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SECONDARY METABOLITES ISOLATED FROM THE FUNGUS *MONASCUS PILOSUS*

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Investigation of the 95% EtOH extract of red mold rice fermented with the yellow mutant of the fungus *Monascus pilosus* BCRC 38072 led to the isolation of one new pyran-2-one derivative, namely monascuspyrone (**1**), together with nine known compounds. The structure of the new natural compound was determined as 6-(2-hydroxydodecan-2-yl)-3-(hydroxymethyl)-4-methoxy-2*H*-pyran-2-one. The known compounds were identified as monascin (**2**), ankaflavin (**3**), monasfluore B (**4**), 3-*epi*-betulinic acid (**5**), 3-*epi*-betulinic acid acetate (**6**), α-tocospiro B (**7**), methyl isovanillate (**8**), *p*-dihydrocoumaric acid (**9**) and methylparaben (**10**). Interesting, this is the first report of *Monascus* metabolites with a pyran-2-one derivative Compounds **5–9** were isolated from this species for the first time. Their structures were elucidated by 1D and 2D-NMR spectroscopy together with HR-ESI-MS analysis, and comparison of the spectroscopic data with those reported in the literatures.

INTRODUCTION

The filamentous fungi of the *Monascus* species have been used for a long time as a meat colorant, health food, and a Chinese folk medicine.[1] They comprise four represent species: *M. pilosus*, *M. purpureus*, *M. rubber*, and *M. anka*. These species belonged to the class Ascomycetes and the family Monascaceae.[1] Red mold rice (also called red yeast rice), which is also known as "koji", "red koji", "anka", "Ang-kak", and "ben-koji", is obtained by the fermentation of rice (*Oryza sativa*) with fungi of the genus *Monascus*, mainly *M. pilosus*, *M. purpureus*, and *M. anka*, to produce a red-colored product. Red mold rice has long been used in East Asia for over 1000 years to color (as a natural food colorant, such as for red rice wine, red soy bean cheese), aromatize and conserve meat, fish and soybean products.[1] *M. purpureus*, *M. pilosus*, and *M. anka* (Eurotiaceae) are representatives natural colorant of the *Monascus* fungi traditionally used in East Asia as a source of pigments.[1] Red mold rice was a great invention in ancient China and it is also applied for medicinal purposes like promoted digestion and blood circulation, strengthened the spleen, and removed blood stasis.[2] The above-mentioned representative molds can produce several pigments and some physiological biological active metabolites when grown on cooked rice.[1] Several secondary metabolites useful as food additives and pharmaceuticals have been reported by *Monascus* spp.[1-3] Polyketides, furanoisophthalides, amino acids, azaphilones, pyranoindole alkaloids, benzenoids, furans, and fatty acids[2-13] are widely distrusted in the fungus *Monascus* species. Some major pigments of red mold rice and some secondary metabolites have been identified, but knowledge of their biological effects is limited and partial. Some minor compounds like pyrone derivatives or other type of compounds produced by *Monascus* sp. have received less attention. Hence, it is still worthy to explore and investigate the components of red mold rice and their functionality in further studies. In the course of our search for potential diverse secondary metabolites from natural fungal sources,

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we have screened over 50 species of fungal materials by using TLC profile analyses. As a result, one of the species, *M. pilosus* BCRC 38072, has been found to contain more abundant metabolites by TLC profile analyses.

Careful examination on the EtOAc-soluble fraction of a 95% EtOH extract of the red mold rice produced by *M. pilosus* BCRC 38072 has resulted in the isolation of ten constituents, including a new pyran-2-one derivative, monascuspyrone (**1**), together with nine known compounds. In this paper, we describe the isolation and structural elucidation of the new compound (Figure 1).

