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Monaphilones A−C, Three New Antiproliferative Azaphilone Derivatives from *Monascus purpureus* NTU 568

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Monascus purpureus NTU 568 was a mutant strain from *M. purpureus* HM105. The methanol extract of red mold rice fermented by this strain exhibited four major yellow pigment signals on HPLC profile. By repeated chemical chromatography methods, three new azaphilone derivatives, namely, monaphilone A (**1**), B (**2**) and C (**3**), along with the known pigments ankaflavin (**4**) and monascin (**5**), were isolated and characterized. Based on spectroscopic analyses, mainly 1D and 2D NMR data, the structures of compounds **1**−**3** were completely elucidated; in addition, **1**−**3** were determined to be new azaphilone structures, due to the decrease of carbon monoxide for producing a γ-lactone ring, compared with other azaphilone derivatives. Biological evaluations showed that monaphilone A (**1**) and B (**2**) exhibited an antiproliferative effect against HEp-2 (human laryngeal carcinoma cell line) and WiDr (human colon adenocarcinoma cell line), and none of the five compounds had toxicity to normal human lung cell lines (WI-38 and MRC-5) at 70 μM.

KEYWORDS: Monaphilones A−C; azaphilone; *Monascus purpureus*; HPLC; ankaflavin; monascin

INTRODUCTION

Red mold rice (RMR), a fermented product of *Monascus* species, has been used as a traditional Chinese medicine and natural food colorant for thousands of years in Asia. RMR is also a common food additive for preserving fish and meat. Some bioactive metabolites, such as pigments, dimerumic acid, and γ-aminobutyric acid (GABA) etc., have been isolated from RMR in addition to monacolin K and its analogues via pharmacognosical technologies. These functional constituents derived from RMR also had been deemed to be provided with various health benefits in preventive medicine. Monacolin K, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, can reduce cholesterol biosynthesis in human liver (*1*, *2*). The azaphilonoidal pigments of RMR, including different colors such as yellow (monascin and ankaflavin), orange (rubropunctatin and monascorburin), and red (rubropunctamine and monascorburamine), have exhibited anti-inflammatory (*3*) and cytotoxic effects (*4*). One of the ingredients in RMR, dimerumic acid, is an antioxidant exhibiting a protective effect against CCl_4-induced liver injury (*5*). GABA was known as a neurotransmitter and hypotensive agent (*6*).

Monascus purpureus NTU 568 was mutated from *M. purpureus* HM105. The RMR fermented by *M. purpureus* NTU 568 had been verified to possess several functions including antihypolipidemic, antihypertensive, antioxidative, and anti-inflammatory effects, as well as antitumor activity in vivo (*7*−*11*). The known metabolites of *M. purpureus* fermented RMR, such as monascin, ankaflavin, and several yellow pigments, have anti-inflammatory, immunosuppressive, and cytotoxic activities (*12*, *13*). In our previous studies, the methanol extracts of RMR possessed a cytotoxic effect on Caco-2 colorectal adenocarcinoma cells and mitigated oral carcinogenesis in 7,12-dimethyl-1,2-benz[*a*]-anthracene-induced oral carcinogenesis in hamsters (*14*, *15*). The incidence and mortality rates of colon and laryngeal tumor in Taiwan have been increasing tremendously within 10 years. Recently, a preliminary screening assay showed that methanol extracts of RMR inhibited the proliferation of human colon adenocarcinoma [(WiDr) IC_{50} = 27.86 μg/mL] and human laryngeal carcinoma [(HEp-2) IC_{50} = 33.53 μg/mL]. Furthermore, ankaflavin possessed an antiproliferative effect on A549 (human lung epithelial carcinoma) and Hep-G2 (human hepatocellular carcinoma), while it was ineffective for MRC-5 and WI-38 (human lung noncarcinogenic cell lines) (*4*). These findings motivated us to investigate the antiproliferative components isolated from *M. purpureus* NTU 568 fermented RMR, by using human cancer cell lines, HEp-2 and WiDr, as well as human lung cell lines, MRC-5 and WI-38. In this study, we report the isolation, structural elucidation, and antiproliferative activity of these azaphilone constituents from the extracts of RMR. The HPLC profile of the methanol extract of RMR is also provided for these isolated yellow pigments.

MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer (Thermo Nicolet,

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Madison, WI). Optical rotations were determined on a JASCO P-1020 polarimeter (Jasco Co., Tokyo, Japan). Electrospray ionization mass spectrometry (ESIMS) data were obtained on a LCQ mass spectrometer (Finnigan MAT LCQ, San Jose, CA). High resolution electronic ionization mass spectrometry (HREIMS) data were measured on a Finnigan MAT-95XL mass spectrometer (San Jose, CA). Nuclear magnetic resonance (NMR) spectra were run on Bruker NMR (Unity Plus 400 and 600 MHz) (Bruker BioSpin, Rheinstetten, Germany) and Varian NMR spectrometers (Varian Gemini 200 MHz, Varian Inc., Palo Alto, CA) using acetone-d_6 as solvent. Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) and silica gel 60 (70−230 mesh and 230−400 mesh, Merck, Darmstadt, Germany) were used for column chromatography, and silica gel 60 GF_{254} (Merck, Darmstadt, Germany) was used for thin layer chromatography (TLC). The spots on TLC were detected under ultraviolet (UV) lamps (254 nm) and by spraying with an anisaldehyde−sulfuric acid solution followed by heating. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a SPD-6AV UV detector, equipped with a 250 × 20 mm i.d. preparative Cosmosil AR-II column (Nacalai Tesque, Inc., Kyoto, Japan).

Reagents. Methanol and acetonitrile (HPLC grade), acetone, ethyl acetate, *n*-hexane and methanol (analytical grade) were purchased from ECHO (Miaoli, Taiwan). Trifluoroacetic acid (TFA), anisaldehyde and sulfuric acid were purchased from Merck. Fetal bovine serum, minimum essential medium (MEM), phosphate buffered saline (PBS) and trypan blue were purchased from Biological Industries (Kibbutz Beit Haemek, Isreal). Other chemicals, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Co. (St. Louis, MO).

Preparation of Red Mold Rice. *Monascus purpureus* NTU 568 was fermented on long-grain rice (*Oryza sative*) as described previously (*16*), and the red mold rice (RMR) was obtained. The fermented material was further dried and crushed to provide the substances for the extraction and HPLC analysis.

Isolation and Identification of Azaphilone Metabolites. The dried RMR powder (5 kg) was soaked three times with methanol (25 L) in a 100 L extractor equipped a temperature controller for 24 h at 50 °C, and the methanol extracts (ca. 70 L) were obtained. After filtering through filter paper, those were combined and then concentrated under reduced pressure. The dried residue mixed with silica gel was subjected to chromatography by a silica gel column eluting with gradient solvent systems (*n*-hexane/ethyl acetate, 10:0, 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 0:10, v/v) and 8 fractions were obtained according to the results of TLC. Fractions 4 and 5 displayed clear yellow spots on an analytical TLC plate with *n*-hexane/ethyl acetate (4:1, v/v). Fraction 4 was separated again by silica gel column eluting with *n*-hexane/ethyl acetate (9:1) to give 3 subfractions (Fr. 4-1−4-3). Then, Fr. 4-2 (164 mg) was subjected to a Sephadex LH-20 column chromatography to remove other impurities. Finally, a partially purified fraction (45 mg) was further purified via a semipreparative HPLC system equipped with Cosmosil $5C_{18}$ packing column (250 × 20 mm) with 87% MeOH as mobile phase solvent at a flow rate of 7 mL/min and compound **1** (10 mg) and compound **2** (15 mg) were obtained, respectively (**Figure 1**). By repeating silica and LH-20 gel column chromatography, fraction 5 was separated to five subfractions (Fr. 5-21−5-25). After treatment as for Fr. 4, compounds **3** (8 mg), **4** (monascin, 75.8 mg) and **5** (ankaflavin, 99.4 mg) were isolated from a part of Fr. 5-23 (250 mg).

Monaphilone A (1). Yellowish oil. $[\alpha]_D^{25}$: +260.62° (*c* 0.32, acetone). IR: ν_{max} (KBr) 3468, 2954, 2926, 2852, 1708, 1644, 1535, 1454, 1405, 1373, 1275, 1194 cm^{-1}. ESIMS: *m*/*z* 383 $[M + Na]^+$. HREIMS: *m*/*z* 360.2302 $[M]^+$ (calcd 360.2301, $C_{22}H_{32}O_4$). ^1H and ^{13}C NMR data are shown in **Table 1**.

Monaphilone B (2). Yellowish oil. $[\alpha]_D^{25}$: +440.00° (*c* 0.15, acetone). IR: ν_{max} (KBr) 3464, 2957, 2926, 2848, 1708, 1644, 1528, 1451, 1409, 1377, 1271, 1194 cm^{-1}. ESIMS: *m*/*z* 355 $[M + Na]^+$. HREIMS: *m*/*z* 332.1999 $[M]^+$ (calcd 332.1988, $C_{20}H_{28}O_4$). ^1H and ^{13}C NMR data are shown in **Table 1**.

