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Cytotoxic steroids from *Monascus purpureus*-fermented rice

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ABSTRACT

Bioassay-guided fractionation of an EtOH extract of *Monascus purpureus*-fermented rice led to the isolation of two new steroids (22*S*, 23*R*, 24*S*)-20β,23α,25α-trihydroxy-16,22-epoxy-4,6,8(14)-trienergosta-3-one (**1**), the first example of a steroid possessing both a conjugated triene ketone system and a fused 4H-furan ring side chain within one molecule, and (22*E*, 24*R*)-3β,5α-dihydroxyergosta-23-methyl-7,22-dien-6-one (**2**), as well as two known compounds (22*E*, 24*R*)-3β,5α-dihydroxyergosta-7,22-dien-6-one (**3**) and (22*E*, 24*R*)-6β-methoxy-ergosta-7,22-diene-3β,5α-diol (**4**). Their structures were assigned by detailed interpretation of HRESIMS, 1D and 2D NMR spectroscopic data. The absolute stereochemistry of **1** was determined by single-crystal X-ray crystallography while the absolute stereochemistry of **2** was established by CD. Compounds **1**-**4** showed cytotoxic activity against the lung adenocarcinoma (A549) with IC_{50} values of 0.08, 0.94, 12.6 and 13.5 μM, respectively. In addition, compounds **1** and **2** exhibited moderate activities against human ovarian cancer (A2780), with IC_{50} values of 2.8 and 5.1 μM.

1. Introduction

Monascus purpureus-fermented rice has been extensively used as a folk medicine for thousands of years. Many types of secondary metabolites with various biological activities, such as pigments, monacolin analogs, γ-aminobutyric acid (GABA), citrinin, and dimerumic acid. [1–4], were produced by *Monascus*. The extract of *Monascus*-fermented red rice and its secondary metabolites (monacolin K [5], monascorubrin [6], and ankaflavin [4]) have been reported to possess anti-proliferative effects on cancer cells [4].

In the course of our ongoing search for potential antitumor agents from natural products, we found that the ethanol extract of *M. purpureus*-fermented rice showed potent cytotoxic activity. Chemical and biological investigation of this material were carried out, and the petroleum ether-soluble fraction of the ethanolic extract showed potent cytotoxic activity in vitro assays. Initial bioassay-guided fractionation of the extract led to the isolation of three cytotoxic azaphilones, monapurones A–C [7]. As part of our continuing investigation of the antitumor constituents from this material, four cytotoxic steroids (**1**–**4**) (22*S*, 23*R*, 24*S*)-20β,23α,25α-trihydroxy-16,22-epoxy-4,6,8(14)-trienergosta-3-one (**1**), (22*E*, 24*R*)-3β,5α-dihydroxyergosta-23-methyl-7,22-dien-6-one (**2**), (22*E*, 24*R*)-3β,5α-dihydroxyergosta-7,22-dien-6-one (**3**) [8] and (22*E*, 24*R*)-6β-methoxy-ergosta-7,22-diene-3β,5α-diol (**4**). [9] were obtained from this fraction, including two (**1** and **2**) new compounds. Compound **1** showed the most potent cytotoxic activity against the lung adenocarcinoma (A549) with IC_{50} values of 0.08. This also represents the first example of a steroid possessing both a conjugated triene ketone system and a fused 4*H*-furan ring side chain within one molecule. Herein, details of the isolation, structural elucidation and cytotoxic activities of these compounds are described. This is also the first determination of the absolute configuration of **1** by single-crystal X-ray crystallography.

2. Experimental

2.1. General

Optical rotations were measured with a Perkin-Elmer Model 343 spectropolarimeter. UV spectra were taken on a Thermo Spectronic spectrophotometer, Vision32 software V1.25. IR spectra were recorded as microscope transmission on a Nicolet 5700 FT-IR spectrophotometer. CD spectral data were determined on a JASCO J-810 circular dichroism spectrometer. NMR spectra were acquired in CD_3COCD_3 or $CDCl_3$ with TMS as an internal standard on Varian 500 MHz spectrometer. ESIMS and HRESIMS data

