Screening of Azaphilone Derivatives From *Monascus pilosus*-Fermented Rice (Red Yeast Rice) and Their Evaluation as Nonsteroidal Androgen Receptor Antagonists

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Abstract

*Monascus pilosus* BCRC 38093 is a mutant strain of *M. pilosus* BCRC 38072. The 95% ethanol extract of red yeast rice fermented by *M. pilosus* BCRC 38093 showed 4 major signals on high performance liquid chromatography (HPLC) examination. The extraction of metabolites and chromatography of the ethyl acetate crude extract on silica gel yielded 2 azaphilone derivatives, monascin (1) and monascinol (2), and 2 monacolin-type derivatives, acid-form monacolin K (3) and lactone-form monacolin K (4). Their structural characterization was elucidated by spectroscopic techniques ultraviolet-visible (UV), infrared spectroscopy (IR), and two dimensional-nuclear magnetic resonance (2D-NMR) and mass spectrometry. These compounds were assayed for their anti-androgen activity; monascinol (2) exhibited strong activity.

Keywords

*Monascus pilosus*, red yeast rice, azaphilone, androgen receptor, hormone receptor

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The androgen receptor (AR) is a member of the steroid hormone receptor subfamily of nuclear receptors, which also includes the estrogen, progesterone, mineralocorticoid, and glucocorticoid receptors.¹ The natural steroid androgens, namely testosterone, the circulating hormone, and dihydrotestosterone (DHT), its active metabolite, are responsible for the development and maintenance of normal prostate cells.

Either the overexpression of an AR or expression of mutated AR genes has been found in several diseases, such as cancer, including prostate and breast cancers, as well as other disorders such as polyglutamate disease, androgen-dependent alopecia, hirsutism, acne, prostatic hyperplasia, spinal and muscular atrophy, and Kennedy's disease. However, this condition can also promote the malignant growth of the prostate gland, and it is known that most prostate cancers (PCas) are androgen dependent.²³ The mechanism of action of the androgens involves an interaction with a specific AR (AR), and this receptor is postulated to play a crucial role in the development of PCa.⁴

Red yeast rice (ang-kak, red koji), made by filamentous *Monascus* species, has traditionally been used in East Asia in the production of natural food colorant, such as for red rice wine, red soybean cheese, meat, meat products, and fish, to aromaticize and conserve meat, fish, and soybean products.⁵ It is also used as a folk medicine.⁵ There are 4 representative species: *Monascus pilosus*, *Monascus purpureus*, *Monascus rubber*, and *Monascus anka* (class Ascomycetes and family Monascaceae).⁵ Red yeast rice is employed for medicinal purposes like promotion of digestion and blood circulation, strengthening of the spleen, and removal of blood stasis.⁶ The above-mentioned yeasts can produce several pigments and biologically active metabolites when grown on cooked rice.⁵

In a series of studies on anti-androgen activity of natural products, we were especially interested to study the chemical composition of red yeast rice, and 1 specific pink mutant, *M. pilosus* BCRC 38093, which is different from other traditional red mutant *Monascus* species, and which has been found to be one of the active ones. Careful examination of the EtOAc-soluble fraction of a 95% EtOH extract of the red yeast rice

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produced by *M. pilosus* BCRC 38093 has resulted in the isolation of 2 azaphilone derivatives, monascin (1) and monascinol (2), together with 2 monacolin-type derivatives, acid-form monacolin K (3) and lactone form monacolin K (4). (Figure 1) The structures of these isolates were established by means of spectroscopic and mass spectrometric (MS) experiments. This paper describes the isolation and structure elucidation of compound 2, as well as the evaluation of the anti-androgen activity of 2 of the pure isolated compounds (1 and 2). The fingerprinting analysis method by HPLC is also discussed.

Monascus pilosus BCRC 38093 was mutated from *M. pilosus* BCRC 38072. The red yeast rice produced by fermentation with *M. pilosus* BCRC 38093 showed high monacolin K production. The red yeast rice was extracted with 95% ethanol, successively. The crude ethyl acetate layer showed strong antitumor activity. Further bioassay-directed fractionation of the active fraction led to the isolation of 2 yellow pigments, monascin (1) and monascinol (2). Compound 2, isolated as yellowish oil, was assigned the molecular formula C_{21}H_{28}O_{5}Na by ESI-MS ([M+Na]+, m/z 383) and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) ([M+Na]+, m/z 383.1832). IR (Neat) absorptions were observed at 3400 cm−1, pointing to the presence of hydroxyl (OH) and an ester carbonyl group (C═O). The UV spectrum (λ max (MeOH) nm (log ε)) showed maximum absorption at 230 (4.32) and 388 (4.11) nm, and a bathochromic shift in alkaline solution indicated the presence of a phenol derivative. This was confirmed by the 1H NMR spectrum, which showed 1 proton at δ_H 4.20 (1H, m) assigned to OH-1’, which disappeared upon addition of D_2O. The 1H NMR spectrum of compound 2 was similar to that of the known compound, monascin (1), except that the substitution at C-11 in 2 was an octan-2-ol moiety in place of an octan-2-one group in monascin [δ_H 0.85 (3H, t, J = 5.8 Hz, CH_{3}β′), 1.23 (4H, m, CH_{2}δ′, 5′), 1.50 (2H, m, H-3′), 2.66 (1H, t, J = 6.9 Hz, CH_{2}α′), and 2.77 (1H, t, J = 7.2 Hz, CH_{2}α′)]. The structure was further confirmed by 13C NMR, distortionless enhancement by polarization transfer (DEPT), 1H-1H correlation spectroscopy (COSY), and 1H-1H nuclear overhauser effect spectroscopy (NOESY), hetero-nuclear single quantum coherence (HSQC), and hetero-nuclear multiple-bond connectivity (HMBC), experiments. Thus, compound 2 was determined to be (3S,3aR,9aR)-3a,4-dihydro-3-[(S)-1-hydroxyhexyl]-9a-methyl-6-[(E)-prop-1-enyl]-3H-furo[3,2-g]isochromene-2,9(8H,9aH)-dione. Compound 2 was designated as monascinol.

