Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 2569–2575
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Azaphilone pigments from a yellow mutant of the fungus *Monascus kaoliang*

Suchada Jongrungruangchok [a], Prasat Kittakoop [b,*], Busaba Yongsmith [c], Rapepol Bavovada [d], Somboon Tanasupawat [a], Nattapat Lartpornmatulee [b], Yodhathai Thebtaranonth [b]

[a] *Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technical Development Agency (NSTDA), Thailand Science Park, 113, Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand*
[c] *Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[d] *Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

Received 12 May 2004; received in revised form 21 July 2004
Available online 18 September 2004

Abstract

Azaphilone pigments, monascusones A (**1**) and B (**2**), together with two known azaphilones, monascin (**3**) and FK17-P2b2 (**4**), were isolated from the CH_2Cl_2 extract of a yellow mutant of the fungus *M. kaoliang* grown on rice. Structures of the isolated compounds were elucidated by analyses of spectroscopic data. Monascusone A (**1**), the major metabolite of *M. kaoliang*, showed no antimalarial (against *Plasmodium falciparum*), antitubercular (against *Mycobacterium tuberculosis* H37Ra), and antifungal (toward *Candida albicans*) activities. Compound **1** exhibited no cytotoxicity against BC (breast cancer) and KB (human epidermoid carcinoma of cavity) cell lines.

Keywords: Monascus kaoliang; Azaphilone pigments; Monascusone A; Monascusone B; Food colorant

1. Introduction

Red yeast rice, also known as ''Red Koji'' or ''Angkak'', obtained as a culture of a fungal species of the genus *Monascus* on rice, has long been used in East Asia as a natural food colorant, e.g. for red rice wine, red soy bean cheese, meat products and fish (Blanc et al., 1994; Ma et al., 2000; Wild et al., 2002). Its use is increasing, especially in western countries, because of the growing interest in natural food supplements. *Monascus* pigments are secondary metabolites possessing an azaphilone skeleton, examples are: orange pigments, e.g. monascorubrin and rubropunctatin, which possess the oxo-lactone ring; red pigments, e.g. monascorubramine and rubropunctamine, the nitrogen analogues of the orange pigments; and yellow pigments such as monascin and ankaflavin (Sato et al., 1997). In addition, other non-pigment compounds have also been isolated (Endo et al., 1986; Hossain et al., 1996; Su et al., 2003). However, a great number of *Monascus* metabolites have not yet been characterized chemically, and knowledge of their pharmacological and toxicological effects is very limited (Akihisa et al., 2004; Hsieh and Tai, 2003; Sabater-Vilar et al., 1999). Previously, the yellow pigment producing mutants by secondary mutation of a local

* Corresponding author. Tel: +662 5646700x3560; fax: +662 5646707.
E-mail address: prasat@biotec.or.th (P. Kittakoop).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.08.032

cassava starch utilizing *Monascus kaoliang* have been screened in our laboratory. One yellow mutant, *M. kaoliang* KB20M10.2, exhibited remarkable yellow pigmentation in submerged culture (Yongsmith et al., 1994) as well as in rice solid culture (Yongsmith et al., 2000). We have chemically explored the constituents of a yellow mutant of the red yeast rice of *M. kaoliang* KB20M10.2, and herein report the isolation, structural elucidation, and biological activities of the fungal secondary metabolites.

2. Results and discussion

A crude CH_2Cl_2 extract of *M. kaoliang* KB20M10.2 grown on rice (1 kg) was sequentially subjected to Sephadex LH-20 and Si gel chromatography, to yield two new yellow pigments, monascusone A (**1**) and monascusone B (**2**) together with two known compounds, monascin (**3**) and FK17-P2b2 (**4**). Spectral data of compounds **3** and **4** were identical in all respects to those published by Chen et al. (1971); Takayuki and Hiroshi (1994); Martinkova et al. (1999).

