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New Bioactive Orange Pigments with Yellow Fluorescence from *Monascus*-Fermented Dioscorea

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ABSTRACT: Red mold dioscorea (RMD) is a fermented product of *Monascus purpureus* NTU 568 using dioscorea as culture substrate. To investigate the bioactive components of RMD, six orange pigments including four new azaphilones with yellow fluorescence, monapilol A−D (**1**−**4**), and known monascorubrin (**5**) and rubropunctatin (**6**) were isolated and characterized. Structural elucidation of new isolates was based on nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC) and other spectroscopic analyses. The structures of monapilols (**1**−**4**) were similar to those of monascorubrin (**5**) and rubropunctatin (**6**); however, the hydroxyl group (8-OH) in compounds **1**−**4** substituted for the C-8 carbonyl in compounds **5** and **6**. Biological evaluation indicated that compounds **1**−**4** inhibited nitric oxide (NO) production on lipopolysaccharide-stimulated RAW 264.7 cells. Compounds **1**−**4** also exhibited antiproliferative activities against human laryngeal carcinoma (HEp-2) and human colon adenocarinoma (WiDr).

KEYWORDS: red mold dioscorea, bioactive orange pigments, human laryngeal carcinoma, human colon adenocarinoma

INTRODUCTION

The fermented product of *Monascus* genus fungi has been used as a food additive and in Chinese traditional medicine for thousands of years. *Monascus*-fermented red rice, called red mold rice (RMR), becomes a popular functional food for treating hyperlipidemia because of the well-known second metabolite, monacolin K. Monacolin K is a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that results in reducing the biosynthesis of cholesterol.[1,2] RMR exhibited the regulation of obesity-related factors,[3] the mitigation of oral carcinogenesis in 7,12-dimethyl-1,2-benz[*a*]anthracene (DMBA)-induced oral tumor,[4] and the amelioration of memory impairment [5] *in vivo*. The extracts of RMR have a broad range of biological activities including antioxidant, anti-inflammatory, and antitumor activities.[6,7] Phytochemical studies [8] revealed that RMR possesses six major azaphilonoid pigments, including yellow pigments, ankaflavin and monascin, orange pigments, monascorubrin and rubropunctain, and the red pigments, monascorubramine and rubropuctamine.

Chronic inflammation involves all three steps of tumor development: initiation, progression, and metastasis. Colorectal and oral cancers have a fast rising incidence and mortality rate in Taiwan. In epidemiological studies, anti-inflammatory therapies showed effectiveness in reducing tumor incidence. The strategies for controlling the incidence of colorectal and oral cancers have targeted anti-inflammatory and antiproliferative actions.[9] Recently, we found that red mold dioscorea (RMD), a fermented product of *Monascus* using dioscorea as substrate, has a greater hypolipidemic, antiatherosclerotic, antihypertensive, and antidiabetic effect than traditional RMR.[10−12] In addition, the extract of RMD not only induced apoptosis in human oral cancer cells but also possessed anti-inflammatory and antioxidative potentials against DMBA-induced oral injury in hamster.[13] RMD contained a higher amount of anti-inflammatory and antiproliferative yellow pigments (monascin and ankaflavin) than RMR by analysis of the extract of RMD.[14−16] Recently, azaphilonoid derivatives from RMR have been demonstrated to have anti-inflammatory and antitumor effects on HEp-2 (human laryngeal carcinoma) and WiDr (human colon adenocarcinoma) cell lines by us and the other group;[17−19] whereas there is a lack of phytochemical study on RMD. This motivated us to investigate the constituents of anti-inflammation and antiproliferation against HEp-2 and WiDr cell lines from RMD. Here, we report the isolation and structural elucidation of six bioactive azaphilonoidal pigments from RMD. In addition, these isolated orange pigments were tested for anti-inflammatory and antiproliferative activities.

MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer (Thermo Nicolet, Madison, WI, USA). Optical rotations were determined on a JASCO P-1020 polarimeter (Jasco Co., Tokyo, Japan) in acetone. Fluorescence spectra were recorded on a SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA, USA). Electrospray ionization mass spectrometry (ESIMS) and high resolution electrospray ionization mass spectrometry (HRESIMS) data were measured on a LCQ mass spectrometer (Finnigan MAT LCQ, San Jose, CA, USA) and a Finnigan MAT 95S mass spectrometer (San Jose, CA, USA), respectively. Nuclear magnetic resonance (NMR) spectra were run on a Bruker NMR (Unity Plus 400 MHz) (Bruker BioSpin, Rheinstetten, Germany) 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)