To study the anti-androgen activity of the azaphilone derivatives isolated from *M. pilosus*, luminescent secreted alkaline phosphatase (SEAP) assays were used of MDA-MB-453 cells transiently transfected with SEAP reporter construct containing prostate-specific antigen (PSA) promoter. Transfected cells were treated with 10 nM DHT and azaphilone compounds for 48 hours and then harvested and assayed for SEAP activity. Chemiluminescence readout was normalized relative to the readout from cells treated with DHT alone. Monascinol (2) showed potent anti-androgen activity with an IC_{50} value of 7.0 µM, while bicalutamide (Casodex, AstraZeneca, United States) exhibited an IC_{50} of 4.2 µM (Figure 2). Monascin (1) is a less potent anti-androgen with an estimated IC_{50} value of 67.5 µM.

In this study, we developed a HPLC technique using a gradient of phosphoric acid and acetonitrile, which allows quantitative analysis of the red yeast rice of *M. pilosus* and identification of 4 compounds.

From the bioactive fraction, we isolated 2 azaphilone derivatives, monascin (1) and monascinol (2), and 2 monacolin-type derivatives, acid-form monacolin K (3) and lactone-form monacolin K (4). The compounds were identified by the comparison of spectroscopic and MS data with that in the literature. Compound 2 exhibited strong anti-androgen activity with an IC_{50} value of 7.0 µM (bicalutamide, the control, IC_{50} 4.2 µM). Compound 1 showed weak inhibitory activity (IC_{50} 67.5 µM).

The major anti-androgen active components were identified as monascin (1) and monascinol (2). In this study, we found that the azaphilone compounds in the red yeast rice of *Monascus* sp. have growth inhibitory and anti-androgenic properties that contribute to their protection against the development and progression of prostate malignancy.

**Experimental**

**General**

Optical rotations were measured on a Jasco P-1020 digital polarimeter, UV spectra were obtained on a Jasco UV-240 spectrophotometer in MeOH, and IR spectra (KBr or neat)
were taken on a Perkin-Elmer System 2000 FT-IR spectrometer. 1D (1H, 13C, and DEPT) and 2D (COSY, NOESY, HSQC, and HMBC) NMR spectra using CDCl₃ and CD₃COCD₃ as solvents were recorded on Varian Unity Plus 400 (400 MHz for 1H NMR and 100 MHz for 13C NMR) and Varian INOVA-500 (500 MHz for 1H NMR and 125 MHz for 13C NMR) spectrometers. Chemical shifts were internally referenced to the solvent signals in CDCl₃ (1H, δ 7.26; 13C, δ 77.0) with tetramethylsilane (TMS) as the internal standard. Low-resolution ESI-MS were obtained on an API 3000 (Applied Biosystems) and high-resolution ESI-MS on a Bruker Daltonics APEX II 30e spectrometer. Silica gel (70-230 and 230-400 mesh; Merck) was used for column chromatography, and silica gel 60 F-254 (Merck) was used for thin layer chromatography (TLC) and prep. TLC. The prep-HPLC separations were performed using a Lab Alliance series III with a Model V520 UV detector on an Inertsil ODS (Octa Decyl Silane)-3 column, 5 µm, 250 mm × 10 mm i.d. (GL Sciences, Tokyo, Japan). All organic solvents used for partition and column chromatography were of analytical grade. Acetonitrile for HPLC analysis was of HPLC grade from Merck (Darmstadt, Germany). Cell lines were obtained from BCRC (Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsin-Chu). Cell culture medium RPMI 1640, penicillin, streptomycin, and FBS were purchased from Gibco BRL (Invitrogen Corporation, Carlsbad, CA, USA).

**Microorganism and Preparation of Red Yeast Rice**

*Monascus pilosus* BCRC 38093 was used throughout in this study, and specimens were deposited at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute. *Monascus pilosus* BCRC 38093 was inoculated on Potato Dextrose Agar (PDA, Difco, United States) plates and incubated at 30°C for 7 days. The spores were washed from the PDA plate using sterile water (7 mL per plate) and the concentration of the spore suspension was calculated. Sterile zailai rice (50 g) was put in a jar and mixed with 8 mL of spore suspension (2 × 10⁶/mL) and 12 mL of sterile water. The rice was incubated at 25°C and left for 14 days to obtain red yeast rice.