The ESITOF mass spectrum of monascusone A (**1**) deduced a molecular formula of **1** as $C_{13}H_{18}O_5$ [observed *m/z* at 277.1041 $(M + Na)^+$, calcd. for $C_{13}H_{18}O_5Na$, 277.1052]. The ^1H NMR spectral data ($CDCl_3$) of monascusone A (**1**) prominently showed signals of two methyls (as a singlet and a doublet), three methylene groups, a singlet olefinic proton (at δ_H 5.28), and two oxygenated methines (at δ_H 4.00 and 4.08). Analyses of ^{13}C, DEPT, and HMQC spectral data of monascusone A (**1**) revealed the presence of three methine, three methylene, two methyl, and five quaternary carbons. The HMQC spectral data of monascusone A (**1**) assisted in the assignment of protons attached to their corresponding carbons (Table 1). Attachment of the unsaturated sp^2 C-3 to an oxygen atom in **1** was evident from a downfield shift of its ^{13}C resonance (at δ_C 165.7). The IR absorption at 1650 cm^{-1}, together with ^{13}C resonance at δ_C 197.6, confirmed the presence of a conjugated ketone carbonyl in **1**. The ^1H–^1H COSY of monascusone A (**1**) spectrum established the connectivity from H-1′ through H-3′ and the coupling between H-6 and H-5, and also demonstrated the allylic coupling between H-1′ and H-4. The HMBC spectrum of monascusone A (**1**) showed correlations from H-1 to C-3, C4a, and C8a; H-4 to C-3, C-5, and C-8a; H-5 to C-4a and C-8a; H-1′ to C-3 and C-4; 7-Me protons to C-6, C-7, and C-8. On the basis of these spectral data, the structure of monascusone A (**1**) was therefore identified as 6,7-dihydroxy-3-(2-hydroxy-propyl)-7-methyl-1,5,6,7-tetrahydro- isochromen-8-one. The coupling constants between H-6 and the two adjacent methylene protons of 10.4 and 5.7 Hz indicated an axial orientation of this proton. The absence of a cross peak between H-6 and 7-Me protons in the NOESY spectrum of monascusone A (**1**) implied an antirelationship between H-6 and 7-Me. The absolute configuration of monascusone A (**1**) was addressed by

Table 1
^1H (400 MHz) and ^{13}C NMR (100 MHz) spectral data ($CDCl_3$) of monascusone A (**1**) and monascusone B (**2**)

C	Monascusone A (**1**)		Monascusone B (**2**)	
	δ_C, mult.[a]	δ_H, mult., *J* in Hz	δ_C, mult.[a]	δ_H, mult., *J* in Hz
1	64.6, *t*	4.83, *dt*, 12.7, 1.3 4.93, *dd*, 12.6, 1.7	63.8, *t*	4.76, *dd*, 12.6,1.4 5.11, *dd*, 12.6, 1.2
2	–	–	–	–
3	165.7, *s*	–	160.3, *s*	–
4	103.5, *d*	5.28, *s*	103.3, *d*	5.32, *s*
4a	150.2, *s*	–	150.8, *s*	–
5	33.9, *t*	2.43, *ddd*, 15.1, 10.6, 1.5 2.61, *dd*, 15.2, 5.5	29.5, *t*	2.50, *m* 2.76, *dd*, 17.6, 4.1
6	72.7, *d*	4.00, *dd*, 10.4, 5.7	42.8, *s*	3.22, *ddd*, 17.3, 13.2, 4.1
7	77.6, *s*	–	83.1, *s*	–
8	197.6, *s*	–	189.7, *s*	–
8a	112.9, *s*	–	113.9, *s*	–
9	–	–	–	–
10	–	–	169.3, *s*	–
11	–	–	55.6, d	3.70, *d*, 13.2
12	–	–	200.2, *s*	–
13	–	–	30.0, *q*	2.53, *s*
1′	43.8, *t*	2.34, *d*, 6.2	124.4, *d*	5.95, *dd*, 15.4, 1.6
2′	66.1, *d*	4.08, *m*	135.5, *d*	6.56, *m*
3′	18.3, *q*	1.24, *d*, 6.5	18.5, *q*	1.90, *dd*, 7.0, 1.5
7-Me	23.5, *q*	1.25, *s*	17.7, *q*	1.50, *s*

[a] Multiplicity was determined by analyses of DEPT spectra.