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Table 1. ^{1}H NMR Spectroscopic Data for Monaphilol A (1), B (2), C (3), and D (4) [a]

no.	1	2	3	4
1	7.52 (d, J = 2.0)	7.52 (d, J = 2.0)	7.55 (s)	7.55 (s)
4	6.42 (s)	6.41 (s)	6.45 (s)	6.46 (s)
5	6.67 (s)	6.67 (s)	6.68 (s)	6.68 (s)
8	4.72 (dd, J = 5.2, 2.0)	4.72 (dd, J = 5.2, 2.0)		
8-OH	5.51 (d, J = 5.2)	5.52 (d, J = 5.2)	5.80 (s)	5.82 (s)
8b			2.42 (d, J = 15.2)	2.43 (d, J = 15.2)
			2.95 (d, J = 15.2)	2.96 (d, J = 15.2)
8d			2.13 (3H, s)	2.14 (3H, s)
9	6.23 (d, J = 15.6)	6.23 (d, J = 16.0)	6.23 (d, J = 15.6)	6.24 (d, J = 15.6)
10	6.58 (dq, J = 15.6, 6.8)	6.57 (dq, J = 16.0, 6.8)	6.59 (dq, J = 15.6, 6.8)	6.60 (dq, J = 15.6, 6.8)
11	1.91 (3H, d, J = 6.8)	1.90 (3H, d, J = 6.8)	1.91 (3H, d, J = 6.8)	1.92 (3H, d, J = 7.2)
12	1.32 (s)	1.32 (s)	1.41 (s)	1.42 (s)
15	2.83 (t, J = 7.2)	2.83 (t, J = 7.2)	2.85 (t, J = 7.2)	2.85 (t, J = 7.2)
16	1.56 (m)	1.56 (m)	1.57 (m)	1.58 (m)
17	1.29 (m)	1.29 (m)	1.30 (m)	1.31 (m)
18	1.29 (m)	1.29 (m)	1.30 (m)	1.31 (m)
19	1.29 (m)	0.87 (t, J = 6.8)	1.30 (m)	0.88 (t, J = 6.8)
20	1.29 (m)		1.30 (m)	
21	0.87 (t, J = 6.8)		0.88 (t, J = 6.8)	

[a] Assignments were confirmed by ^{1}H−^{1}H COSY, HMQC, and HMBC. ^{1}H NMR spectroscopic data of compounds **1**−**4** were measured at 400 MHz in acetone-d_6. m: multiple signal.

were used for column chromatography and silica gel 60 GF_{254} (Merck) for thin layer chromatography (TLC). The spots on TLC were detected under ultraviolet (UV) lamps (254 and 365 nm) and by spraying with an anisaldehyde−sulfuric acid solution and then 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 (HPLC grade), acetone, ethyl acetate, *n*-hexane and methanol (analytical grade) were purchased from ECHO (Miaoli, Taiwan). Acetone-d_6 (CD_3COCD_3) were purchased from CIL Co. (Andover, MA, USA). 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, USA).

Preparation of Red Mold Dioscorea. Red mold dioscorea (RMD) was prepared from *Monascus purpureus* NTU 568 fermented on dioscorea (*Dioscorea batatas* Decne), supplied by the Sunway Biotechnology Company Limited. The fermented material was further dried and crushed to provide the substances for the extraction.

Isolation and Identification of Azaphilone Metabolites. The dried RMD powder (5 kg) was extracted three times with acetone (25 L × 3) in a 100 L extractor equipped a temperature controller for 24 h, at 40 °C, and the acetone extracts (ca. 70 L) were obtained. After filtering through filter paper, the extracts were combined and then concentrated under reduced pressure. The dried residue mixed with silica gel was subjected to a 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) to obtain 8 fractions (fr 1 to fr 8). Fractions 3 and 4 displayed clear orange spots on an analytical TLC plate with *n*-hexane/ethyl acetate (7:3, v/v). Fraction 4 was separated again by Sephadex LH-20 column chromatography to give 3 subfractions (fr 4-1−4-3). Then, fr 4-2 (164 mg) was subjected to a silica gel column eluting with dichloromethane/ethyl acetate (95:5, 90:10, and 85:15) to obtain 3 partial purified fractions (fr 4-2-1−4-2-3). Using a semipreparative HPLC system equipped with Cosmosil $5C_{18}$ packing column (250 × 20 mm) with 85% MeOH as mobile phase solvent at a flow rate of 7 mL/min, compounds **5** (10 mg) and **6** (15 mg) were yielded from fr 4-2-1, compounds **3** (1.3 mg) and **4** (5.7 mg) from fr 4-2-2, and compounds **1** (2.5 mg) and **2** (1.5 mg) from fr 4-2-3.

