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Azaphilones, Furanoisophthalides, and Amino Acids from the Extracts of *Monascus pilosus*-Fermented Rice (Red-Mold Rice) and Their Chemopreventive Effects

TOSHIHIRO AKIHISA,*,‡ HARUKUNI TOKUDA,§ KEN YASUKAWA,⊥ MOTOHIKO UKIYA,‡ AYAKA KIYOTA,‡ NAOYUKI SAKAMOTO,§ TAKASHI SUZUKI,⊥ NOBUKAZU TANABE,# AND HOYOKU NISHINO§

College of Science and Technology, Nihon University, 1-8 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8308, Japan, Department of Biochemistry, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi-shi, Chiba 274-8555, Japan, and Research Center, Gunze, Co., Ishiburo 1, Ikurashinmachi, Ayabe-shi, Kyoto, 623-8512, Japan

Six azaphilones, monascin (**1**), ankaflavin (**2**), rubropunctatin (**3**), monascorburin (**4**), rubropunctamine (**5**), and monascorburamine (**6**), two furanoisophthalides, xanthomonasin A (**7**) and xanthomonasin B (**8**), and two amino acids, (+)-monascumic acid (**9**) and (−)-monascumic acid (**10**), isolated from the extracts of *Monascus pilosus*-fermented rice (red-mold rice) were evaluated for their inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, on the induction of Epstein−Barr virus early antigen (EBV-EA) by TPA in Raji cells, and on the activation of (±)-(*E*)-methyl-2[(*E*)-hydroxy-imino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor. Among the compounds tested, seven compounds (**1**−**6** and **10**) on TPA-induced inflammation, and six compounds (**1**, **3**−**5**, **9**, and **10**) on EBV-EA activation, exhibited potent inhibitory effects. All of the compounds tested showed moderate inhibitory effects on NOR 1 activation.

KEYWORDS: ***Monascus pilosus*; red-mold rice; azaphilones; furanoisophthalides; amino acids; TPA-induced ear edema; antitumor-promoter; Epstein−Barr virus early antigen**

INTRODUCTION

Species of the fungi *Monascus* (Eurotiaceae) have been utilized for making fermented food and preserving meat for hundreds of years. Red-mold rice fermented using *Monascus* spp. is effective in decreasing blood pressure (*1*) and lowering plasma cholesterol levels (*2*−*4*) and has antibacterial activity (*5*). γ-Aminobutyric acid (GABA) (*6*), possessing antihypertensive effects for humans, and monacolin K (lovastatin; mevinolin) (*3*), which functions as an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, have been isolated from red-mold rice. In the course of our search for potential antitumor-promoters from natural sources (*7*), we have isolated and characterized two new azetidine-type amino acids, (+)-[**9**; (+)-monascumic acid] and (−)-*syn*-2-isobutyl-4-methylazetidine-2,4-dicarboxylic acids [**10**; (−)-monascumic acid], from the extract of red-mold rice fermented with *Monascus pilosus* (*8*). In this paper, we report the isolation and characterization of further eight compounds (**1**−**8**) from the red-mold rice extracts and the inhibitory effects of 10 compounds (**1**−**10**) (**Figure 1**) on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, on the induction of Epstein−Barr virus early antigen (EBV-EA) by TPA, and on activation of (±)-(*E*)-methyl-2[(*E*)-hydroxy-imino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor. Compounds **1**−**6** along with a new nonpigment, monascodilone, and citrinin have recently been detected in the red-mold rice obtained as cultures of *M. purpureus* DSM1379 on rice (*9*).