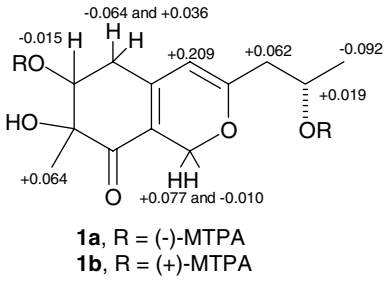


Fig. 1. $\Delta\delta$ values $[\delta_{(-)}-\delta_{(+)}]$ for the MTPA esters (**1a** and **1b**) of **1**.

the use of Mosher ester (Dale and Mosher, 1973; Sullivan et al., 1973). However, we found that (*R*) and (*S*)-methoxy trifluoromethylphenyl acetate (MTPA) esters (compounds **1a** and **1b** respectively), of monascusone A (**1**) were not stable and easily degraded to unidentified products during purification of the reaction mixtures. We therefore employed a convenient procedure for the Mosher ester formation, which was carried out in an NMR tube as recently reported by Kinghorn and his colleagues (Su et al., 2002). The $\Delta\delta$ values $[\delta(-)-\delta(+)]$ are shown in Fig. 1, indicating that the absolute configurations at C-2′ and C-6 are *S*. It should be noted that one of the H-5 methylene protons (−0.064 and +0.036) possessed a positive $\Delta\delta$ value (+0.036), and this may raise an uncertainty concerning the absolute stereochemistry of H-6. However, considering the similarity of positive optical rotations for both monascusone A (**1**) and monascin (**3**), as well as, in part, from the biosynthetic pathway of these metabolites (Fig. 2), supported the proposed stereochemistry of H-6 and H-7 in **1**. It is known that the hexaketide chromophore of azaphilone pigments, e.g. monascusone A (**1**) and FK17-P2b2 (**4**), are derived from the condensation of acetate and malonate by polyketide synthase (Fig. 2) while the side chain of these pigments originated from a medium-chain fatty acid synthesized via fatty acid synthetic pathway (Hajjaj et al., 2000). The azaphilone chromophore is subsequently esterified with a medium-chain fatty acid to furnish the observed products as shown in Fig. 2.

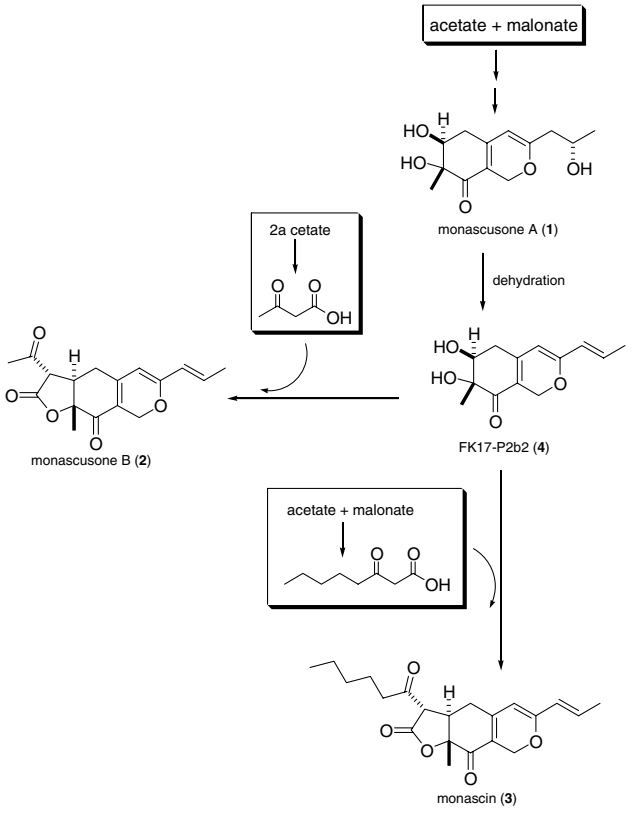
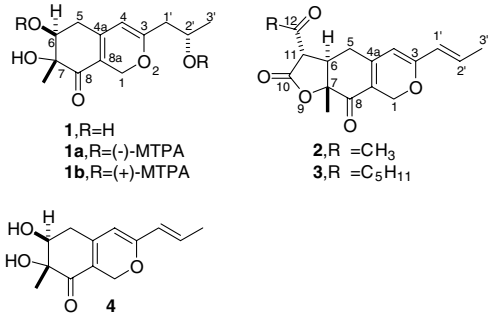


Fig. 2. Possible biosynthetic pathway of azaphilone pigments **1**–**4** by the fungus *M. kaoliang*.