*Monaphilol A (**1**):* dark orange oil; $[\alpha]_D^{25}$ −2648.8° (*c* 1.23, acetone); UV (MeOH) λ_{max} (log ε) 469 (4.0), 302 (3.4); IR ν_{max} (KBr) 3401, 2954, 2918, 2859, 1742, 1655, 1528, 1437, 1239, 1168, 1073, 902, 827 cm^{-1}; ESIMS *m*/*z* 385 $[M + H]^+$; HRESIMS *m*/*z* 385.2016 $[M + H]^+$ (calcd 385.2015, $C_{23}H_{29}O_5$). ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

*Monaphilol B (**2**):* dark orange oil; $[\alpha]_D^{25}$ −2581.8° (*c* 0.33, acetone); UV (MeOH) λ_{max} (log ε) 469 (4.0), 303 (3.4); IR ν_{max} (KBr) 3398, 2958, 2930, 2851, 1734, 1655, 1524, 1441, 1263, 1239, 1172, 1073, 906, 796 cm^{-1}. ESIMS *m*/*z* 357 $[M + H]^+$; HRESIMS *m*/*z* 357.1698 $[M + H]^+$ (calcd 357.1702, $C_{21}H_{25}O_5$). ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

*Monaphilol C (**3**):* dark orange oil; $[\alpha]_D^{25}$ −2095.7° (*c* 3.49, acetone); UV (MeOH) λ_{max} (log ε) 470 (3.9), 307 (3.4); IR ν_{max} (KBr) 3366, 2958, 2926, 2855, 1746, 1663, 1528, 1449, 1358, 1239, 1073, 958, 835 cm^{-1}; ESIMS *m*/*z* 441 $[M + H]^+$; HRESIMS *m*/*z* 441.2256 $[M + H]^+$ (calcd 441.2277, $C_{26}H_{33}O_6$). ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

*Monaphilol D (**4**):* dark orange oil; $[\alpha]_D^{25}$ −1491.1° (*c* 0.45, acetone); UV (MeOH) λ_{max} (log ε) 470 (4.0), 307 (3.4); IR ν_{max} (KBr) 3366, 2958, 2926, 2859, 1738, 1667, 1528, 1441, 1362, 1235, 1168, 1069, 891, 807 cm^{-1}; ESIMS *m*/*z* 413 $[M + H]^+$; HREIMS *m*/*z* 413.1997 $[M + H]^+$ (calcd 413.1964, $C_{24}H_{29}O_6$). ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

TLC Analysis. TLC was performed on silica gel 60 plates (Merck). The solvent systems were *n*-hexane/ethyl acetate (7:3, v/v) and dichloromethane/methanol (95:5, v/v). The yellow fluorescence properties of compounds were detected under ultraviolet light at 365 nm.

Cell Lines and Culture. HEp-2 (human laryngeal carcinoma), WiDr (human colon adenocarcinoma), and RAW 264.7 (murine

Table 2. ^{13}C NMR Spectroscopic Data for Monaphilol A (1), B (2), C (3), and D (4) [a]

position	1	2	3	4
1	147.0	147.0	148.7	148.7
3	158.1	158.1	158.1	158.1
4	111.6	111.6	109.5	109.5
4a	145.9	145.9	145.5	145.6
5	103.5	103.5	102.7	102.7
6	173.9	173.9	173.7	173.8
7	83.6	83.6	85.8	85.8
8	71.9	71.9	77.3	77.3
8a	122.6	122.6	124.7	124.7
8b			44.5	44.5
8c			209.3	209.3
8d			32.5	32.5
9	124.2	124.2	124.0	124.0
10	135.3	135.3	135.7	135.7
11	18.5	18.5	18.6	18.6
12	19.7	19.7	21.9	21.9
13	111.6	111.6	111.0	111.0
13a	171.2	171.2	171.2	171.2
14	197.0	197.0	196.8	196.8
15	41.7	41.6	41.7	41.7
16	24.7	24.3	24.7	24.4
17	29.6	32.3	29.9	32.2
18	29.6	23.2	29.9	23.2
19	32.5	14.2	32.5	14.3
20	23.3		23.2	
21	14.3		14.3	

[a] Assignments were confirmed by ^{1}H−^{1}H COSY, HMQC, and HMBC. ^{13}C NMR spectroscopic data of compounds **1**−**4** were measured at 100 MHz in acetone-d_6.

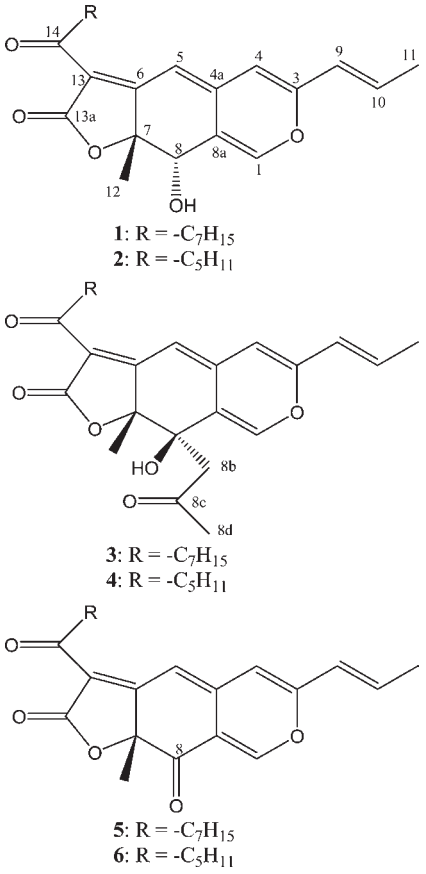

Figure 1. The structures of compounds **1**−**6** (**1**, monaphilol A; **2**, monaphilol B; **3**, monaphilol C; **4**, monaphilol D; **5**, monascorubrin; **6**, rubropunctatin).

macrophage) cell lines 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 RAW 264.7 cell line was maintained in DMEM containing 10% fetal bovine serum. All of them were cultured in a 37 °C incubator with 5% CO_2.