MATERIALS AND METHODS

Ultraviolet and visible light (UV−vis) spectra were recorded on a Shimadzu UV-2200 spectrometer in MeOH. Electron-impact mass spectra (EIMS) (70 eV) and electrospray ionization mass spectra (ESIMS) (positive mode) were recorded on JEOL JMS−BU20 spectrometer and on Agilent 1100MSD SL spectrometer, respectively, using a direct inlet system. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a JEOL LA-400 spectrometer at 400 MHz in $CDCl_3$ with tetramethylsilane as internal standard. Silica gel (Silica gel 60, 220−400 mesh, Merck) and octadecyl silica (Chromatorex-ODS, 100−200 mesh; Fuji Silysia Chemical, Ltd., Aichi, Japan) were used for open column chromatography. Reversed-phase preparative HPLC was carried out on a 25 cm × 10 mm i.d. Pegasil ODS II (Senshu Scientific Co., Ltd., Tokyo, Japan) C_{18} silica column at 25 °C. A refractive index detector was used for reversed-phase HPLC.

* To whom correspondence should be addressed (fax +81-3-3293-7572; e-mail akihisa@chem.cst. nihon-u.ac.jp).
‡ College of Science and Technology, Nihon University.
§ Kyoto Prefectural University of Medicine.
⊥ College of Pharmacy, Nihon University.
Gunze, Co.

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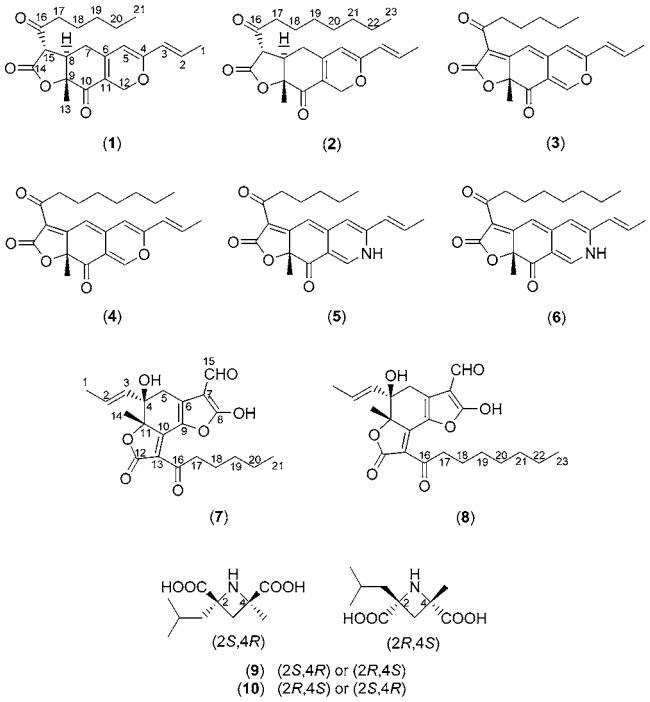

Figure 1. Structures of compounds isolated from the extracts of red-mold rice.

Chemicals and Materials. Red-mold rice was prepared from cooked paddy rice inoculated with *Monascus pilosus* IFO 4520, obtained from the Institute for Fermentation (IFO), Osaka, Japan, as described previously (*6*). (+)-Monascumic acid (**9**) and (−)-monascumic acid (**10**) were isolated from red-mold rice recently (*8*). The following chemicals were purchased: TPA from ChemSyn Laboratories (Lenexa, KS), indomethacin, β-carotene, and glyzyrrhizin from Sigma Chemical Co. (St. Louis, MO), the EBV cell culture reagents and *n*-butyric acid from Nacalai Tesque, Inc. (Kyoto, Japan), and NOR 1 and carboxy-PTIO from Dojindo Laboratories (Kumamoto, Japan).