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were measured with a Q-Trap LC/MS/MS (Turbo Ionspray Source) and an AccuToFCS JMST100CS spectrometer. Column chromatography was performed with silica gel (160-200 mesh, Qingdao Marine Chemical Inc. China), RP-18 reverse phase silica gel (43–60 μm) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala Sweden). LPLC separation was performed with Combiflash (ISCO Companion). HPLC was performed with a Waters 600 system with 2996 DAD-detector, with an Alltima C_{18} (250 mm × 10 mm i.d., 5 μm) column. TLC was carried out with glass precoated silica gel GF_{254} plates. Spots were visualized under UV light or by spraying with 8% H_2SO_4 in 95% EtOH followed by heating.

2.2. Biological material

Monascus-purpureus fermented rice was prepared from cooked paddy rice inoculated with *M. purpureus* B0708, purchased from the Beijing Dawn Aerospace Bio-tech Company. The sample was deposited at Beijing Union University, Beijing Key Laboratory of Bioactive Substances and Functional Foods, Beijing 100191, China.

2.3. Extraction and isolation

The dried red yeast rice powder (4.5 kg) was extracted with 50 L of 95% EtOH, 80% EtOH and 60% EtOH, respectively, at room temperature (3×, each for 3 h). After removal of the solvent under reduced pressure, the residue (850.0 g) was suspended in 3 L of H_2O, and then partitioned sequentially with petroleum ether (3 times with 5 L each) and EtOAc (4 times with 5 L each) to yield petroleum ether (38.0 g), EtOAc (223.5 g) and H_2O (588.5 g) fractions, respectively.

The EtOH extract showed cytotoxic activity with IC_{50} value of less than 50 μg/mL The EtOAc (223.5 g) and H_2O (588.5 g) fractions showed no cytotoxic activity (IC_{50} > 50 μg/mL). The petroleum ether-soluble portion (38.0 g) showing cytotoxic activity (IC_{50} = 38.6 μg/mL) was fractionated via silica gel column chromatography eluted with a gradient of increasing acetone (0–100%) in petroleum ether to give 12 fractions F_1–F_{12}. The fraction F_{10} (13.2 g) with cytotoxic activity (IC_{50} = 13.4 μg/mL) was purified by normal phase LPLC (flash column) using a gradient increasing acetone in petroleum ether (60–90 °C) to give 8 (A1–A8) subfractions. A6 subfraction (2.3 g) was purified by gradient reversed phase MPLC using 25–80% MeOH in H_2O as mobile phase, and then further purified by gel chromatography over Sephadex LH-20 eluted with $CHCl_3$–MeOH (2:1) to afford **1** (22.5 mg). A3 subfraction (1.8 g) was separated via Sephadex LH-20 eluted with petroleum ether-chloroform-acetone (5:5:1), and further purified by half-preparative reversed phase HPLC using 87% MeOH in H_2O as mobile phase to afford **2** (11.0 mg) and **3** (15.6 mg). A1 subfraction (1.1 g) was purified by normal phase LPLC (flash column) using petroleum ether-acetone (15:1), and further purified by preparative reversed phase HPLC using 90% MeOH in H_2O as mobile phase to afford **4** (35.8 mg).

(22*S*, 23*R*, 24*S*)-20β,23α,25α-trihydroxy-16,22-epoxy-4,6,8(14)-trienergosta-3-one (1): colorless block; $[\alpha]_D^{20}$ + 421.4° (*c* 0.035, CH_3OH); UV λ_{max}^{MeOH} nm (log ε): 206 (0.156), 343 (0.513); CD (CH_3OH) **Δε =** 269 nm (−0.16), 363 nm (+1.70); IR (KBr) ν_{max} 3448, 3355, 2968, 2940, 2893, 2857, 1717, 1631, 1580, 1455, 1360, 1324, 1273, 1223, 1143, 1032, 946, 874, 822; ^{1}H NMR spectral data (CD_3OCD_3, 500 MHz): see Table 1; ^{13}C NMR spectral data (CD_3OCD_3, 125 MHz): see Table 1. HRESI-MS (*m*/*z*):465.3308 $[M+H]^+$ (calcd. for $C_{28}H_{41}O_5H$ 457.2954).