Monascusone B (**2**) possessed a molecular formula $C_{17}H_{18}O_5$ as established from the ESITOF mass spectrum [observed *m/z* at 303.1235 $(M+H)^+$, calcd. for $C_{17}H_{19}O_5$, 303.1232]. In general, the ^1H and ^{13}C NMR spectra of monascusone B (**2**) were similar to those of monascin (**3**), except that the aliphatic hydrocarbon signals of the hexoyl moiety in **3** were replaced by a downfield singlet methyl (δ_H 2.53 with δ_C 30.0) in **2**. The HMBC spectrum of monascusone B (**2**) well assembled its gross structure; key long-ranged ^1H–^{13}C correlations were observed from H-1 to C-3, C-4a, and C-8a; H-4 to C-3, C-5, C-8a, and C-1′; H-5 to C-8a; 7-Me protons to C-6, C-7, and C-8; H-11 to C-6, C-10, and C-12; H-13 to C-12; and both H-1′ and H-2′ to C-3. Based upon these spectral data, the structure of monascusone B (**2**) was established as shown. Monascusone B (**2**) exhibited a positive optical rotation similar to that of monascin (**3**), implying that monascusone B (**2**) possessed the same stereochemistry as that of monascin (**3**). The biosynthetic route of monascusone B (**2**) also logically supported the proposed configuration of **2** (Fig. 2). The hexaketide chromophore (e.g. compound **4**) in **2** is possibly synthesized, via monascusone A (**1**), from the condensation of acetate and malonate, which is in turn esterified with 3-oxo-bytyrate, while that of monascin (**3**) is coupled with a medium-chain fatty acid as discussed earlier (Fig. 2).

Monascusone A (**1**), the major metabolite of *M. kaoliang* KB20M10.2, was inactive against the malarial parasite (*Plasmodium falciparum*), *Mycobacterium tuberculosis* H37Ra, and *Candida albicans*. Compound **1** showed no cytotoxicity against BC (breast cancer) and KB (human epidermoid carcinoma of cavity) cell lines. Unfortunately, due to the limited amount of materials, the minor metabolites, compounds **2**, **3**, and **4**, were not tested for their biological activities. A previous study reported that monascin (**3**) and its derivatives possessed mild antibiotic activity against *Bacillus subtilis* and *Candida pseudotropicalis*, and also showed immunosuppressive activity on mouse T-splenocytes (Martinkova et al., 1999). Results from this present investigation and from that reported by Martinkova et al. (1999) confirmed that monascin (**3**) and its derivatives are not toxic, and this may be the reason why pigments of the *Monascus* species have been widely used as natural food colorant in East Asia since ancient times particularly for Chinese food and cosmetics. FK17-P2b2 (**4**) was previously patented as a novel UV-absorbing compound, and its excellent near ultraviolet region absorbing ability is very useful for protecting various kinds of industrial products from deterioration and can be used as an additive in cosmetics (Takayuki and Hiroshi, 1994).

3. Experimental

3.1. General

^{1}H and ^{13}C, DEPT, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC experiments were carried out on Bruker DRX 400 NMR spectrometer, operating at 400 MHz for protons and 100 MHz for carbons. ESITOF mass spectra were obtained from a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass (Eckers et al., 2000). Optical rotations were measured on a JASCO DIP 370 polarimeter, while UV spectra were recorded on a Cary 1E UV–Vis spectrophotometer. The IR spectra were measured on a Perkin–Elmer 2000 spectrophotometer.

3.2. Fungal material

A yellow mutant of the fungus *M. kaoliang* KB20M10.2 was isolated and identified after Iizuka and Lin (1981) by B.Y., and deposited (Registration No. KB20M10.2) at the Department of Microbiology, Faculty of Science, Kasetsart University. The fungus was initially grown in a Conidia medium (Hiroi et al., 1979), and inoculated into boiled rice (1 kg). The culture was subsequently incubated at 30 °C, for 14 days, and harvested for further study.