Isolation and Identification of Azaphilones. Red-mold rice (1.5 kg) was extracted with 12 L of 70% ethanol (EtOH) (EtOH−H_2O, 7:3, v/v) for 30 min under stirring. The mixture was suction-filtered, and the residue was washed with 3 L of 70% EtOH. The combined filtrate and wash were evaporated in vacuo at 50 °C to leave the 70% EtOH extract (152.9 g). The extract was suspended in 1 L of H_2O, and the suspension was extracted with ethyl acetate (EtOAc) (five times with 0.5 L each). The EtOAc solution was evaporated in vacuo at 50 °C to yield the EtOAc fraction (26.1 g). A portion (11.9 g) of the EtOAc fraction was chromatographed on silica gel (200 g) which was eluted successively with solvent of increasing polarity (*n*-hexanes−EtOAc = 1:0, 9:1, 4:1, 1:1, 3:7, 1:9, 0:1; EtOAc-MeOH = 9:1, 1:1, 0:1; v/v) to afford 11 fractions, Fr. A∼K. A portion (250 mg) of Fr. C was subjected to HPLC [eluting solvent, methanol (MeOH)−H_2O−acetic acid (AcOH), 75:25:3, v/v/v; flow rate, 2.5 mL/min] which yielded monascin (**1**) [18 mg, retention time (t_R) 27 min] and ankaflavin (**2**) (4 mg, t_R 40 min). A portion of Fr. D (200 mg) gave, upon HPLC (eluting solvent, MeOH−H_2O−HCOOH, 75:25:0.1, v/v/v; flow rate, 2.5 mL/min), rubropunctatin (**3**) (2 mg, t_R 18 min) and monascorubrin (**4**) (2 mg, t_R 27 min). Furthermore, HPLC (eluting solvent, MeOH−H_2O−AcOH, 60:40:3, v/v/v; flow rate, 2.5 mL/min) of a portion of Fr. E (500 mg) gave rubropunctamine (**5**) (22 mg, t_R 8 min) and monascorubramine (**6**) (11 mg, t_R 19 min). Identification of compounds **1**−**6** was performed by UV−vis, MS, and ^{1}H NMR comparison with the literature.

Monascin (1): yellow amorphous solid; UV−vis λ_{max} 382, 460 nm; ESIMS *m*/*z*: 359 (MH^+); ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, J = 6.8 Hz, CH_3CH$_2$-), 1.45 (3H, s, H-13), 1.88 (3H, d, J = 6.8 Hz, H-1), 2.44 (1H, dd, J = 7.6, 11.6 Hz, H_a-17), 2.61 (1H, ddd, J = 7.6, 7.6, 18.0 Hz, H_a-7), 2.68 (1H, dd, J = 4.0, 17.6 Hz, H_b-17), 3.02 (1H, ddd, J = 7.6, 7.6, 18.0, H_b-7), 3.24 (1H, ddd, J = 4.0, 13.2, 13.2 Hz, H-8), 3.66 (1H, d, J = 13.2 Hz, H-15), 4.71 (1H, d, J = 12.8 Hz, H_a-12), 5.06 (1H, d, J = 12.8 Hz, H_b-12), 5.27 (1H, s, H-5), 5.91 (1H, d, J = 15.2 Hz, H-3), 6.51 (1H, dq, J = 15.2, 6.8 Hz, H-2). The spectral data are consistent with those of compound **1** (*10*−*12*).

Ankaflavin (2): yellow amorphous solid; UV−vis λ_{max} 382, 460 nm; ESIMS *m*/*z* 387 (MH^+). The ^{1}H NMR ($CDCl_3$) signals for this compound were essentially the same as those of compound **1**, and the spectral data are consistent with those of compound **2** (*10*−*12*).

Rubropunctatin (3): red amorphous solid; UV−vis λ_{max} 300, 410, 530 nm; EIMS *m*/*z* 353 (M^+); ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 6.8 Hz, CH_3CH$_2$-), 1.71 (3H, s, H-13), 1.95 (3H, d, J = 7.2 Hz, H-1), 2.47 (1H, br dd, J = 7.2, 9.0 Hz, H_a-17), 2.94 (1H, ddd, J = 5.2, 7.2, 7.2, H_b-17), 6.03 (1H, d, J = 16.7 Hz, H-3), 6.14 (1H, s, H-7), 6.58 (1H, dq, J = 16.7, 7.2 Hz, H-2), 6.89 (1H, s, H-5), 7.86 (1H, s, H-12). The spectral data are consistent with those of compound **3** (*12*).

Monascorubrin (4): red amorphous solid; UV−vis λ_{max} 300, 410, 530 nm; EIMS *m*/*z* 381 (M^+). The ^{1}H NMR ($CDCl_3$) signals for this compound were essentially the same as those of compound **3**, and the spectral data are consistent with those of compound **4** (*12*).