Assay for Antiproliferation. The antiproliferative effects of compounds against cancer cells were measured by MTT assay.[20,21] Cells (3×10^3 per well) were seeded in 96-well plates containing 180 μL of MEM and allowed to attach for 4 h. Cells were incubated with 20 μL of vehicle or test agents (1.25, 2.5, 5, 10, 25, 50, and 100 μg/mL) under 37 °C incubator with 5% CO_2. All test agents were dissolved in DMSO, and the final concentration of DMSO was <0.05% (v/v). After 3 days, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h to make the 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 ELISA reader in the absorbance at 570 nm and provided a relative estimate of cell proliferation. Mitomycin C was used as a positive control to ensure that the assay was working properly. [22]

Measurement of Nitric Oxide in RAW 264.7 Macrophage Cells. RAW 264.7 macrophage cells (5×10^4 per well) were seeded and maintained with 90 μL of DMEM in 96-well plates. The cells were preincubated for 12 h for cell attachment. The attached cells were treated with or without LPS (1 μg/mL) in the absence or presence of the test compounds (5.0, 2.50, 1.25, 0.62, 0.31 μg/mL) dissolved in DMEM. To determine the accumulation of nitric oxide (NO) in the culture medium 24 h after simulation with LPS, the NO concentrations were determined by Griess reagent kit (Promega, Madison, WI, USA). The viability of the cells was assessed by MTT assay.

Data Analysis. The data of antiproliferative experiment were presented as mean ± standard deviation for three independently performed experiments (n = 3). Significant difference was analyzed by Student's t-test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Isolation of Compounds. The concentrated crude extracts exhibited a group of orange pigments on a silica gel TLC plate developed with n-hexane/ethyl acetate (7/3, v/v) at the R_f values of 0.36−0.56. After isolation by silica and LH-20 gel column chromatography, the orange pigment fraction (fr 4-2) was furnished. Further purification of fr 4-2 by silica gel column chromatography and semipreparative HPLC yielded compounds **1**−**6** (Figure 1). Fr 4-2 was also analyzed by TLC using dichloromethane/ethyl acetate (95/5, v/v) as the solvents to obtain three groups of orange spots on the TLC plate. The first group, containing **5** and **6**, was clearly orange at the R_f values of 0.65−0.73; the second, containing **3** and **4**, and the third, containing **1** and **2**, at the R_f values of 0.36−0.42 and 0.17−0.22, respectively, were brightly orange and demonstrated yellow fluorescence under UV-light irradiation (λ = 365 nm).

Structural Elucidation. Compound **1** was obtained as a dark orange oil. Its HRESIMS showed a molecular formula of $C_{23}H_{28}O_5$, indicating 10 double bond equivalents (DBE) as