Monaphilone C (3). Colorless oil. $[\alpha]_D^{25}$: +92.00° (*c* 0.25, acetone). IR: ν_{max} (KBr) 3481, 2954, 2930, 2871, 1710, 1663, 1461, 1409, 1378, 1263, 1100, 1037, 803 cm^{-1}. ESIMS: *m*/*z* 359 $[M + Na]^+$. HREIMS: *m*/*z* 336.2292 $[M]^+$ (calcd 336.2301, $C_{20}H_{32}O_4$). ^1H and ^{13}C NMR data are shown in **Table 1**.

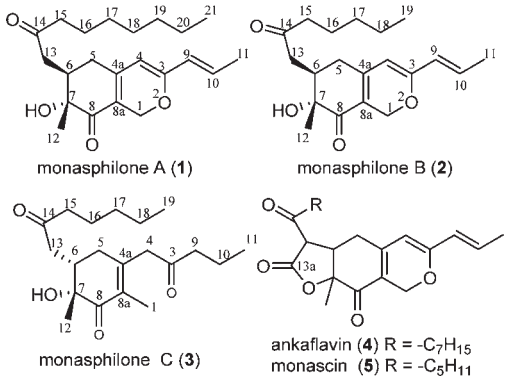

Figure 1. The structures of compounds **1**−**5** (**1**, monaphilone A; **2**, monaphilone B; **3**, monashexenone; **4**, ankaflavin; **5**, monascin).

Ankaflavin (4). Yellow amorphous solid. ESIMS: *m*/*z* 387 $[M + H]^+$. ^1H NMR (d_6-acetone, 400 MHz): δ 0.87 (3H, t, J = 6.8, H-21), 1.29 (8H, m, H-17−20), 1.45 (3H, s, H-12), 1.60 (2H, m, H-16), 1.82 (3H, d, J = 7.2, H-11), 2.66 (2H, m, H-5), 2.70 (1H, m, H-15a), 2.92 (1H, m, H-15b), 3.15 (1H, m, H-6), 4.27 (1H, d, J = 13.2, H-13), 4.68 (1H, d, J = 12.4, H-1a), 4.90 (1H, d, J = 12.4, H-1b), 5.50 (1H, s, H-4), 6.01 (1H, d, J = 15.6, H-9), 6.41 (1H, dt, J = 15.6, 7.2, H-10). ^{13}C NMR (d_6-acetone, 100 MHz): δ 14.2 (C-21), 17.7 (C-12), 18.3 (C-11), 23.2 (C-20), 23.6 (C-16), 29.2 (C-5), 29.9 (C-17, C-18), 32.3 (C-19), 43.4 (C-15), 44.4 (C-6), 55.3 (C-13), 64.2 (C-1), 84.0 (C-7), 104.6 (C-4), 115.1 (C-8a), 125.5 (C-9), 134.6 (C-10), 151.6 (C-4a), 160.2 (C-3), 171.3 (C-13a), 190.6 (C-8), 203.6 (C-14).

Monascin (5). Yellow amorphous solid. ESIMS: *m*/*z* 359 $[M + H]^+$. ^1H NMR ($CDCl_3$, 200 MHz): δ 0.88 (3H, t, J = 6.6, H-19), 1.30 (4H, m, H-17, H-18), 1.43 (3H, s, H-12), 1.61 (2H, m, H-16), 1.84 (3H, d, J = 6.0, H-11), 2.49 (1H, m, H-15a), 2.64 (1H, m, H-5a), 2.70 (1H, m, H-15b), 2.99 (1H, m, H-5b), 3.14 (1H, m, H-6), 3.64 (1H, d, J = 9.0, H-13), 4.67 (1H, d, J = 12.6, H-1a), 5.02 (1H, d, J = 12.6, H-1b), 5.26 (1H, s, H-4), 5.85 (1H, d, J = 15.4, H-9), 6.47 (1H, dt, J = 15.4, 6.0, H-10). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 12.9 (C-19), 16.7 (C-12), 17.5 (C-11), 21.4 (C-18), 21.8 (C-16), 28.4 (C-17), 30.1 (C-5), 41.9 (C-6, C-15), 53.9 (C-13), 62.8 (C-1), 82.2 (C-7), 102.3 (C-4), 113.0 (C-8a), 123.4 (C-9), 134.4 (C-10), 149.8 (C-4a), 159.5 (C-3), 168.5 (C-13a), 188.8 (C-8), 201.5 (C-14).

