Postprint: Study on Metabolites of Endophytic Fungus Pestalotiopsis heterocornis from Podocarpus

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Abstract

Ten metabolites were isolated from the fermentation broth of the endophytic fungus Pestalotiopsis heterocornis derived from Podocarpus macrophyllus and identified as jesterone (1), hydroxy-jesterone (2), ambuic acid (3), 6β-hydroxy-stigmast-4-en-3-one (4), (24S)-ergost-5-en-3β,7α-diol (5), 7,22-diene-3β,5α,7β-trihydroxy-ergosterol (6), ergosta-7,22-dien-3-one (7), (4E,8E,2S,3R,2 R)-N-2 -hydroxypalmitoyl-9-methyl-4,8-sphingadienine (8), batyl alcohol (9), and palmitic acid (10) by modern spectroscopic methods including mass spectrometry and nuclear magnetic resonance. All of these compounds were isolated from the metabolites of the endophytic fungus P. heterocornis for the first time, while compounds 4, 6, and 7 were isolated from the metabolites of the genus Pestalotiopsis for the first time.

Full Text

Preamble

Title: Metabolites of Endophytic Fungus *Pestalotiopsis heterocornis* Isolated from *Podocarpus macrophyllus*

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Abstract

Ten metabolites were isolated from the fermentation broth of the endophytic fungus *Pestalotiopsis heterocornis* derived from *Podocarpus macrophyllus*. Their structures were elucidated using modern spectroscopic methods including mass spectrometry and nuclear magnetic resonance as jesterone (1), hydroxy-jesterone (2), ambuic acid (3), 6β-hydroxystigmast-4-en-3-one (4), (24S)-ergosta-5-en-3β,7α-diol (5), ergosta-7,22-dien-3β,5α,7β-triol (6), ergosta-7,22-dien-3-one (7), (4E,8E,2S,3R,2 R)-N-2 -hydroxyhexadecanoyl-9-methyl-4,8-sphingadienin (8), batyl alcohol (9), and palmitic acid (10). All compounds were isolated from *P. heterocornis* for the first time, and compounds 4, 6, and 7 were obtained from the genus *Pestalotiopsis* for the first time.

Keywords: endophytic fungi, *Pestalotiopsis heterocornis*, metabolites

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Introduction

Endophytic fungi are ubiquitous in healthy plant tissues, exhibiting remarkable diversity and broad distribution. The metabolic products of plant endophytic fungi are abundant and possess numerous biological activities, including plant growth regulation, insecticidal, antimicrobial, and antitumor properties. In recent years, endophytic fungi have become an important source for discovering novel bioactive compounds and lead compounds, attracting increasing attention to the study of active substances in their metabolites. *Pestalotiopsis* is a significant group of plant endophytic fungi capable of producing various metabolites, including anticancer and antimicrobial substances. The taxol production capacity of *P. microspora* strains has initially demonstrated commercial potential, thereby stimulating research interest in endophytic *Pestalotiopsis*.

This paper reports the investigation of metabolites from *Pestalotiopsis heterocornis*, an endophytic fungus isolated from *Podocarpus macrophyllus*. Ten compounds were isolated and identified as jesterone (1), hydroxy-jesterone (2), ambuic acid (3), 6β-hydroxystigmast-4-en-3-one (4), (24S)-ergosta-5-en-3β,7α-diol (5), ergosta-7,22-dien-3β,5α,7β-triol (6), ergosta-7,22-dien-3-one (7), (4E,8E,2S,3R,2 R)-N-2 -hydroxyhexadecanoyl-9-methyl-4,8-sphingadienin (8), batyl alcohol (9), and palmitic acid (10).

Materials and Methods

Instruments and Reagents

The following instruments were used: Bruker 500 AVANCE III NMR spectrometer with TMS as internal standard (^{1}H-NMR and ^{13}C-NMR measured at 500 MHz and 125 MHz, respectively), X-4 digital display micro-melting point apparatus (thermometer uncorrected), Nicolet NEXUS-470 infrared spectrometer (KBr pellet), Thermo LCQ FLEET electrospray ionization mass spectrometer (ESI-MS, APCI-MS), and Waters 600 Pre-HPLC (Waters Corporation, USA). Chromatographic materials included silica gel (Qingdao Marine Chemical Factory), Sephadex LH-20 (Pharmacia, USA), and GF254 high-performance thin-layer plates (Yantai Huiyou Silica Gel Development Co., Ltd.). All reagents used were analytically pure. The fungal strain was isolated from *Podocarpus macrophyllus* (Thunb.) in Nanjing, Jiangsu, and identified as *Pestalotiopsis heterocornis* by Professor Tan Xianhe from the School of Pharmacy, Nanjing University of Chinese Medicine. The specimen is preserved in our laboratory.