(22*E*, 24*R*)-3β,5α-dihydroxyergosta-23-methyl-7,22-dien-6-one (2): white amorphous powder; $[\alpha]_D^{20}$ + 12.7° (*c* 0.05, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε): 201 (0.072), 252 (0.082); CD ($CHCl_3$) Δε = 253 nm (−1.64), 346 nm (+0.43); IR (KBr) ν_{max} 3483, 3445, 3301, 2955, 2924, 2871, 1725, 1672, 1627, 1457, 1387, 1369, 1248, 1161, 1101, 992, 971, 869; ^{1}H NMR spectral data ($CDCl_3$, 500 MHz): see Table 1; ^{13}C NMR spectral data ($CDCl_3$, 125 MHz): see Table 1. HRESI-MS (*m*/*z*):465.3333 $[M+Na]^+$ (calcd. for $C_{29}H_{46}O_3Na$ 465.3344).

2.4. X-ray analysis of 1

X-ray crystallographic of 1: crystals of compound **1** were grown via the slow evaporation of a acetone solution. A colorless block of appropriate dimensions was mounted on a sealed tube. The room temperature (293 ± 2° K), single-crystal X-ray experiments were performed on a Rigaku MicroMax 002+ diffractometer equipped with ConfocalLaser monochromatized Cu K_α radiation by using the ω and κ scan technique to a maximum 2θ value of 144.24°. The structure was established by direct methods (SHELXL-97) and expanded using Fourier techniques (SHELXL-97). 33 non-hydrogen atoms were refined anisotropically using the least-squares method, and all the hydrogen atoms were positioned by geometrical calculations and difference Fourier overlapping calculation. The reliable factor is R_1 = 0.0329, wR_2 = 0.0904, S = 1.039 ($|F|^2 \geq 2\sigma|F|^2$). The absolute configuration was assigned on the basis of the the Flack parameter 0.11(15).

Crystal data of 1: colorless block; crystal size 0.17 mm × 0.27 mm × 0.64 mm; molecular formula $C_{28}H_{40}O_5$; fw 456.62; orthorhombic system; space group $P2_12_12_1$; unit cell dimensions a = 11.254 (4), b = 12.157 (5), c = 18.161 (6) Å, V = 2484.7 (16) Å^3; Z = 4; d_{calc} = 1.221 g/cm^3; reflections collected 4237 and independent reflections 4687. Crystallographic data of **1** have been deposited at the Cambridge Crystallographic Data Centre (deposition no. 794794). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/deposit or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK. [fax: +44 1223 336 033; or email: deposit@ccdc.cam.ac.uk].

2.5. In vitro cytotoxicity bioassays

HCT-8, Bel7402, BGC-823, A549, A2780 and WISH cell lines were obtained from ATCC. Cells were maintained in RRMI 1640 medium supplemented with 10% fetal newborn bovine serum (FBS), 100 units/mL of penicillin, and 100 g/mL of streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. All the cells were seeded in 96-well microtiter plates at 1200 cells/well. After 24 h, the tested compounds were added to the systems. After 96 h of drug treatment, cell viability was determined by measuring the metabolic conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple formazan crystals by active cells. Topotecan was the positive control. MTT assay results were read using a MK 3 Wellscan (Labsystem Drogon) plate reader at 570 nm. All compounds were tested at five concentrations and were dissolved in 0.1% DMSO each well. Each concentration of the compounds was tested in three parallel wells. IC_{50} values were calculated using Microsoft Excel software.

3. Results and discussion

Compound **1**, a colorless crystal (in acetone), showed a molecular formula of $C_{28}H_{40}O_5$ as determined by HRESIMS at *m*/*z* 457.3008 $[M+H]^+$(calcd 457. 2954) with nine degrees of unsaturation. The IR absorptions revealed the presence of hydroxyl (3355 cm^{-1}), conjugated carbonyl (1717 cm^{-1}), and double bond (1631 and 1580 cm^{-1}) functionalities. The UV absorption at λ_{max} = 343 nm (log ε 3.06) was suggestive of the presence of a conjugated trienone chromophore.

The ^{13}C NMR spectrum displayed 28 carbon signals, which were classified as six methyls, five methylenes, nine methines (three