HPLC Analysis. The RMR powder (1 g) was extracted with 10 mL of methanol at 50 °C for 30 min. The extract was further filtered with a 0.45 μm filter and analyzed by HPLC. HPLC analysis was performed on a Hitachi L-2130 series apparatus with a diode array detector L-2450 (Tokyo, Japan). An Agilent TC-C18 column, 250 × 4.6 mm i.d., 5 μm (Agilent Technologies, Santa Clara, CA), was used as analytical column. The analytical method employed was linear gradient elution (acetonitrile/0.05 TFA water, 30/70 to 80/20, v/v) for 20 min followed by isocratic elution (acetonitrile/0.05 TFA water, 80/20, v/v) from 20 to 35 min.

TLC Analysis. TLC was performed on silica gel 60 plates (Merck). The solvent systems were *n*-hexane/ethyl acetate (4:1, v/v) and dichloromethane/methanol (95:5, v/v). Yellow pigments were detected under visible light.

Cell Lines and Culture. HEp-2 (human laryngeal carcinoma), WiDr (human colon adenocarcinoma), WI-38 (human lung fibroblast) and MRC-5 (human embryonal lung fibroblast) were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). HEp-2 and WiDr cell lines were maintained in MEM containing 5% fetal bovine serum, and WI-38 and MRC-5 cell lines were maintained in MEM containing 10% fetal bovine serum. All of them were cultured in a 37 °C incubator with 5% CO_2.

Assay for Antiproliferation. For determining the antiproliferative efficiencies, MTT assay was adapted according to the reported methods (*17*, *18*). Cells (3×10^3 per well) were seeded in 180 μL of MEM in 96-well plates. After 4 h, 20 μL of test agents were dissolved in PBS solution and added at final concentrations of 5, 10, 25, 50, and 100 μg/mL under 37 °C incubator with 5% CO_2. After 3 days, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h to make cellular conversion of a tetrazolium salt into a formazan product. Then the supernatant was removed and 200 μL of DMSO was added to dissolve the formazan. Finally, the formazan could be detected by an ELISA reader

Table 1. ^{1}H NMR and ^{13}C NMR Spectroscopic Data for Monaphilone A (**1**), Monaphilone B (**2**), and Monaphilone C (**3**)[a−c]

	1		2		3	
no.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4. 71 (d, *J* = 12.4) 4.88 (d, *J* = 12.4)	64.3	4.69 (d, *J* = 12.4) 4.87 (d, *J* = 12.4)	64.0	1.70 (s)	11.7
3		160.3		160.0		206.3
4	5.41 (s)	104.3	5.41 (s)	104.1	3.47 (d, *J* = 16.2) 3.60 (d, *J* = 16.2)	48.9
4a		152.4		152.1		152.0
5	2.21 (m) 2.49 (m)	32.9	2.21 (m) 2.50 (m)	32.6	2.15 (m) 2.43 (m)	37.1
6	2.54 (m)	40.3	2.54 (m)	40.0	2.47 (m)	39.9
7		74.8		74.5		74.6
8		198.6		198.3		203.0
8a		114.2		113.9		130.2
9	6.01 (d, *J* = 15.2)	125.7	5.99 (d, *J* = 15.2)	125.4	2.51 (t, 7.2)	45.1
10	6.39 (dq, *J* = 15.2, 7.2)	134.1	6.38 (dq, *J* = 15.2, 7.2)	133.8	1.56 (m)	17.6
11	1.82 (3H, d, *J* = 7.2)	18.3	1.81 (3H, d, *J* = 7.2)	18.0	0.87 (3H, t, *J* = 7.8)	14.3
12	1.15 (s)	20.0	1.10 (s)	19.7	1.10 (s)	19.7
13	2.47 (m) 2.91 (d, *J* = 15.2)	42.4	2.52 (m) 2.86 (d, *J* = 15.2)	42.1	2.49 (m) 2.86 (d, *J* = 14.4)	42.3
14		209.8		209.5		209.7
15	2.49 (m)	43.4	2.48 (m)	43.0	2.46 (m)	43.3
16	1.54 (m)	24.4	1.54 (t, 7.2)	23.8	1.52 (m)	24.0
17	1.27 (m)	29.9	1.29 (m)	32.6	1.24 (m)	32.0
18	1.27 (m)	29.9	1.29 (m)	22.7	1.27 (m)	23.0
19	1.27 (m)	32.4	0.86 (t, 6.8)	13.8	0.86 (t, 7.8)	14.1
20	1.27 (m)	23.2				
21	0.87 (t, 6.8)	14.2				

[a] Assignments were confirmed by $^{1}H-^{1}H$ COSY, HMQC, HMBC. [b] ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds **1**−**3** were measured at 400 and 100 MHz in acetone-d_6. [c] m: multiple signal.