Fermentation Medium Preparation

PDA fermentation medium was prepared as follows: 200 g of potatoes were washed, peeled, cut into small pieces, boiled in 1000 mL water for 30 min, and filtered through gauze. Twenty grams of glucose were added to the filtrate, dissolved thoroughly, and distributed into containers.

Extraction and Isolation

The fermentation broth of *P. heterocornis* (20 L, with approximately 500 mL medium per 1 L conical flask, statically fermented at room temperature for 20 days, 40 flasks per batch) was extracted three times with ethyl acetate. The combined extracts were concentrated under reduced pressure to yield 3.5 g of crude extract. This extract was subjected to silica gel column chromatography with a chloroform-methanol gradient elution to obtain six fractions (Fr. A–Fr. F).

Fraction B (eluted with chloroform:methanol 95:5) was further separated by Sephadex LH-20 column chromatography using chloroform:methanol (1:1) to yield two subfractions, Fr. B1 and Fr. B2. Fr. B1 was purified by silica gel column chromatography with petroleum ether:ethyl acetate (10:1) to afford compound **4** (15.3 mg). Fr. B2 was recrystallized to give compound **7** (11.2 mg).

Fraction C (eluted with chloroform:methanol 90:10) was separated by silica gel column chromatography using petroleum ether:acetone (8:1) to obtain two subfractions. Fr. C1 was further purified by silica gel column chromatography with pure chloroform to yield a mixture of compounds **1** and **2** (32.2 mg), which

was separated by Pre-HPLC (mobile phase methanol:water 80:20) to obtain compound **1** (8.0 mg) and compound **2** (12.1 mg). Fr. C2 was recrystallized to afford compound **5** (6.9 mg).

Fraction D (eluted with chloroform:methanol 85:15) was separated by silica gel column chromatography using chloroform:acetone (15:1) to yield three subfractions. Fr. D1 was further purified by silica gel column chromatography with petroleum ether:acetone (5:1) to give compound **8** (7.2 mg). Fr. D2 was subjected to Sephadex LH-20 column chromatography with chloroform:methanol (1:1) to afford compound **6** (16.2 mg). Fr. D3 was purified by Pre-HPLC (mobile phase methanol:water:acetic acid 7:3:0.1) to obtain compound **3** (8.2 mg).

Fraction E was further separated by silica gel column chromatography to yield compounds **9** (10.1 mg) and **10** (6.5 mg).

Structure Identification

Compound 1 was obtained as a pale yellow liquid with molecular formula $C_{15}H_{20}O_4$. ESI-MS m/z: 263 $[M-H]^-$. ^1H NMR ($CDCl_3$) δ: 1.80 (1H, d, J = 6.6 Hz, Me-1), 5.82 (1H, dt, J = 6.6, 17.3 Hz, H-2), 6.01 (1H, d, J = 17.3 Hz, H-3), 4.83 (1H, d, J = 1.3 Hz, H-6), 3.64 (1H, d, J = 1.3 Hz, H-7), 2.75 (1H, dd, J = 8.0, 16.2 Hz, H-9a), 2.56 (1H, dd, J = 7.0, 16.2 Hz, H-9b), 5.00 (1H, m, H-10), 4.68 (1H, d, J = 15.4 Hz, H-12a), 4.40 (1H, d, J = 15.4 Hz, H-12b), 1.60 (3H, s, Me-14), 1.67 (3H, s, Me-15). ^{13}C NMR ($CDCl_3$) δ: 19.3 (C-1), 135.2 (C-2), 122.1 (C-3), 131.7 (C-4), 145.4 (C-5), 65.5 (C-6), 59.4 (C-7), 60.2 (C-8), 26.7 (C-9), 116.7 (C-10), 136.2 (C-11), 63.1 (C-12), 195.0 (C-13), 18.2 (C-14), 25.8 (C-15). These data are consistent with those reported in the literature (Li & Strobel, 2001b), identifying compound **1** as jesterone.