3.3. Extraction and isolation

Culture of *M. kaoliang* KB20M10.2 (grown on 1 kg of rice) was macerated in CH_2Cl_2 for two days, and a CH_2Cl_2 extract was evaporated to dryness. The crude CH_2Cl_2 extract (16.5 g) was subsequently subjected to Sephadex LH-20 cc, eluted with MeOH, to provide 11 fractions (A1–A11). Fractions (A4–A7) were pooled and repeatedly subjected to Sephadex LH-20 column (MeOH as eluent). Finally the fraction with yellow color was subjected to Si gel cc (CH_2Cl_2:acetone, 99:1) to afford monascusone A (**1**) (80 mg), monascusone B (**2**) (2.8 mg), monascin (**3**) (20 mg), and FK17-P2b2 (**4**) (4.2 mg).

Monascusone A (**1**); yellow viscous liquid; $[\alpha]_D^{30} + 71.95°$ (c 0.93, $CHCl_3$); IR ν_{max} cm^{-1}: 3350, 2974, 2932, 1650, 1554, 1409, 1290, 1193, 1072, 978, 898; UV ($CHCl_3$) λ_{max} nm: 243, 346; ESITOF MS: *m/z* 277.1041 $(M + Na)^+$, calcd. for $(C_{13}H_{18}O_5 + Na)^+$, 277.1052; for ^{1}H and ^{13}C NMR spectra, see Table 1.

Monascusone B (**2**); yellow viscous liquid; $[\alpha]_D^{30} + 205.47°$ (c 0.28, $CHCl_3$); IR ν_{max} cm^{-1}: 3447, 2927, 1784, 1791, 1380, 1526, 1459, 1382, 1263, 1219, 1088, 866, 804; UV ($CHCl_3$) λ_{max} nm: 243, 375; ESITOF MS: *m/z* 303.1235 $(M + H)^+$, calcd. for $C_{17}H_{19}O_5$, 303.1232; for ^{1}H and ^{13}C NMR spectra, see Table 1.

3.4. Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of monascusone A (*1*) in NMR tubes (Su et al., 2002)

Monascusone A (**1**) (2 mg) was dissolved in pyridine-d_5 (0.5 mL) in an NMR tube, and this solution was subjected to ^{1}H NMR analysis. (*S*)-(+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride [(*S*)-(+)-MTPA chloride] (6 μL) was added into the solution, the mixture was left at room temperature for 45 min and the progress of the esterification reaction was monitored by ^{1}H NMR analysis where upon a downfield shift of H-2′ and H-6 was observed. After complete formation of (*R*)-(+)-MTPA ester of **1**, the reaction mixture was then subjected to ^{1}H NMR analysis. ^{1}H NMR data (pyridine-d_5) of the (*R*)-(+)-MTPA ester derivative of monascusone A (**1**): δ_H 7.185–7.854 (aromatic protons of MTPA), 5.912 (1H, *dd*, J = 10.3, 5.2 Hz, H-6), 5.510 (1H, *m*, H-2′), 5.100 (1H, *s*, H-4), 5.058 (1H, *d*, J = 12.5 Hz, H-1a), 4.860 (1H, *d*, J = 12.7 Hz, H-1b), 3.427 (6H, *s*, OMe of MTPA), 2.884, (1H, *dd*, J = 15.2, 5.1 Hz, H-5a), 2.722 (1H, *m*, H-5b), 2.515 (2H, *d*, J = 6.3 Hz, H-1′), 1.428 (3H, *s*, 7-Me), and 1.346 (3H, *d*, J = 6.1 Hz, H-3).

(*S*)-(−)-MTPA ester derivative of monascusone A (**1**) was prepared accordingly. ^{1}H NMR data (pyridine-d_5) of the (*S*)-(−)-MTPA ester derivative of monascusone A (**1**): δ_H 7.185–7.854 (aromatic protons of MTPA), 5.897 (1H, *dd*, J = 10.4, 5.7 Hz, H-6), 5.529 (1H, *m*,

H-2′), 5.309 (1H, *s*, H-4), 5.048 (1H, *d*, *J* = 12.3 Hz, H-1a), 4.937 (1H, *d*, *J* = 12.4 Hz, H-1b), 3.428 (6H, *s*, OMe of MTPA), 2.920 (1H, *dd*, *J* = 15.5, 5.3 Hz, H-5a), 2.658 (1H, *m*, H-5b), 2.577 (2H, *d*, *J* = 6.2 Hz, H-1′), 1.492 (1H, *s*, 7-Me), and 1.254 (3H, *d*, *J* = 6.18 Hz, H-3′).