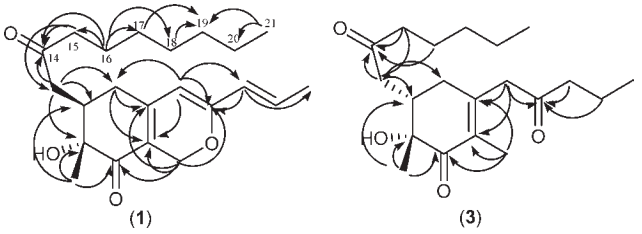

Figure 2. Key HMBC correlations of monaphilone A (**1**) and monaphilone C (**3**).

in the absorbance at 570 nm and provided a relative estimate of cell proliferation.

Data Analysis. The data of antiproliferative experiment were presented as mean ± standard deviation for three independently performed experiments (n = 3).

RESULTS AND DISCUSSION

Structural Elucidation. Compound **1** (monaphilone A) gave a molecular ion peak at m/z 360.2302 $[M]^+$ by HREIMS corresponding to a molecular formula of $C_{22}H_{32}O_4$. The IR spectra displayed absorption bands for hydroxyl (3468 cm^{-1}), conjugated ketone (1708 cm^{-1}) and vinyl groups (1644, 1535, and 1454 cm^{-1}). The ^{1}H NMR spectral data of **1** (**Table 1**) showed signals for three methyls (a singlet, a doublet and a triplet), nine methylenes, one methine, and three olefinic protons [a singlet and a *trans*-olefinic group (J = 15.2 Hz)]. The ^{13}C NMR spectrum (**Table 1**) indicated the presence of two carbonyl carbons, six olefinic carbons and fourteen sp^3 carbons. Inspection of the HMBC spectrum of **1** (**Figure 2**) revealed cross peaks of H-1 [δ_H 4.71 (d, J = 12.4) and 4.88 (d, J = 12.4)]/C-3 (δ_c 160.3), C-4a (δ_c 152.4), C-8 (δ_c 198.6), C-8a (δ_c 114.2), H-4 [δ_H 5.41 (s)]/C-3, C-5 (δ_c 32.9), C-8a (δ_c 114.2), C-9 (δ_c 125.7), H-5 [δ_H 2.21 (m) and 2.49 (m)]/C-4a, C-8a, H-9 [δ_H 6.01 (d, J = 15.2)]/C-3, C-11 (δ_c 18.3), and the methyl protons 12-CH_3 [δ_H 1.15 (s)/ C-6 (δ_c 40.0), C-7 (δ_c 74.8), C-8]. These spectral data suggested that **1** possessed an azaphilone structure, a second six-membered ring with ketone (C-8) fused to the 2*H*-pyran with a propenyl substituent (*19*). Based on long-range correlations, the pendant signals including a methyl group (12-CH_3) substituted of an oxygenated quaternary carbon, and a propenyl group with a pair of *trans*-olefinic protons (H-9 and H-10), were assigned to C-7 and -3, respectively. In addition, sequential cross peaks for an *n*-heptane moiety (H-15 to H-21) were found in the $^{1}H-^{1}H$ COSY spectrum. This finding, together with the HMBC spectral correlations between a pair of methylene protons (H-13a,b) and the C-14 ketone, C-15 and C-6, determined that the side chain oxononyl moiety was located at C-6. Moreover, after the irradiation of the methyl group (H-12) in the 1D NOE (nuclear Overhauser effect) experiment, no enhancement of H-6 was found, suggesting the *trans*-configuration between H-6 and H-12 in **1**.

Based on the foregoing evidence, the structure of **1** was elucidated as 6,7-dihydro-7-hydroxy-7-methyl-6-(2-oxononyl)-3-[(*E*)-prop-1-enyl]-1*H*-isochromen-8(5*H*)-one. Compound **1** lacks a γ-lactone ring resulting from the loss of a carbon monoxide, compared with the similar azaphilone structures ankaflavin (**4**) and monascin (**5**), revealing that **1** is a new type of azaphilone in nature as shown.

The HREIMS of **2** revealed the elemental formula $C_{20}H_{28}O_4$ from a molecular ion at m/z 332.1999 $[M]^+$. The IR, UV, ^{1}H and ^{13}C NMR spectra showed that **2** possesses a similar azaphilone derivative as **1**. The molecular weight of **2** is 28 units less than **1**, suggesting the absence of two methylene groups in **2**. The NMR spectrum of **2** showed the presence of a 2-oxoheptyl moiety in **2**, rather than a 2-oxononyl moiety in **1**. Thus, the structure of **2** was elucidated as 6,7-dihydro-7-hydroxy-7-methyl-6-(2-oxoheptyl)-3-[(*E*)-prop-1-enyl]-1*H*-isochromen-8(5*H*)-one, and has been named monaphilone B.