Compound 2 was isolated as a pale yellow liquid with molecular formula $C_{15}H_{20}O_5$. ESI-MS m/z: 279 $[M-H]^-$. ^1H NMR ($CDCl_3$) δ: 1.81 (1H, d, J = 6.4 Hz, Me-1), 5.80 (1H, dd, J = 6.4, 15.3 Hz, H-2), 6.00 (1H, d, J = 15.3 Hz, H-3), 4.80 (1H, s, H-6), 3.82 (1H, s, H-7), 4.92 (1H, d, J = 8.1 Hz, H-9), 5.08 (1H, d, J = 8.1 Hz, H-10), 4.64 (1H, d, J = 15.0 Hz, H-12a), 4.40 (1H, d, J = 15.0 Hz, H-12b), 1.68 (3H, s, Me-14), 1.70 (3H, s, Me-15). ^{13}C NMR ($CDCl_3$) δ: 19.2 (C-1), 135.0 (C-2), 122.1 (C-3), 131.5 (C-4), 145.6 (C-5), 64.4 (C-6), 58.2 (C-7), 60.4 (C-8), 65.5 (C-9), 120.5 (C-10), 139.2 (C-11), 61.1 (C-12), 195.1 (C-13), 18.5 (C-14), 25.8 (C-15). These data match those reported in the literature (Li & Strobel, 2001b), identifying compound **2** as hydroxy-jesterone.

Compound 3 was obtained as needle-like crystals ($CH_3OH/CHCl_3$) with molecular formula $C_{19}H_{26}O_6$. ESI-MS m/z: 349 $[M-H]^-$. ^1H NMR ($CDCl_3$) δ: 6.58 (1H, t, J = 7.3 Hz, H-3), 2.80 (1H, dd, J = 15.8, 7.3 Hz, H-4), 3.78 (1H, d, J = 3.2 Hz, H-6), 4.82 (1H, d, J = 3.2 Hz, H-7), 6.17 (1H, d, J = 16.4 Hz, H-11), 5.82 (1H, m, H-12), 2.12 (1H, m, H-13), 1.43 (2H, m, H-14), 1.33 (2H, m, H-15), 1.32 (2H, m, H-16), 0.95 (3H, t, J = 8.0 Hz, Me-17), 1.83 (3H, s,

Me-18), 4.40 (1H, d, J = 13.4 Hz, H-19a), 4.52 (1H, d, J = 13.4 Hz, H-19b). ^{13}C NMR ($CDCl_3$) δ: 171.7 (C-1), 132.2 (C-2), 136.4 (C-3), 28.5 (C-4), 61.4 (C-5), 61.1 (C-6), 66.4 (C-7), 150.3 (C-8), 132.5 (C-9), 196.5 (C-10), 122.2 (C-11), 140.1 (C-12), 34.2 (C-13), 30.3 (C-14), 32.8 (C-15), 23.8 (C-16), 14.4 (C-17), 13.2 (C-18), 60.8 (C-19). These data are consistent with those reported in the literature (Li et al., 2001a), identifying compound **3** as ambuic acid.

Compound 4 was isolated as colorless needle-like crystals with molecular formula $C_{29}H_{48}O_2$, melting point 213–215 °C. APCI-MS m/z: 427 $[M-H]^-$, 451 $[M+Na]^+$. ^{1}H NMR ($CDCl_3$) δ: 5.80 (1H, s, H-4), 4.34 (1H, s, H-6), 0.75 (3H, s, Me-18), 1.38 (3H, s, Me-19), 0.93 (3H, d, J = 6.5 Hz, Me-21), 0.82 (3H, d, J = 6.1 Hz, Me-26), 0.84 (3H, d, J = 6.1 Hz, Me-27), 0.85 (3H, t, J = 6.7 Hz, Me-29). ^{13}C NMR ($CDCl_3$) δ: 37.3 (C-1), 34.5 (C-2), 200.6 (C-3), 126.6 (C-4), 168.7 (C-5), 73.5 (C-6), 38.8 (C-7), 30.0 (C-8), 53.9 (C-9), 38.2 (C-10), 21.2 (C-11), 38.9 (C-12), 42.8 (C-13), 56.3 (C-14), 24.4 (C-15), 28.4 (C-16), 56.1 (C-17), 12.2 (C-18), 19.7 (C-19), 36.3 (C-20), 19.0 (C-21), 34.6 (C-22), 26.4 (C-23), 46.1 (C-24), 29.4 (C-25), 20.0 (C-26), 19.3 (C-27), 23.3 (C-28), 12.2 (C-29). These data are consistent with those reported in the literature (Arai et al., 1998), identifying compound **4** as 6β-hydroxystigmast-4-en-3-one.