Rubropunctamine (5): purple amorphous solid; UV−vis λ_{max} 250, 280, 480 nm; ESIMS *m*/*z*: 355 (MH^+); ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, J = 6.6 Hz, CH_3CH$_2$-), 1.75 (3H, s, H-13), 2.00 (3H, d, J = 6.8 Hz, H-1), 2.94 (1H, br dd, J = 6.1, 6.4 Hz, H_a-17), 3.02 (1H, br dd, J = 6.1, 6.4 Hz, H_b-17), 3.28 (1H, s, >NH), 6.35 (1H, d, J = 15.6 Hz, H-3), 6.37 (1H, s, H-5), 6.72 (1H, s, H-7), 6.78 (1H, s, H-12), 7.05 (1H, dq, J = 15.6, 6.8 Hz, H-2). The spectral data are consistent with those of compound **5** (*12*).

Isolation and Identification of Furanoisophthalides. Extraction of the dried red-mold rice (1.0 g) with 1 M solution of HCl in MeOH yielded an extract (245 mg) which was subjected to HPLC on an ODS column (eluting solvent, MeOH−H_2O−AcOH, 65:35:3, v/v/v; flow rate, 3.0 mL/min) to give xanthomonasin A (**7**) (67 mg, t_R 5.2 min) and xanthomonasin B (20 mg, t_R 8.4 min). Identification of **7** and **8** was performed by UV−vis, MS, and ^{1}H NMR comparison with the literature.

Xanthomonasin A (7): yellow amorphous solid; UV−vis λ_{max} 230, 460 nm. ESIMS *m*/*z*: 389 (MH^+); ^{1}H NMR [dimethyl sulfoxide (DMSO)-d_6]: δ 0.85 (3H, t, J = 7.2 Hz, CH_3CH$_2$), 1.41 (3H, s, H-14), 1.53 (3H, dd, J = 1.5, 6.4 Hz, H-1), 5.31 (1H, dq, J = 15.6, 1.5 Hz, H-3), 5.58 (1H, dq, J = 15.6, 6.4 Hz, H-2), 9.41 (1H, s, H-15). The spectral data are consistent with those of compound **7** (*13*).

Xanthomonasin B (8): yellow amorphous solid; UV−vis λ_{max} 230, 460 nm. ESIMS *m*/*z*: 417 (MH^+). The ^{1}H NMR (DMSO-d_6) signals for this compound were essentially the same as those of compound **7**, and the spectral data are consistent with those of compound **8** (*13*).

Animals. Specific pathogen-free female ICR mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed, five per polycarbonate cage, in an air-conditioned specific pathogen-free room at 24 ± 2 °C. Food and water were available ad libitum.

Assay of TPA-Induced Ear Edema Inflammation in Mice. TPA (1 nM) dissolved in acetone (20 μL) was applied to the right ear only of ICR mice by means of a micropipet. A volume of 10 μL was delivered to both the inner and outer surfaces of the ear. The samples or their vehicle, MeOH−$CHCl_3$−H_2O (2:1:1, 20 μL), as a control, were topically applied about 30 min before the TPA treatment. Ear thickness was determined with a pocket thickness gauge with a range of 0−9 mm, graduated at 0.01 mm intervals, and modified so that the contact surface area was increased to reduce the loading, which was applied to the tip of the ear. The ear thickness was measured before the treatment (*a*) and 6 h after the TPA treatment (*b* = TPA alone; *b*′ = TPA plus sample). The following values were then calculated:

$$\text{edema A induced by TPA alone } (b - a)$$

$$\text{edema B induced by TPA plus a sample } (b' - a)$$

$$\text{inhibitory ratio (\%)} = [(\text{edema A} - \text{edema B})/\text{edema A}] \times 100$$

Each value was the mean of individual determinations from five mice. The 50% inhibitory dose (ID_{50}) values were determined by the method of probit-graphic interpolation for four dose levels. A statistical