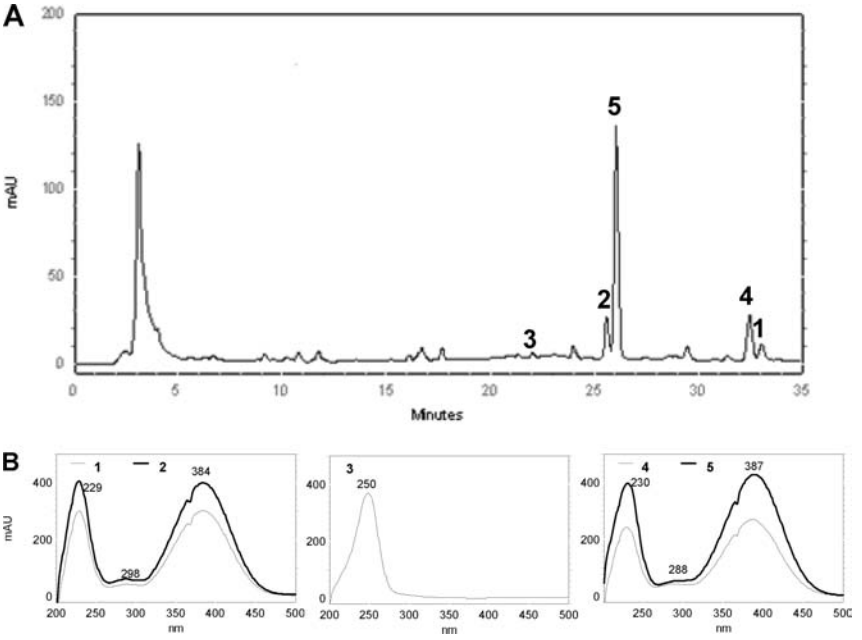

Figure 3. (**A**) The HPLC profile of red mold rice detected at 250 nm. (**B**) UV spectra of peaks **1**−**5** (**1**, monaphilone A; **2**, monaphilone B; **3**, monaphilone C; **4**, ankaflavin; **5**, monascin) detected by PDA.

The molecular formula $C_{20}H_{32}O_4$ of **3** was deduced from a molecular ion peak at m/z 336.2292 $[M]^+$ in its HREIMS. The IR absorption bands (3481, 1710, and 1663 cm^{-1}) indicated the presence of hydroxyl and conjugated carbonyl groups. The ^1H NMR spectrum of **3** (**Table 1**) showed signals for four aliphatic methyls (two singlets and two triplets), nine methylenes, one methane, and one hydroxyl groups. The ^{13}C NMR spectrum showed the presence of three carbonyl carbons, two olefinic carbons and fifteen sp^3 carbons. The $^1H-^1H$ COSY (H-9/H-10, H-10/H-11; H-15/H-16, H-16/H-17, H-17/H-18, H-18/H-19) together with HMBC experiments {**Figure 2**: H-9 [δ_H 2.51 (t, 7.2)]/C-3 (δ_C 206.3), H-10 [δ_H 1.56 (m)]/C-3 and H-4 [δ_H 3.47 (d, J = 16.2) and 3.60 (d, J = 16.2)]/C-3; H-15 [δ_H 2.46 (m)]/C-14 (δ_C 209.7), H-16 [δ_H 1.52 (m)]/C-14) and H-13 [δ_H 2.49 (m) and 2.86 (d, J = 14.4)]/C-14} established the presence of 2-oxopentyl ($C_3H_7COCH_2-$, from C-4 to C-11) and 2-oxoheptyl moieties ($C_5H_{11}COCH_2-$, from C-13 to C-19).

Further confirmation by COSY (H-5/H-6) and HMBC {H-12 [δ_H 1.10 (s)]/C-8 (δ_C 203.0), C6 (δ_C 39.9), C-7 (δ_C 74.6); H-1 [δ_H 1.70 (s)] /C-8, C-8a (δ_C 130.2), C-4a (δ_C 152.0)} NMR data allowed the skeleton of **3** to be determined as 6-hydroxy-2,6-dimethylcyclohex-2-enone. Moreover, the HMBC spectrum of **3** displayed correlations between H-4 and the olefinic carbon (C-4a), and between H-13 and C-5 (δ_C 37.1) and C-6, confirming that the 2-oxopentyl group was located at C-4a of in **3**. The connection between the 2-oxoheptyl group and C-6 was also verified by HMBC spectral data (H-13 to C-5 and C-6). The relative configuration of **3** was determined by NOESY (nuclear Overhauser effect spectroscopy) correlations. Thus, the *syn*-relation for H-6 [δ_H 2.47 (m)]/H-12 [δ_H 1.10 (s)] was deduced from NOE correlation between H-6 and H-12. From the above evidence, the structure of **3** was determined as 6-hydroxy-2,6-dimethyl-5-(2-oxoheptyl)-3-(2-oxopentyl)cyclohex-2-enone, and has been named monaphilone C. The other isolated compounds, **4** and **5**, were identified as ankaflavin (**4**) and monascin (**5**), respectively, by comparison with authentic samples and literature data (*4*).