Compound 5 was obtained as colorless needle-like crystals (acetone) with molecular formula $C_{28}H_{48}O_2$, melting point 216–218 °C. APCI-MS m/z: 415 $[M-H]^-$, 439 $[M+Na]^+$. ^{1}H NMR ($CDCl_3$) δ: 3.55 (1H, m, H-3), 5.60 (1H, d, J = 5.5 Hz, H-6), 3.83 (1H, d, J = 5.5 Hz, H-7), 0.68 (3H, s, Me-18), 0.98 (3H, s, Me-19), 0.91 (3H, d, J = 6.5 Hz, Me-21), 0.83 (3H, d, J = 6.8 Hz, Me-26), 0.76 (3H, d, J = 6.8 Hz, Me-27), 0.74 (3H, d, J = 6.9 Hz, Me-28). ^{13}C NMR ($CDCl_3$) δ: 37.7 (C-1), 31.5 (C-2), 71.7 (C-3), 42.0 (C-4), 146.5 (C-5), 125.7 (C-6), 65.4 (C-7), 38.4 (C-8), 42.3 (C-9), 37.8 (C-10), 21.5 (C-11), 39.0 (C-12), 42.4 (C-13), 50.0 (C-14), 24.4 (C-15), 28.9 (C-16), 55.8 (C-17), 18.6 (C-18), 11.6 (C-19), 36.4 (C-20), 20.0 (C-21), 33.6 (C-22), 30.5 (C-23), 39.4 (C-24), 31.3 (C-25), 17.6 (C-26), 20.7 (C-27), 15.7 (C-28). These data are consistent with those reported in the literature (Zhang et al., 2003), identifying compound **5** as (24S)-ergosta-5-en-3β,7α-diol.

Compound 6 was isolated as colorless needle-like crystals (methanol) with molecular formula $C_{28}H_{46}O_3$, melting point 224–226 °C. ESI-MS m/z: 429 $[M-H]^-$, 453 $[M+Na]^+$. ^{1}H NMR (DMSO-d_6) δ: 3.75 (1H, m, H-3), 3.40 (1H, s, H-6), 5.10 (1H, s, H-7), 5.15 (1H, dd, J = 15.5, 3.0 Hz, H-22), 5.20 (1H, dd, J = 15.5, 3.0 Hz, H-23), 0.58 (3H, s, Me-18), 0.92 (3H, s, Me-19), 0.97 (3H, d, J = 6.5 Hz, Me-21), 0.81 (3H, d, J = 6.9 Hz, Me-26), 0.83 (3H, d, J = 6.7 Hz, Me-27), 0.89 (3H, d, J = 7.1 Hz, Me-28). ^{13}C NMR ($CDCl_3$) δ: 32.7 (C-1), 31.3 (C-2), 65.7 (C-3), 40.0 (C-4), 74.5 (C-5), 72.3 (C-6), 119.4 (C-7), 139.4 (C-8), 42.3 (C-9), 36.8 (C-10), 21.4 (C-11), 39.0 (C-12), 42.8 (C-13), 54.3 (C-14), 22.4 (C-15), 27.7 (C-16), 55.5 (C-17), 12.3 (C-18), 17.6 (C-19), 39.4 (C-20), 20.8 (C-21), 135.6 (C-22), 131.5 (C-23), 42.2 (C-24), 32.3 (C-25), 19.6 (C-26), 19.7 (C-27), 17.5 (C-28). These data are consistent with those reported in the literature (Lü et al., 2008), identifying compound **6** as ergosta-7,22-dien-3β,5α,7β-triol.