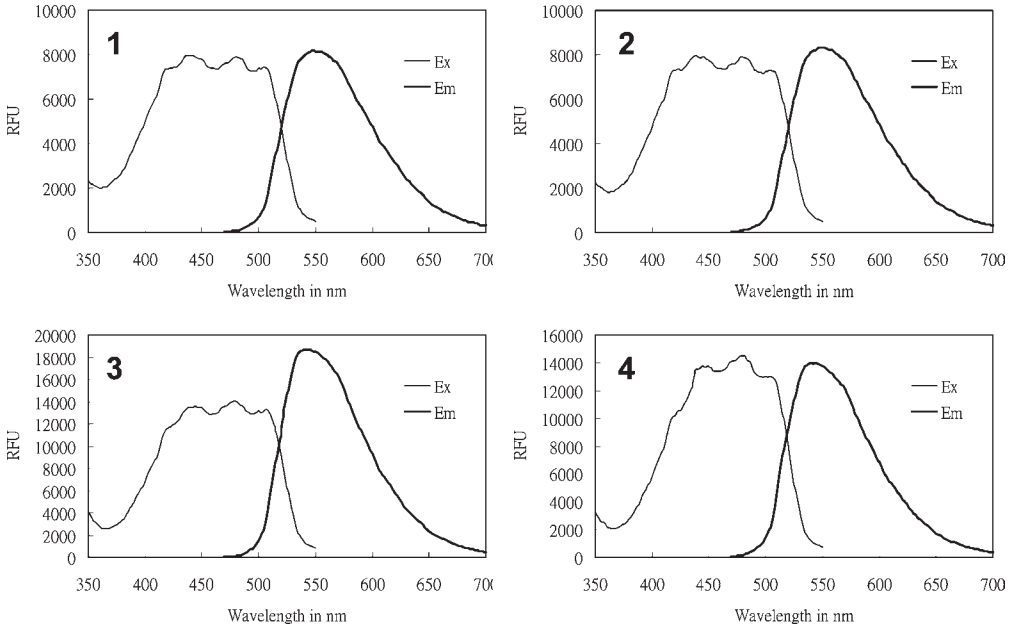

Figure 2. The excitation (Ex) and emission (Em) spectra of (**1**) monaphilol A, (**2**) monaphilol B, (**3**) monaphilol C, and (**4**) monaphilol D measured by fluorescence spectrophotometer (**1** and **2**, λ_{ex} = 438 nm, λ_{em} = 554 nm; **3** and **4**, λ_{ex} = 480 nm, λ_{em} = 540 nm).

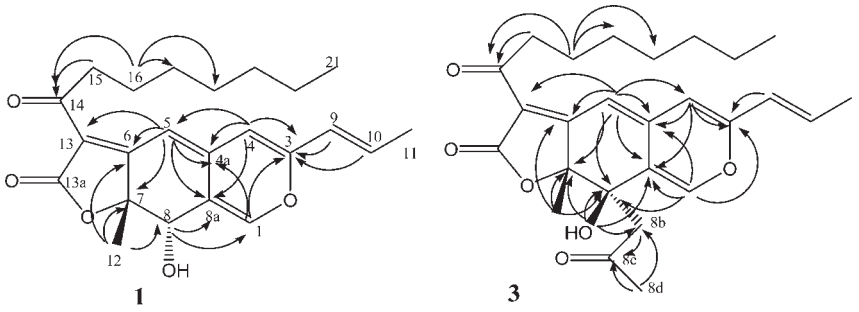

Figure 3. Key HMBC correlations of monaphilol A (**1**) and monaphilol C (**3**).

determined by $[M + H]^+$ ion at m/z 385.2015. The fluorescence spectrum of **1** presented maximum excitation and emission at 438 and 554 nm, respectively, as shown in Figure 2, indicating the presence of an extended conjugated system. The IR spectrum exhibited absorption bands at 3401 (hydroxyl group), 1742 (α,β-unsaturated γ-lactone group), and 1655 (conjugated ketone group) cm^{-1}. The ^1H NMR spectroscopic data (Table 1) of **1** revealed five olefinic [δ 6.23 (d), 6.42 (s), 6.58 (dq), 6.67 (s) and 7.52 (d, J = 2.0)], one methine [δ 4.72 (dd, J = 5.2, 2.0)] and three methyl [δ 0.87 (t), 1.32 (s) and 1.91 (d)] protons. The ^{13}C NMR spectrum (Table 2) showed ten olefinic (δ 103.5, 109.1, 111.6, 122.6, 124.2, 135.3, 145.9, 147.0, 158.1.2, and 173.9), one conjugated carbonyl (δ 197.0), one lactone carbonyl (δ 171.2) and eleven sp^3 carbons. This evidence, together with HMBC spectral cross peaks of H-4/C-3, C-4a, C-5, C-8a, H-5/C-4a, C-6, C-7, C-8a, C-13, H-8/C-1, C-7, C-8a, H-1/C-3, C-4a, C-8, C-8a, and 12-CH_3/C-6, C-7, C-8, suggested the presence of an azaphilone bearing a five-membered unsaturated lactone ring,[23] which was confirmed as 9-hydroxy-9a-methyl-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one.

In addition, proton signals for one pair of *trans*-vinyl protons [δ 6.23 (H-9) and 6.58 (H-10)] with a methyl group [δ 1.91 (H-11)] were observed in the ^1H NMR and COSY spectra of **1**. The pendant signals for an octanoyl substituent were deduced based on the correlations of H-15/H-16 and H-20/H-21 in the COSY spectrum and long-range correlations of H-15/C-14 and H-16/C-14 in the HMBC spectra (Figure 3). Moreover, HMBC cross peaks of H-9, H-10/C-3 and H-15/C-13 confirmed that the *trans*-propenyl group and octanoyl unit were connected to C-3 and C-13, respectively, in a 9-hydroxy-9a-methyl-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one moiety. The relative configuration between OH-8 and CH_3-12 was determined as the *trans* form due to the correlation between H-8 and CH_3-12 in the NOESY spectrum. Furthermore, **1** exhibited a negative specific rotation [−2648.8°], suggesting an *R* configuration at C-7 compared with the reference data.[24] Thus, **1** was assigned as (9*S*,9a*R*)-9-hydroxy-9a-methyl-3-octanoyl-6-((*E*)-prop-1-enyl)-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one, and was named monaphilol A.