TLC and HPLC Analysis. The concentrated methanol extract of RMR obtained from *M. purpureus* MTU 568 was analyzed by silica gel TLC with *n*-hexane/ethyl acetate (4:1, v/v). Two major yellow pigment groups, including compounds **1**−**5**, were observed on a TLC plate. Their R_f values were 0.58 (**1**), 0.63 (**2**), 0.30 (**3**), 0.39 (**4**), and 0.49 (**5**) under the absorbance of 254 nm, respectively. Furthermore, four major peaks (**1**, **2**, **4** and **5**) were exhibited in the HPLC chromatogram of crude extract of RMR (**Figure 3A**) responding to the retention time from 25 to 34 min. The minor component, compound **3**, was also observed at 22 min.

The UV absorption spectra (**Figure 3B**) at 229−228 nm, 384−387 nm provided the characteristics of compounds **1**, **2**, **4**, and **5**. However, the maximum absorption signal of **3** appears at 250 nm due to the absence of a pyran ring in comparison with other yellow pigments from *M. purpureus* MTU 568. On the basis of the foregoing spectroscopic evidence, it is implicated that the hydrophobic constituents of RMR mainly should be composed of azaphilone derivatives including compounds **1**−**5**.

Table 2. Antiproliferation Effects of Compounds **1**-**5** on HEp-2 and WiDr Cell Lines

	IC_{50}[a] (μM)	
compound	HEp-2	WiDr
1	72.1 ± 2.5	55.8 ± 1.8
2	77.6 ± 4.4	55.3 ± 3.5
3	124.1 ± 11.9	142.4 ± 5.7
4	94.7 ± 2.7	111.6 ± 9.1
5	59.8 ± 8.7	*b*
mitomycin C[c]	0.4 ± 0.0	0.4 ± 0.1

[a] IC_{50}: inhibitory concentration 50%. [b] IC_{50} > 250 μM. [c] Positive control. All values are presented as mean ± SD (n = 3).

Antiproliferative Activities. Compounds **1**−**5** were evaluated for inhibitory effects on the proliferation on human tumor cell lines, HEp-2 (human laryngeal carcinoma) and WiDr (human colon adenocarcinoma), as well as human lung noncarcinogenic cell lines (WI-38 and MRC-5). Mitomycin C, a clinic antitumor agent, was used as a positive control. The biological results (**Table 2**) revealed that monaphilone A (**1**) and B (**2**) displayed higher antiproliferative effects than monaphilone C (**3**), ankaflavin (**4**), and monascin (**5**) on HEp-2 (IC_{50} = 72.1 and 77.6 μM) and WiDr (IC_{50} = 55.8 and 55.3 μM), respectively. Monascin (**5**) had no significant inhibitory effect toward WiDr cells. As shown