Compound 7 was obtained as colorless needle-like crystals (chloroform) with molecular formula $C_{28}H_{44}O$, melting point 179–181 °C. APCI-MS m/z: 419 $[M+Na]^+$. ^1H NMR ($CDCl_3$) δ: 0.55 (3H, s, Me-18), 0.81 (3H, d, J = 6.4 Hz, Me-26), 0.83 (3H, d, J = 6.4 Hz, Me-27), 0.90 (3H, d, J = 6.6 Hz, Me-28), 1.03 (3H, s, Me-19), 1.04 (3H, d, J = 7.4 Hz, Me-21), 5.21 (3H, m, H-7, H-22, and H-23). ^{13}C NMR ($CDCl_3$) δ: 38.6 (C-1), 38.3 (C-2), 212.1 (C-3), 44.2 (C-4), 42.6 (C-5), 30.4 (C-6), 117.3 (C-7), 139.3 (C-8), 48.6 (C-9), 34.4 (C-10), 21.4 (C-11), 39.3 (C-12), 43.4 (C-13), 55.8 (C-14), 22.7 (C-15), 28.2 (C-16), 55.1 (C-17), 12.1 (C-18), 12.4 (C-19), 40.5 (C-20), 19.7 (C-21), 135.4 (C-22), 132.1 (C-23), 42.7 (C-24), 33.1 (C-25), 19.8 (C-26), 21.0 (C-27), 17.6 (C-28). These data are consistent with those reported in the literature (Wang & Sun, 2007), identifying compound **7** as ergosta-7,22-dien-3-one.

Compound 8 was isolated as a white amorphous powder with molecular formula $C_{35}H_{67}NO_4$. ESI-MS m/z: 564 $[M-H]^-$. ^1H NMR (CD_3COCD_3) δ: 0.89 (6H, t, J = 7.2 Hz, Me-18/16), 1.18–1.43 (40H, m), 1.59 (3H, s, Me-19), 1.76 (1H, m, H-3), 1.98 (2H, t, J = 7.2 Hz, H-10), 2.02 (1H, m, H-3), 2.08 (4H, m, H-6/7), 3.65 (1H, m), 3.81 (2H, m), 4.01 (2H, m), 4.15 (1H, m), 4.40 (1H, d, J = 5.2 Hz), 4.75 (1H, d, J = 5.6 Hz), 5.14 (1H, m, H-8), 5.55 (1H, dd, J = 15.5, 6.3 Hz, H-4), 5.68 (1H, m, H-5), 7.31 (1H, d, J = 7.4 Hz, NH). ^{13}C NMR (CD_3COCD_3) δ: 14.4, 16.1, 23.4, 28.4, 28.5, 29.3–29.9, 32.5, 33.3, 35.7, 40.2, 56.3, 62.3, 72.7, 73.6, 124.7, 131.6, 132.4, 136.2, 175.1. These data are consistent with those reported in the literature (Zhou & Liu, 2009), identifying compound **8** as (4E,8E,2S,3R,2 R)-N-2 -hydroxyhexadecanoyl-9-methyl-4,8-sphingadienin.

Compound 9 was isolated as colorless needle-like crystals ($CH_3OH/CDCl_3$) with molecular formula $C_{21}H_{44}O_3$. ESI-MS m/z: 343 $[M-H]^-$. ^1H NMR ($CDCl_3$) δ: 0.92 (3H, t, J = 5.9 Hz, Me-18), 1.12–1.48 (32H, br s), 2.66 (1H, m), 2.32 (1H, m), 3.3–4.2 (7H, m). ^{13}C NMR ($CDCl_3$) δ: 14.2 (Me-18), 22.7, 22.8, 26.3, 32.0, 29.3–29.9, 64.3, 70.2, 72.2, 72.8. These data are consistent with those reported in the literature (Lan et al., 2003), identifying compound **9** as batyl alcohol.

Compound 10 was obtained as a white powder with molecular formula $C_{16}H_{32}O_2$. ESI-MS m/z: 255 $[M-H]^-$. ^1H NMR ($CDCl_3$) δ: 2.33 (2H, t, J = 7.4 Hz, H-2), 1.62 (2H, m, H-3), 1.22–1.30 (24H, m, H-4–H-15), 0.89 (3H, t, J = 7.4 Hz, Me-16). ^{13}C NMR ($CDCl_3$) δ: 179.9 (C-1), 34.3 (C-2), 31.8 (C-15), 29.4–29.9 (C-3–C-14), 14.2 (C-16). Based on the mass spectrometry and NMR data, compound **10** was identified as palmitic acid (hexadecanoic acid).

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Note: Figure translations are in progress. See original paper for figures.