The HRESIMS of **2** revealed a molecular formula of $C_{21}H_{24}O_5$ as calculated from $[M + H]^+$ ion at m/z 357.1689. Characteristic IR, UV, and fluorescence spectra and ^1H and ^{13}C NMR signals of **2** suggested that **2** possessed a similar skeleton to **1**. The molecular weight of **2** was 28 units fewer than that of **1**,

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suggesting the absence of two methylene groups. Furthermore, in the ^{1}H NMR spectrum of **2**, the decrease of the methylene integrated peak area, together with the loss of carbon signal data in the ^{13}C NMR spectrum, was consistent with a hexanoyl group at C-13 in **2**. Accordingly, the structure of **2** (monaphilol B) was elucidated as (9*S*,9a*R*)-3-hexanoyl-9-hydroxy-9a-methyl-6-((*E*)-prop-1-enyl)-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one.

The molecular formulas of **3** and **4** were determined as $C_{26}H_{32}O_6$ and $C_{24}H_{28}O_6$, respectively, based on the ion peaks at *m*/*z* 441.2256 and *m*/*z* 413.1997 $[M + H]^+$ in the HRESIMS, respectively. The similar IR, UV, and fluorescence spectra of **1**−**4**, together with their close ^{1}H and ^{13}C NMR spectroscopic signals (Tables 1 and 2), indicated that **3** and **4** also contained an azaphilone skeleton. The ^{1}H NMR spectrum of **3** showed signals for a 2-oxopropyl group [δ 2.13 (H-8d), δ 2.42 (H-8b) δ 2.95 (H-8b)] rather than an oxymethine in **1**. The HMBC spectral cross peaks of H-8b/C-8, 8c, H-8d/C-8b, 8c and OH-8/C-8b suggested the location of 2-oxopropyl group at C-8. Furthermore, the relative configuration between OH-8 and CH_3-12 was determined to be the *cis* form according to the correlation between OH-8 and CH_3-12 in the NOESY spectrum. The *R* configuration at C-7 in **3** was deduced by the negative specific rotation as **1**. From the above data, monaphilol C (**3**) was elucidated as (9*R*,9a*R*)-9-hydroxy-9a-methyl-3-octanoyl-9-(2-oxopropyl)-6-((*E*)-prop-1-enyl)-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one.

As for compound **4**, its molecular weight is 28 units less than that of **3**, and it has ^{1}H and ^{13}C NMR spectra very similar to those of **3** except for the signals for a hexanoyl group in **4** instead of an octanoyl group in **3**. Therefore, the structure of **4** was determined as (9*R*,9a*R*)-3-hexanoyl-9-hydroxy-9a-methyl-9-(2-oxopropyl)-6-((*E*)-prop-1-enyl)-9,9a-dihydro-2*H*-furo[3,2-*g*]isochromen-2-one and named monaphilol D.

Compounds **5** and **6** were obtained as orange amorphous solids. They were identified as the known orange pigments, monascorubrin and rubropunctatin, respectively, by comparison with authentic samples and literature data.[25] The above-mentioned monaphilol A−D (**1**−**4**) were new orange pigments and displayed strong yellow fluorescence under UV-light irradiation (λ = 365 nm). Notably, compounds **5** and **6** had no visible fluorescence, probably resulting from a ketone at C-8 instead of a hydroxyl group (8-OH) in **1**−**4**. This is the first report that yellow fluorescence compounds have been isolated from *Monascus*-fermented products.

Inhibition on NO Production in LPS-stimulated RAW 264.7 Cells. The LPS-stimulated RAW 264.7 cells were initially treated with compounds **1**−**6** at 5 μg/mL. The inhibitory rate of NO production for compounds **5** and **6** was less than 50%; however, compounds **1**−**4** inhibited over 90% NO production (preliminary tested data are not shown). Compounds **1**−**4** were further tested at 2.5, 1.25, 0.625, 0.313 μg/mL (Figure 4). The results indicated that compounds **1**−**4** significantly inhibited the LPS-stimulated NO production in a dose-dependent manner (Figure 4). The IC_{50} values of **1**−**4** were 1.0, 3.8, 2.8, and 1.7 μM, respectively. Moreover, compounds **1**−**4** more strongly reduced LPS-stimulated NO production than a positive control, quercetin (IC_{50} = 13.2 μM). All above isolated azaphilonoids (**1**−**6**) did not exhibit cytotoxicity against RAW 264.7 cells at tested concentrations. Thus, inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells by compounds **1**−**4** did not result from cytotoxicity.