3.5. Bioassays

3.5.1. Cytotoxicity

The cytotoxic assay employed the colorimetric method reported by Skehan et al. (1990). KB (human epidermoid carcinoma of cavity, ATCC CCL-17) and BC (breast cancer cell line) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan et al. (1990). Elliptine was used as positive control. DMSO (10%) was used as negative control. Briefly, cells at a logarithimic growth phase were harvested and diluted to 10^5 cells/mL with fresh medium and gently mixed. Testing compound was dissolved in DMSO (concentration at 20 mg/mL), and this solution was then diluted with distilled water to obtain a stock solution at 0.4 mg/mL (with 10% DMSO). The stock solution (10 μL) and cell suspension (190 μL) were transferred into microtiter plates (concentration at 20 μg/mL with 0.05% DMSO). If the compound is active at 20 μg/mL, a series of solutions were prepared by twofold dilution of the stock solution (diluted with 10% DMSO solution), and exposed to cells as mentioned above, in order to obtain IC_{50} value. Plates were incubated at 37°C under 5% CO_2 atmosphere for 72 h. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4°C for 30 min, washed with water, and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B (SRB) dissolved in 1% acetic for 30 min. After staining period, SRB was removed with 1% acetic acid. Plated were air-dried before bound dye was solubilized with 10 mM Tris base for 5 min on shaker. Optical density was read in a microtiter plate reader at wavelength 510 nm. Ellipticine, the reference substance, exhibited activity toward BC and KB cell lines, both with the IC_{50} of 0.3 μg/mL.

3.5.2. Antimalarial activity

The parasite *P. falciparum* (K1, multidrug resistant strain) was cultured continuously according to the method of Trager and Jensen (1976). Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al. (1979). Briefly, a mixture of 200 μL of 1.5% of erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 μL of the medium containing a test sample dissolved in DMSO (0.1% final concentration) for 24 h employing the incubation conditions described above. Subsequently, 25 μL of [^{3}H]hypoxanthine (Amersham, USA) in culture medium (10 μCi) was added to each well and plates were incubated for an additional 24 h. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the TopCount microplate scintillation counter (Packard, USA). An IC_{50} value of 1.2 ± 0.02 μg/mL (*n* = 3) was observed for the standard compound, dihydroartemisinin.

3.5.3. Antifungal activity

The antifungal activity was assessed against a clinical isolate of *C. albicans*, employing a colorimetric method (Hawser et al., 1998). Briefly, 100 μL of 2×10^6 CFU/mL of *C. albicans* in RPMI 1640 medium, containing 34.53 g/mL of 3-[*N*-morpholino]propanesulfonic acid was added to each well of 96-well microculture plates containing 100 μL of test compound diluted in 10% DMSO. Plates were incubated at 37°C for 4 h before adding 50 μL of a solution containing 1 mg/mL of 2,3-bis-[2-methoxy-4-nitro-5-sulfonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT tetrazolium) and 0.025 mM of *N*-methylphenazonium methyl sulfate. After additional 4 h incubation at 37°C, the number of living cells was determined by measuring the absorbance of XTT formazan at 450 nm. Amphotericin B and 10% DMSO were used as a positive and a negative control, respectively. In our system, the IC_{50} value of the standard drug, amphotericin B, was 0.04 ± 0.01 μg/mL (*n* = 3).