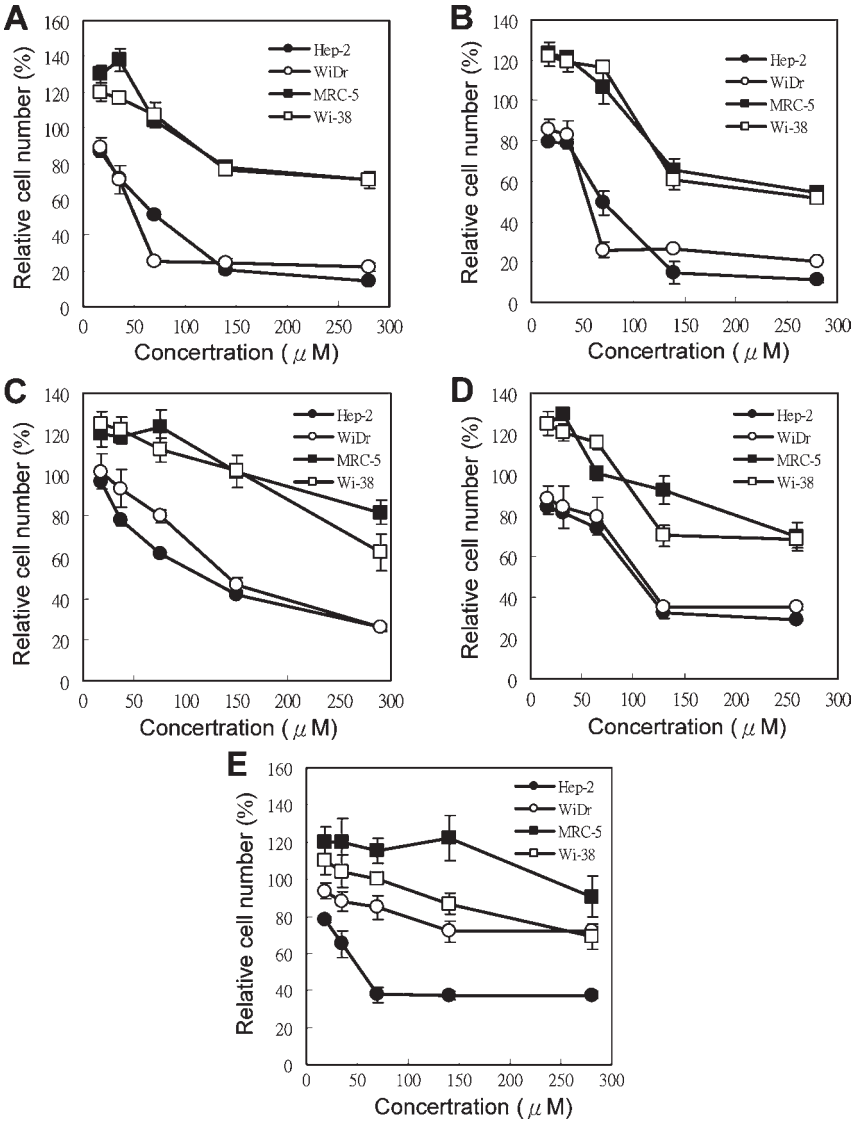

Figure 4. The inhibitory effects of proliferation on four cell lines (HEp-2, WiDr, MRC-5, and WI-38) by monaphilone A (**A**), monaphilone B (**B**), monaphilone C (**C**), ankaflavin (**D**), and monascin (**E**). All values are presented as mean ± SD (n = 3).

in **Figures 4A** and **4B**, compounds **1** and **2** exhibited promising effects against WiDr cells and HEp-2 at 70 μM (exceeding 75% inhibition), whereas high dosages (140 and 280 μM) of them have very weak and low dosages (70 and 35 μM) have no inhibitory activities for WI-38 and MRC-5. The foregoing results proved that antiproliferative effects of compounds **1** and **2** were selective to HEp-2 and WiDr cancer cell lines. Although the antiproliferative effects of tumor cells for all isolates (**1**−**5**) are not very promising (IC_{50} 55.3 to 142.4 μM) compared with mitomycin C, however, these isolated azaphilone derivatives (**1**−**5**) might have potential for development as functional health food, due to their moderate blocking of tumor cell proliferation and nontoxicity for WI-38 and MRC-5. These findings are consistent with reported yellow pigment derivatives which are not significantly toxic to human lung noncarcinogenic cells (*4*, *13*).

The structural elucidation of three new isolates (**1**−**3**) obtained from RMR are based on 1D and 2D NMR, MS, UV, and IR spectroscopic analyses. These new azaphilone derivatives (**1**−**3**) lack a γ-lactone ring, compared with the known pigments of *Monascus*, including rubropunctamin, monsacorubramin, monascin, rubrpunctatin ankaflavin, and monascorubrin. We also observed that the UV absorption spectra of monaphilone A (**1**) and B (**2**), two new yellow pigments, were similar to those of ankaflavin and monascin. Therefore, compounds **1**−**3** were further affirmed as new azaphilone derivatives in natural products. Biological assays showed that monaphilone A (**1**) and B (**2**) exhibited higher antiproliferative effect than monaphilone C (**3**), which lacks a pyran ring, revealing that the pyran ring in the new type of azaphilone analogues may play a crucial role for their antiproliferative effect. In addition, monascin showed no cytotoxic effect on WiDr cells in our study, as well as A549 and HepG2 cells (*4*). However, monascin had higher antiproliferative activity (59.8 μM) against HEp-2 cells than ankaflavin. This result suggested that antiproliferative effects of monascin possessed tissue specificity and especially selectivity to human laryngeal carcinoma cell line. The evidence also supports our previous study (*11*) that RMR comprised other potential anticancer components, in addition to ankaflavin and monacolin K. It was reported that RMR extracts and ankaflavin can induce tumor cell death through apoptosis (*4*, *14*). However, further experiments of the antiproliferative mechanism for monaphilone A (**1**) and B (**2**), as well as other azaphilone derivatives, remain to be investigated.

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