Antiproliferative Activities on Human Cancer Cells. The six orange pigments (**1**−**6**) isolated from RMD were evaluated for

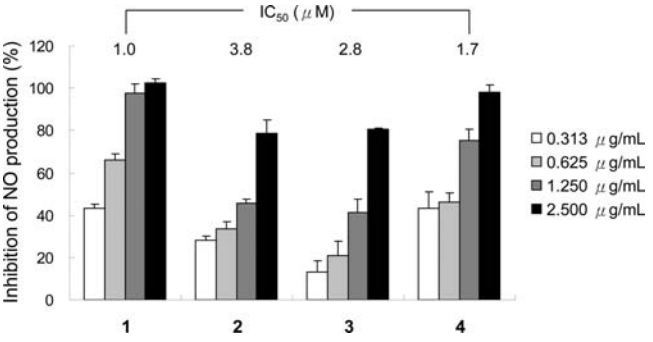

Figure 4. Effects of compounds **1**−**4** on NO production in LPS stimulated RAW 264.7 cell (%) at concentrations of 0.313, 0.625, 1.25, 2.5 μg/mL. Quercetin was used as positive control, and its IC_{50} value was 3.9 μg/mL (**1**, monaphinol A; **2**, monaphinol B; **3**, monaphinol C; **4**, monaphinol D). All values are presented as mean ± SD (n = 3).

the antiproliferative activities on HEp-2 and WiDr cell lines (Figure 5). As shown in Figure 6, compounds **1** and **4**−**6** possessed inhibitory effects against HEp-2 and WiDr cell lines in time-dependent manners. The inhibitory concentrations (IC_{50}) of compounds were calculated and are shown in Table 3. Notably, the isolated azaphilones with OH group at C-8 as **1**−**4** possessed higher antiproliferative activities ($p < 0.05$) compared with **5** and **6**. The structure of **5** was similar to that of **6** except for the different length of the saturated side chain (C_7H_{15} in **5** and C_5H_{11} in **6**) on the ketonic carbonyl group; however, **5** had more potent antiproliferative effects on HEp-2 and WiDr cell lines than **6**. Like **5** and **6**, compound **1** (with C_7H_{15} side chain) had higher antiproliferative efficiency than **2** (with C_5H_{11} side chain). These results could conclude that the length of the saturated side chain on the ketonic carbonyl group of azaphilones may play a crucial role for the antiproliferative activities.[15]

This study revealed that RMD contained greater quantities of orange pigments than RMR. In comparison with previously isolated yellow pigments (ankaflavin and monascin), we found that orange pigments (monascorubrin and rubropunctatin) exerted stronger cytotoxic activities against HEp-2 and WiDr cell lines.[18] The evidence may explain that RMD has more potent inhibitory effects against a panel of cancer cell lines than RMR.[26] The bioassay data showed that compounds **1**−**4** performed anti-inflammation at low dosage (IC_{50} = 1.0−3.8 μM) and antiproliferation of cell lines at high dosage (IC_{50} = 8.6−15.7 μM). These results revealed that the structures of **1**−**4** could prevent tumor formation by anti-inflammatory and antiproliferative effects. It was reported that RMD extracts decreased the DMBA-induced increase in the NO and prostaglandin E_2 (PEG_2) levels in the hamster buccal pouch and induced apoptosis of oral cancer cells by caspase-8 and caspase-9 pathways.[13,26] Moreover, the anti-inflammatory and antiproliferative mechanisms of ankaflavin, an azaphilone structure, were similar to RMD extracts.[4,16] Compounds **1**−**6**, isolated from RMD and belonged to azaphilone structures, might inhibit cancer by the same mechanisms as ankaflavin. However, further experiments of anti-inflammatory and antiproliferative mechanisms for compounds **1**−**6** remain to be investigated.

In summary, six orange pigments (**1**−**6**) were isolated from RMD and elucidated for their structures on the basis of 1-D and 2-D NMR, MS, UV, and IR spectroscopic analyses. Monaphilol A−D (**1**−**4**) were verified to be new orange pigments, which had structures similar to those of monascorubrin (**5**) and