Table 1. Inhibitory Effects of Compounds **1**–**10** and Reference Compounds on TPA-Induced Inflammation in Mice and on the Induction of Epstein–Barr Virus Early Antigen, and Inhibitory Ratio (IR) on NOR 1 Action

	compound	inhibition of inflammation, ID_{50}[a] (mg/ear)	percentage of EBV-EA induciton[b] concentration (mol ratio/32 pmol TPA) 1000		500		100		10		IC_{50}[c] (mol ratio/32 pmol TPA)	IR of NOR 1 activation[d]
1	monascin	0.16	5.3	(60)	43.9	(>80)	72.1	(>80)	94.2	(>80)	421	1.7
2	ankaflavin	0.26	12.6	(60)	65.2	(>80)	80.1	(>80)	100	(>80)	590	1.7
3	rubropunctatin	0.11	2.4	(60)	38.6	(>80)	67.4	(>80)	91.3	(>80)	401	1.9
4	monascorburin	0.40	5.7	(60)	45.2	(>80)	74.8	(>80)	94.1	(>80)	429	1.9
5	rubropuntamine	0.32	9.8	(60)	45.7	(>80)	73.0	(>80)	96.8	(>80)	433	1.5
6	monascorburamine	0.12	18.3	(60)	67.9	(>80)	82.6	(>80)	100	(>80)	610	1.5
7	xanthomonasin A	0.82	13.5	(50)	66.9	(>80)	81.7	(>80)	100	(>80)	607	1.9
8	xanthomonasin B	0.74	16.1	(50)	69.6	(>80)	83.3	(>80)	100	(>80)	620	1.9
9	(+)-monascumic acid	1.30	4.9	(60)	42.0	(>80)	70.6	(>80)	93.6	(>80)	418	1.7
10	(–)-monascumic acid	0.30	7.8	(60)	43.8	(>80)	72.5	(>80)	95.2	(>80)	427	1.7
indomethacin[e]		0.30										
β-carotene[e]			8.6	(70)	34.2	(>80)	82.1	(>80)	100	(>80)	397	
glyzyrrhizin[e]												2.2
carboxy-PTIO[e]												8.0

[a] ID_{50}: 50% inhibitory dose. [b] Values represent relative percentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [c] IC_{50} represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA. [d] Determined at the concentration of 350 nmol. Inhibitory ratio of NOR 1 (positive control; 350 nmol) was taken as 1.0. [e] Reference compound.

analysis was carried out by Student's *t*-test. Details of the in vivo antiinflammatory test have been described previously (*14*).

In Vitro EBV-EA Activation Experiment. The inhibition of EBV-EA activation was assayed by using Raji cells (EBV genome-carrying human lymphoblastoid cells; nonproducer type) which had been cultivated in a 10% fetal bovine serum RPMI-1640 medium (Sigma, St. Louis, MO). The Raji indicator cells (1×10^6 cells/mL) were incubated in 1 mL of a medium containing 4 mM *n*-butyric acid as an inducer, 32 pM of TPA (20 ng/mL in DMSO), and a known amount (32, 16, 3.2, or 0.32 nmol) of the test compound at 37 °C in a CO_2 incubator. After 48 h, the cell suspension was centrifuged at 1000 rpm for 10 min, and the supernatant was removed. The activated cells were stained with high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients, and the conventional indirect immunofluorescence technique was employed for detection. Details of the in vitro assay of EBV-EA induction have been reported previously (*15*).