**Extraction and Separation**

The dried red yeast rice of *M. pilosus* BCRC 38093 (2.986 kg) was extracted 3 times with 95% EtOH at room temperature. The ethanol syrupy extract was partitioned between EtOAc and H₂O (1:1) to afford 30.1 g of the EtOAc soluble fraction. This was chromatographed over silica gel (250 g, 70-230 mesh), eluting with n-hexane/ethyl acetate: 12:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, acetone, and methanol to produce 36 fractions. Fraction 17 was purified by preparative TLC (n-hexane-EtOAc, 1:5:1) to give monascin (1) (40.2 mg) and monascinol (2) (30.9 mg). Acid-form monacolin K (3) (15.4 mg) and lactone-form monacolin K (4) (8.2 mg) were furnished from fraction 20 by HPLC (Inertsil ODS-3 column, 250 mm × 10 mm, MeOH-H₂O = 65:35).

**Analysis of Red Yeast Rice Compounds by HPLC**

One gram of red yeast rice was extracted with 5 mL of ethanol by sonication. The ethanol crude extract was analyzed by HPLC with UV spectroscopic detection (waters 600 pump and 996 photodiode array detector) on an Inertsil ODS-3 column, 5 µm, 250 mm × 4.6 mm i.d. (GL Sciences, Tokyo, Japan) using isocratic elution with water (0.1% H₃PO₄)/acetonitrile (35:65, v/v) at a flow rate of 1 mL/min. Chromatograms were recorded at a wavelength of 237 nm. In this HPLC analytical method, 4 compounds were identified.
**Assay for Anti-Androgen Activity**

Human breast carcinoma MDA-MB-453 cells were maintained in roswell park memorial institute (RPMI)-1640 medium containing penicillin (25 U/mL), streptomycin (25 mg/mL), and 10% FBS in a humidified 5% CO₂ atmosphere. Transfections were performed using the electroporation method with a pPSA-SEAP plasmid (courtesy of Prof Chi-Huei Wang of Kaohsiung Medical University). Briefly, 1 × 10⁶ cells were resuspended in 0.5 mL medium with 10 µg plasmid DNA and pipetted into a 0.4 cm electroporation cuvette. Cells were incubated on ice for 5 minutes, electroporated at 250 V and 960 µF using a gene pulser (Bio-Rad, Hercules, CA, United States), and incubated for 5 more minutes on ice. Transfected cells were resuspended in growth medium with 10% charcoal-stripped FBS and seeded into 96-well microplates. After an overnight incubation, cells were treated with DHT (10 nM) and samples of interest for 48 hours. Aliquots of culture media were analyzed for SEAP activity using a phospha-light reporter chemiluminescence assay kit (Applied Biosystems, United States) and the resulting luminescence was detected using a VICTOR Light 1420 luminescence counter (PerkinElmer, United States).

Monascinol

Yellowish oil.

[α]D: –42.1 (c 0.21, CHCl₃).

Rf: 0.56 (CHCl₃-MeOH, 15:1).

IR (Neat): 3400, 1738 cm⁻¹.

UV/Vis λmax (MeOH) nm (log ε): 230 (4.32), 388 (4.11).

1H NMR (600 MHz, CDCl₃): 0.90 (3H, t, J = 6.8 Hz, CH₃-6′), 1.25-1.46 (4H, m, CH₂-4′, 5′), 1.42 (3H, s, CH₃-16), 1.50-1.61 (4H, m, CH₂-2′, 3′), 1.86 (3H, dd, J = 6.8 Hz, CH₃-15), 2.53-2.60 (1H, m, H-5), 2.70-2.76 (4H, m, H-5, 11), 2.99-3.06 (4H, m, H-6), 4.20 (1H, m, H-1′), 4.70 (1H, d, J = 12.8 Hz, H-1), 5.05 (1H, d, J = 12.8 Hz, H-13), 5.28 (1H, s, H-5), 5.89 (1H, br. d, J = 12.8 Hz, H-13), 6.49 (1H, m, H-14).

13C NMR (100 MHz CDCl₃): 14.0 (6′-CH₃), 17.6 (16-CH₃), 18.5 (15-CH₃), 22.6 (CH₃), 25.9 (CH₃), 30.9 (CH₃), 31.5 (CH₃), 34.9 (CH₃), 41.2 (C-6), 49.0 (C-11), 63.8 (C-1), 69.4 (C-1′), 83.1 (C-7), 103.3 (C-4), 114.0 (C-9), 124.4 (C-13), 135.3 (C-14), 150.9 (C-10), 160.3 (C-3), 175.1 (C-12), 190.7 (C-8).

HRESI: m/z [M+Na⁺] calcd for C₂₁H₂₈O₅Na: 383.1834; found: 383.1832.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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