3.5.4. Antitubercular activity

The antitubercular activity was assessed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). *M. tuberculosis* H37Ra was grown in 100 mL of 7H9GC containing 0.005% Tween 80. Cultures were incubated in a 500-mL plastic flask on a rotary shaker at 200 rpm and 37 °C until they reached an optical density of 0.4–0.5 at 550 nm. Bacteria were washed and suspended in 20 mL of phosphate-buffered saline and passed through an 8-μm pore-size filter to eliminate clumps. The filtrates were aliquoted and stored at −80 °C. Antimicrobial susceptibility testing was performed in 96-well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration. Initial screened-sample dilutions were prepared in either DMSO or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2% v/v glycerol and 1.0 g/L casitone (7H9GC), and subsequent twofold dilutions were performed in 0.1 mL of 7H9GC in the microplates. Frozen inocula were diluted 1:100 in 7H9GC. Addition of 0.1 mL to the well resulted in final bacteral titers of about 5×10^4 CFU/mL. Wells containing sample only were used to determine whether the test-samples themselves could reduce

the dye or not. Additional control wells consisting of bacteria (B) or medium (M) were included. Plates were incubated at 37 °C. Starting at day six of incubation, 20 μL of Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37 °C. Wells were observed at 24 h for a colour change from blue to pink. If the B wells became pink by 24 h, Alamar Blue solution was added to all testing plates. However, if a colour (blue) of M and B wells did not change, both wells were tested daily until a colour of B wells change from blue to pink. After the change of B well colour, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37 °C for 24 h, and the results were recorded with a fluorescence multi-well reader (Cyto-Fluor, Series 4000) at the excitation and emission wavelengths of 530 and 590 nm, respectively. The standard drugs, isoniazid and kanamycin sulfate, showed respective MIC values of 0.040–0.090 and 2.0–5.0 μg/mL.

Acknowledgements

The student scholarship from the Royal Golden Jubilee Ph.D. Program to S.J. is gratefully acknowledged. We thank the Biodiversity Research and Training Program (BRT) for financial support via the Bioresources Utilization Program (BUP). P.K. acknowledges The Thailand Research Fund (TRF) for financial support, and Y.T. thanks BIOTEC for the Senior Research Fellowship Award. B.Y. appreciates the National Research Council of Thailand (NRCT) for the contribution to *Monascus* pigment project.

References

Akihisa, T., Mafune, S., Ukiya, M., Kimura, Y., Yasukawa, K., Suzuki, T., Tokuda, H., Tanabe, N., Fukuoka, T., 2004. (+)- and (−)-Syn-2-isobutyl-4-methylazetidine-2,4-dicarboxylic acids from the extract of *Monascus pilosus*-fermented rice (red-mold rice). J. Nat. Prod. 67, 479–480.

Blanc, P.J., Loret, M.O., Santerre, A.L., Pareilleux, D., Prome, D., Prome, J.C., Laussac, J.P., Goma, G., 1994. Pigments of *Monascus*. J. Food Sci. 59, 862–865.

Chen, F.C., Manchand, P.S., Whalley, W.B., 1971. The chemistry of fungi. LXIV. The structure of monascin: the relative stereochemistry of the azaphilones. J. Chem. Soc. (C) 21, 3577–3579.

Collins, L., Franzblau, S.G., 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob. Agents Ch. 41, 1004–1009.

Dale, J.A., Mosher, H.S., 1973. Nuclear magnetic resonance enantiomer reagents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *O*-methylmandelate, and α-methoxy-α trifluoromethylphenylacetate (MTPA) esters. J. Am. Chem. Soc. 95, 512–519.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Ch. 16, 710–718.

Eckers, C., Wolff, J.C., Haskins, N.J., Sage, A.B., Giles, K., Bateman, R., 2000. Accurate mass liquid chromatography/mass spectrometry on orthogonal acceleration time-of-flight mass analyzers using switching between separate sample and reference sprays. 1. Proof of concepts. Anal. Chem. 72, 3683–3688.

Endo, A., Komagata, D., Shimada, H., 1986. Monakolin M, a new inhibitor of cholesterol biosynthesis. J. Antibiot. 39, 1670–1673.

Hajjaj, H., Klaebe, A., Goma, G., Blanc, P.J., Barbier, E., Francois, J., 2000. Medium-chain fatty acids affect citrinin production in the filamentous fungus *Monascus ruber*. Appl. Environ. Microbiol. 66, 1120–1125.