In Vitro NOR 1 Inhibition Experiment (*16*). Chang liver cells (normal human hepato cells; 5×10^5/mL), derived from human liver in MEM Eagle medium, were cultured 3 days before treatment. NOR 1 was added into culture dish and incubated for 1 h under CO_2 incubator as control. For screening assay, test samples to culture dish were added before 1 min of NOR 1 treatment. Transformed cells were observed under light-microscopy (× 100). All observed cells count for more than 250. The inhibitory ratio was then calculated:

$$\text{inhibitory ratio (IR)} = \frac{\text{transformed cell \% (NOR 1 alone)}}{\text{transformed cell \% (NOR 1 + test sample)}}$$

RESULTS AND DISCUSSION

Six azaphilones were isolated from the EtOAc soluble fraction of a 70% EtOH extract of red-mold rice and identified as monascin (**1**), ankaflavin (**2**), rubropunctatin (**3**), monascorubrin (**4**), rubropunctamine (**5**), and monascorubramine (**6**). In addition, two furanoisophthalides isolated from the acidic MeOH extract of red-mold rice were identified as xanthomonasin A (**7**) and xanthomonasin B (**8**). These eight compounds (**1**−**8**), along with two amino acids (**9** and **10**) recently isolated (*8*), were evaluated for their inhibitory effects on TPA-induced inflammation in mice, on the induction of EBV-EA by TPA in Raji cells, and on the activation of NOR 1, a NO donor.

TPA-Induced Inflammation in Mice. The inhibitory effects on TPA-induced inflammation in mice examined for the 10 compounds, **1**−**10**, were compared with that of a reference compound, indomethacin, a commercially available antiinflammatory drug (**Table 1**). The inhibitory effects (ID_{50} = 0.11−0.40 mg/ear) of six azaphilones, **1**−**6**, and one amino acid, **10** (0.30 mg/ear), were comparable in potency to the effects of indomethacin (0.30 mg/ear). The inhibitory effect against TPA-induced inflammation has been demonstrated to closely parallel that of the inhibition of tumor promotion in two-stage carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and then by TPA, a well-known promoter, in a mouse skin model (*17*); thus, these antiinflammatory compounds from red-mold rice might be expected to possess a high antitumor-promoting effect in the same animal model. Among the compounds tested in this study, monascorubrin (**4**) has previously been proved to inhibit tumor promotion in two-stage carcinogenesis in mice (*17*).

In Vitro EBV-EA Activation. The inhibitory effects on EBV-EA activation induced by TPA, examined as a preliminary evaluation of the potential antitumor-promoting activities, of the 10 compounds, **1**−**10**, are shown in **Table 1**, together with comparable data for β-carotene, a vitamin A precursor that has been intensively studied in cancer chemoprevention by using animal models (*18*). At a 1×10^2 mol ratio/TPA, all compounds tested in this study showed inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells as shown in **Table 1**. Among these, four azaphilones, **1** and **3**−**5**, and two amino acids, **9** and **10**, showed the most potent inhibitory effects on EBV-EA activation (IC_{50} values of 401−433 mol ratio/TPA; 90−95% inhibition of induction at 1×10^3 mol ratio/TPA, and 3−6% inhibition even at 1×10 mol ratio/TPA) which were almost equivalent to those of β-carotene (IC_{50} values of 397 mol ratio/TPA). The inhibitory effects against EBV-EA induction have been demonstrated to be closely parallel with those against tumor promotion in vivo (*7*), and these azaphilones and amino acids from red-mold rice are, therefore, suggested to be potent cancer chemopreventive agents as antitumor promoters.

In Vitro NOR 1 Inhibition. As a primary screening test for antitumor-initiators (*16*), the 10 compounds, **1**−**10**, were evaluated for their scavenging activity against NO generation by NOR 1 in cultured cell system. **Table 1** shows the inhibitory ratios (IR) of the 10 compounds and two reference compounds: glycyrrhizin, a natural compound, and carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt], a synthetic NO scavenger, on NOR 1

action. Among the compounds tested, six compounds, **1**, **2**, **5**, **6**, **9**, and **10**, exhibited moderate activity (IR = 1.5−1.7), whereas four compounds, **3**, **4**, **7**, and **8**, showed more potent activity (IR = 1.9) which was close to that of glycyrrhizin (IR = 2.2).

From the foregoing, it can be concluded that the azaphilones, furanoisophthalides, and amino acids isolated from the extracts of red-mold rice are valuable as antitumor promoters (potential cancer chemopreventive agents) in chemical carcinogenesis.

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