, and precoated silica gel GF254 plates (Qingdao Marine Chemical Inc., China) were used for TLC spotting. C18 reversed-phase silica gel (40–63 µm, Merck, German), and Sephadex LH-20 gel (Pharmacia Fine Chemical Co., Ltd., Sweden) were also used for column chromatography (CC). TLC spots were visualized under UV light and by dipping into 10% H2SO4 in alcohol followed by heating.

2.2 Fungal material

The endophytic fungal strain B. rhodina A13 was isolated from thirty-year-old of A. sinensis, which was collected at Xinyi city, Guangdong province, China, in June, 2008. The strain was identified by sequence analysis of rDNA ITS (internal transcribed spacer) region. The sequence of the ITS region of B. rhodina A13 has been submitted to GenBank (Accession No. EU781670) (Tao et al, 2012). The strain is preserved at the Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology.

2.3 Fermentation, extraction, and isolation

B. rhodina A13 was grown on potato-dextrose agar (PDA) medium at 28 °C for 5 d and then inoculated into five flasks (500 mL) containing potato-dextrose (PD) medium (250 mL). After 5 d of incubation at 28 °C on a rotary shaker at 120 r/min. The 5 mL seed culture (per flask) was transferred into a total of 100 polypropylene bags, each containing 100 g sawdust with 60% moisture content, and the bags were incubated for 38 d in the dark at 27 °C.

The mycelia culture was extracted with 95% EtOH, and the resultant extract was sequentially partitioned with petroleum ether (PE) and EtOAc. Both the petroleum ether portion (26.7 g) and EtOAc fraction (71.1 g) were then subjected to column chromatography (silica gel) eluting with a solvent mixture of n-hexane-ethyl acetate (100:0–6:100)
and CHCl₃-MeOH (1:1) to afford six fractions (P₁–P₆) for petroleum ether portion and eight fractions (E₁–E₈) for EtOAc portion. P₂ (2.0 g) was purified over Sephadex LH-20 using CHCl₃-MeOH (1:1) and then subjected to column chromatography on silica gel C₁₈ with MeOH-H₂O (60:40) to yield compound 2 (27.3 mg). P₃ (1.5 g) was purified over Sephadex LH-20 using CHCl₃-MeOH (1:1) to afford two fractions P₃₁ and P₃₂. P₃₁ was then subjected to column chromatography on silica gel C₁₈ with MeOH-H₂O (60:40) to yield compound 3 (10 mg). Compound 7 (20 mg) was recrystallized from P₆₂ in MeOH. E₅ (1.0 g) was purified over column chromatography Sephadex LH-20 with CHCl₃-MeOH (1:1), silica gel C₁₈ with MeOH-H₂O (60:40), and then subjected to PLC to yield compound 5 (3.0 mg). E₆ (1.8 g) afforded compound 4 (8.2 mg) by Sephadex LH-20 using CHCl₃-MeOH (1:1) and crystallization technique. E₇ (2.3 g) was purified over Sephadex LH-20 using CHCl₃-MeOH (1:1) first, then submitted to column chromatography on silica gel C₁₈ with MeOH-H₂O (60:40), and finally subjected to PLC to yield compound 1 (9.5 mg). E₈ (2.5 g) was purified over Sephadex LH-20 using CHCl₃-MeOH (1:1) first, and then submitted to column chromatography on silica gel C₁₈ with MeOH-H₂O (20:80) to yield compound 3 (16.6 mg).

3. Results

The solid culture of the strain A13 was exhaustively extracted with 95% EtOH for three times. The solvent was removed under vacuum to give a dark brown gum, which was suspended in water and successively partitioned with petroleum ether and EtOAc. Both the PE portion (26.7 g) and EtOAc fraction (71.1 g) were then subjected to column chromatography (silica gel) eluting with a solvent mixture of n-hexane-ethyl acetate (100:0–0:100) and CHCl₃-MeOH (1:1) to afford six fractions (P₁–P₆) for petroleum ether portion and eight fractions (E₁–E₈) for EtOAc portion. The following separation led to the isolation of seven 2-(2-phenylethyl)chromones (1–7) (Figure 2). All the compounds were isolated for the first time from the genus Botryosphaeria.

Figure 2 Chemical structures of compounds 1–7
m, H-8); $^{13}$C-NMR (125 MHz, DMSO-d$_6$) δ: 178.0 (C-4), 169.7 (C-2), 156.0 (C-6), 151.0 (C-8a), 141.5 (C-1′), 129.7 (C-3′, 5′), 129.7 (C-2′, 6′), 127.6 (C-4′), 125.3 (C-4a), 124.1 (C-7), 120.8 (C-8), 110.0 (C-3), 108.9 (C-5), 36.2 (C-8′), 33.4 (C-7′). It was identified as 6-hydroxy-2-(2-phenylethyl)chromone by comparing with spectral data of the literature (Yagura et al, 2003).