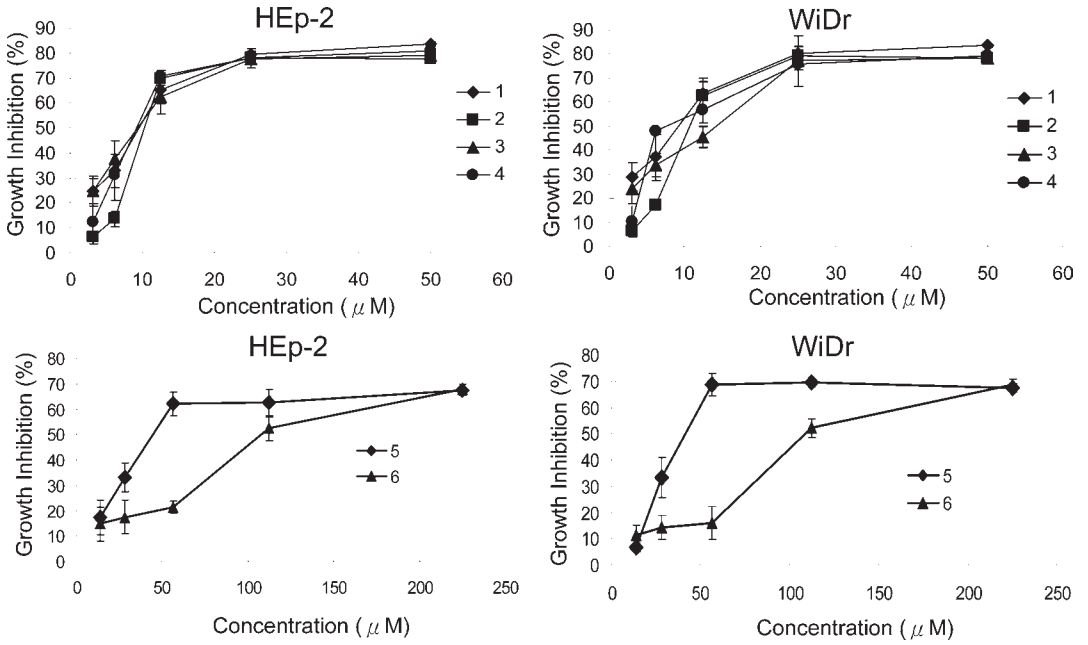

Figure 5. The inhibitory effects of compounds on the proliferation of HEp-2 and WiDr cell lines (**1**, monaphinol A; **2**, monaphinol B; **3**, monaphinol C; **4**, monaphinol D; **5**, monascorubrin; **6**, rubropunctatin). All values are presented as mean ± SD (n = 3).

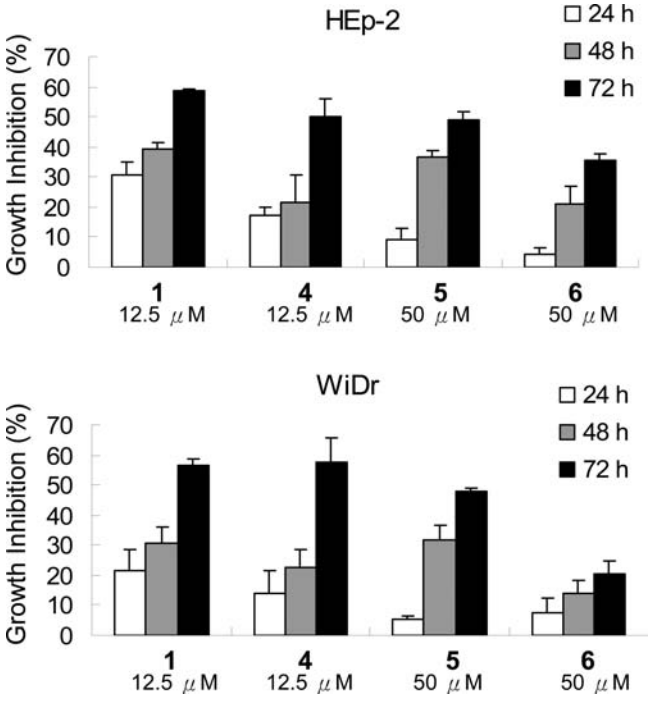

Figure 6. The growth inhibitory effects of compounds on HEp-2 and WiDr cell lines at 24, 48, and 72 h (**1**, monaphinol A; **4**, monaphinol D; **5**, monascorubrin; **6**, rubropunctatin). All values are presented as mean ± SD (n = 3).

rubropunctatin (**6**) except for the reduction of C-8 ketone and displaying strong yellow fluorescence under UV-light irradiation (λ = 365 nm). Bioassays revealed that monaphilols (**1**−**4**) possessed better antiproliferation of cancer cells and anti-inflammatory effect on LPS-stimulated RAW264.7 cells than known orange pigments (**5** and **6**). Currently, chronic inflammation is regarded as the risk factor for developing many types of cancers;[27] thus, the present study demonstrated that the new isolated orange pigments (**1**−**4**) may potentially be developed as chemopreventive drugs.

Table 3. Antiproliferation Effects of Compounds 1−6 for HEp-2 and WiDr Cell Lines

	IC_{50}[a] (μM)	
compd	HEp-2	WiDr
1	9.8 ± 2.0	8.6 ± 2.7
2	14.8 ± 1.5	15.7 ± 1.3
3	8.7 ± 1.0	11.0 ± 3.1
4	10.4 ± 1.0	10.5 ± 1.4
5	55.9 ± 3.4	54.1 ± 2.4
6	106.8 ± 0.5	109.3 ± 6.8
mitomycin C[b]	0.4 ± 0.0	0.4 ± 0.1

[a] IC_{50}: inhibitory concentration 50%. [b] Positive control (**1**, monaphinol A; **2**, monaphinol B; **3**, monaphinol C; **4**, monaphinol D; **5**, monascorubrin; **6**, rubropunctatin).

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