Hawser, S.P., Norris, H., Jessup, C.J., Ghannoum, M.A., 1998. Comparison of a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H- tetrazolium hydroxide (XTT) colorimetric method with the standardized national committee for clinical laboratory standards method of testing clinical yeast isolates for susceptibility to antifungal agents. J. Clin. Microbiol. 36, 1450–1452.

Hiroi, T., Sima, T., Suzuki, M., Tsukioka, M., Ogasawara, N., 1979. Hyperpigment productive mutant of *Monascus anka* for solid culture. Agr. Biol. Chem. 43, 1975–1976.

Hossain, C.F., Okuyama, E., Yamazaki, M., 1996. A new series of coumarin derivatives having monoamine oxidase inhibitory activity from *Monascus anka*. Chem. Pharm. Bull. 44, 1535–1539.

Hsieh, P-S., Tai, Y.-H., 2003. Aqueous extract of *Monascus purpureus* M 9011 prevents and reverses fructose-induced hypertension in rats. J. Agr. Food Chem. 51, 3945–3950.

Iizuka, H., Lin, C.F., 1981. On The Genus *Monascus* Of Asia And Its Specific Characteristics. In: Moo-Young, M., Robinson, C.W., Vezina, C. (Eds.), Advances in Biotechnology, vol. 2. Pergamon Press, Toronto, pp. 555–561.

Ma, J., Li, Y., Ye, Q., Li, J., Hua, Y., Ju, D., Zhang, D., Cooper, R., Chang, M., 2000. Constituents of red yeast rice, a traditional Chinese food and medicine. J. Agr. Food Chem. 48, 5220–5225.

Martinkova, L., Patakova-Juzlova, P., Krent, V., Kucerova, Z., Havlicek, V., Olsovsky, P., Hovorka, O., Rihova, B., Vesely, D., Vesela, D., Ulrichova, J., Prikrylova, V., 1999. Biological activities of oligoketide pigments of *Monascus purpureus*. Food Addit. Contam. 16, 15–24.

Sabater-Vilar, M., Maas, R.F., Fink-Gremmels, J., 1999. Mutagenicity of commercial *Monascus* fermentation products and the role of citrinin contamination. Mutat. Res. 444, 7–16.

Sato, K., Goda, Y., Sakamoto, S.S., Shibata, H., Maitani, T., Yamada, T., 1997. Identification of major pigments containing D-amino acid units in commercial *Monascus* pigments. Chem. Pharm. Bull. 45, 227–229.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer I. 82, 1107–1112.

Su, B.N., Park, E.J., Mbwambo, Z.H., Santarsiero, B.D., Mesecar, A.D., Fong, H.H., Pezzuto, J.M., Kinghorn, A.D., 2002. New chemical constituents of *Euphorbia quinquecostata* and absolute configuration assignment by a convenient Mosher ester procedure carried out in NMR tubes. J. Nat. Prod. 65, 1278–1282.

Su, Y.C., Wang, J.J., Lin, T.T., 2003. Production of the secondary metabolites γ-aminobutyric acid and monakolin K by *Monascus*. J. Ind. Microbiol. Biotechnol. 30, 41–46.

Sullivan, G.R., Dale, J.A., Mosher, H.S., 1973. Correlation of configuration and ^{19}F chemical shifts of αmethoxy-α trifluoromethylphenylacetate derivatives. J. Org. Chem. 38, 2143–2147.

Takayuki, A., Hiroshi, S., 1994. Novel UV-absorbing compounds FK17-P2a, FK-17-P2b1, FK17-P2b2, and FK17-P3 and manufacture of the compounds with *Aspergillus* sp., Patent No. JP 6329576.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Wild, D., Toth, G., Humpf, H.U., 2002. New *Monascus* metabolite isolated from red yeast rice. J. Agr. Food Chem. 50, 3999–4002.

Yongsmith, B., Krairak, S., Bavavoda, R., 1994. Production of yellow pigments in submerged culture of a mutant of *Monascus* spp. J. Ferment. Bioeng. 78, 223–228.

Yongsmith, B., Kitprechavanich, V., Chitradon, L., Chaisrisook, C., Budda, N., 2000. Color mutants of *Monascus* sp. KB9 and their comparative glucoamylases on rice solid culture. J. Mol. Catal. B: Enzymatic 10, 263–272.