Compound 5: colorless oil. EI-MS m/z: 267 [M + H]$^+$; $^{1}H$-NMR (500 MHz, CDCl$_3$) δ: 8.11 (1H, dd, J = 3.1 Hz, H-5), 7.38 (1H, d, J = 3.1 Hz, H-7), 7.30 (2H, m, J = 3.8, 2.2 Hz, H-3′, 5′), 7.24 (1H, J = 3.8 Hz, d, H-4′), 7.22 (2H, m, J = 2.2 Hz, H-2′, 6′), 7.20 (1H, s, H-5′), 6.15 (1H, s, H-3), 3.07 (2H, dd, J = 8.9, 6.7 Hz, H-7), 2.94 (2H, dd, J = 8.9, 6.7 Hz, H-8); $^{13}$C-NMR (125 MHz, CDCl$_3$) δ: 178.3 (C-4), 168.3 (C-2′), 154.2 (C-6), 156.9 (C-8a), 139.9 (C-1′), 128.8 (C-3′, C-5′), 128.4 (C-2′, C-6′), 126.7 (C-4′), 124.4 (C-4a), 123.7 (C-7), 119.4 (C-8′), 109.6 (C-3′), 104.9 (C-5′), 56.1 (6-OCH$_3$), 36.2 (C-8′), 33.2 (C-7′). It was identified as 4′-hydroxy-2-(2-phenylethyl)chromone by comparing with spectral data of the literature (Yang et al, 2013).

Compound 6: yellow powder. EI-MS m/z: 281 [M + H]$^+$; $^{1}H$-NMR (500 MHz, CDCl$_3$) δ: 7.55 (1H, d, J = 3.1 Hz, H-5), 7.38 (1H, d, J = 3.1 Hz, H-7), 7.30 (2H, m, J = 3.8, 2.2 Hz, H-3′, 5′), 7.24 (1H, J = 3.8 Hz, d, H-4′), 7.22 (2H, m, J = 2.2 Hz, H-2′, 6′), 7.20 (1H, s, H-5′), 6.15 (1H, s, H-3), 3.07 (2H, dd, J = 8.9, 6.7 Hz, H-7), 2.94 (2H, dd, J = 8.9, 6.7 Hz, H-8); $^{13}$C-NMR (125 MHz, CDCl$_3$) δ: 178.3 (C-4), 168.3 (C-2′), 154.2 (C-6), 156.9 (C-8a), 139.9 (C-1′), 128.8 (C-3′, C-5′), 128.4 (C-2′, C-6′), 126.7 (C-4′), 124.4 (C-4a), 123.7 (C-7), 119.4 (C-8′), 109.6 (C-3′), 104.9 (C-5′), 56.1 (6-OCH$_3$), 36.2 (C-8′), 33.2 (C-7′). It was identified as 6-hydroxy-2-phenyl-4H-chromen-4-one by comparing with spectral data of the literature (Yang et al, 1989).

Compound 7: yellow powder. EI-MS m/z: 310 [M + H]$^+$; $^{1}H$-NMR (500 MHz, CDCl$_3$) δ: 7.50 (1H, J = 3.1 Hz, H-5), 7.35 (1H, J = 9.1 Hz, H-6), 7.22 (1H, J = 3.11, 9.1 Hz, dd, H-7), 7.17 (2H, m, H-2′, 6′), 6.73 (2H, m, H-2′, 5′), 6.12 (1H, s, H-3), 3.86 (3H, s, 6-OCH$_3$), 3.74 (3H, s, 4′-OCH$_3$), 2.99 (2H, dd, J = 8.9, 6.2 Hz, CH$_2$-2b), 2.90 (2H, dd, J = 8.9, 6.3 Hz, CH$_2$-2a); $^{13}$C-NMR (125 MHz, CDCl$_3$) δ: 178.7 (C-4), 168.6 (C-2), 159.9 (C-4′), 157.0 (C-8a), 151.5 (C-6′), 141.5 (C-1′), 129.8 (C-2′, C-6′), 123.9 (C-4′a), 119.5 (C-8′), 114.3 (C-3′, C-5′), 109.6 (C-3), 105.0 (C-5′), 56.1 (6-OCH$_3$), 55.4 (4′-OCH$_3$), 36.2 (C-8′), 33.2 (C-7′). It was identified as 6-methoxy-2′-(4′-methoxyphenethyl)-4H-chromen-4-one by comparing with spectral data of the literature (Yang et al, 1989).

4. Conclusion

Agarwood is a resinous material collected from Aquilaria trees. And among them, A. sinensis is the main plant resource in China for agarwood. However, agarwood substances were not detected in healthy A. sinensis by GC-MS detection (Tao et al, 2012; Qi et al, 1992). It is generally accepted that the dark resinous material of Aquilaria was produced only when the trees were threatened by externally physical or chemical injury or endophytic fungal irritation. Therefore, the previous artificial agarwood-inducing methods were conducted in the living A. sinensis trees. In the present study, the strain A13 was inoculated to A. sinensis sawdust for solid fermentation, and 2′-(2-phenylethyl)chromones were isolated and identified from the solid culture. Many previous studies have shown that the 2-(2-phenylethyl)chromones were the important components of agarwood (Naef, 2011; Chen et al, 2012; Yoon et al, 2006). Therefore, our investigation will provide an important scientific basis for utilizing endophytic fungal strain to produce agarwood. To the best of our knowledge, this is the first report to produce the characteristic components of agarwood by solid-state fermentation of A. sinensis sawdust with the fungus B. rhodina.

Conflicts of interest statement

The authors declare no conflict of interest.

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