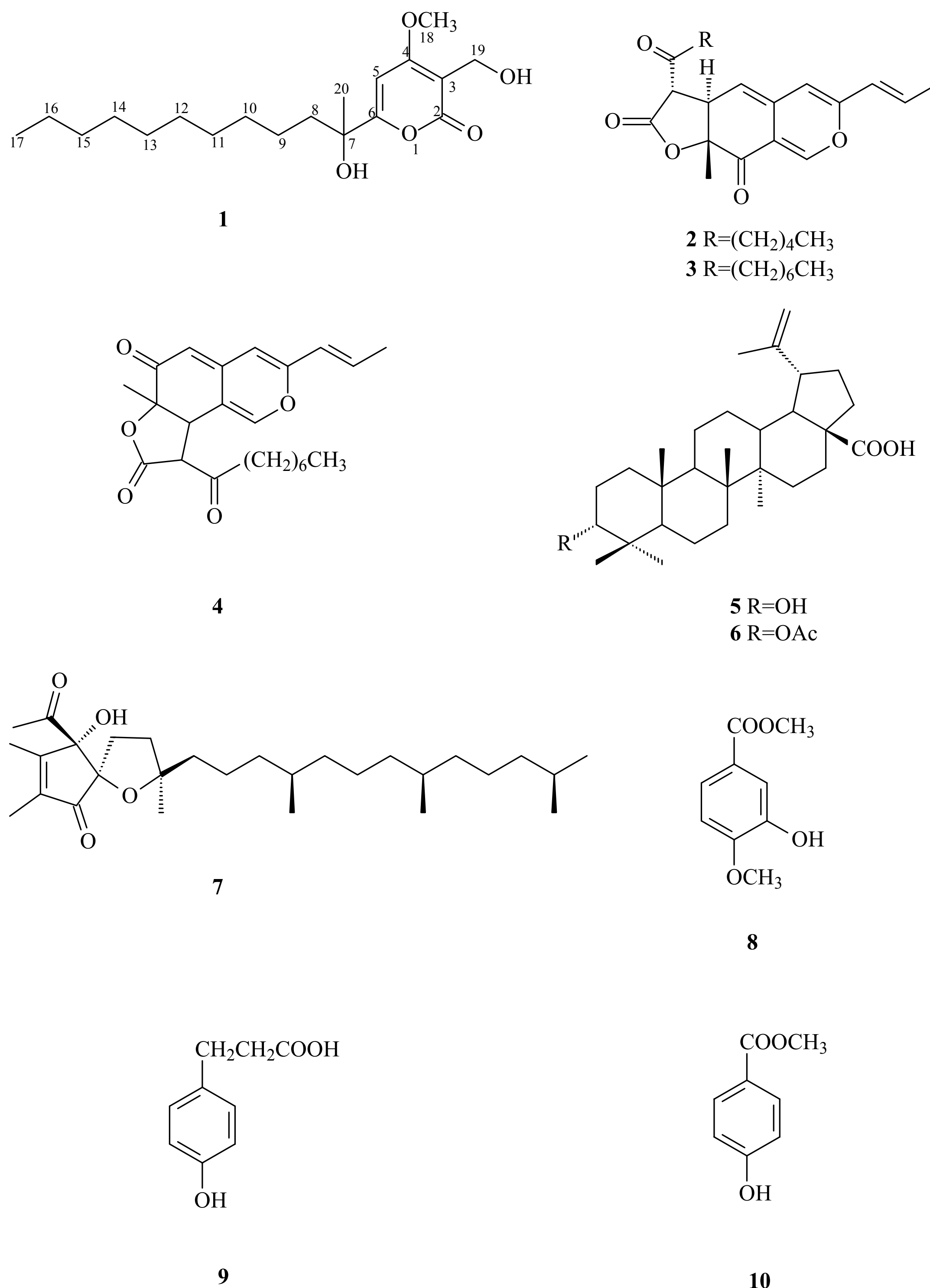

Fig. 1 – Structures of metabolites **1–10** isolated from *M. pilosus* BCRC 38072.

RESULTS AND DISCUSSION

The EtOAc extracts, prepared from the 95% EtOH extract of the red mold rice produced by *M. pilosus* BCRC 38072 were fractionated by a combination of open column chromatography (silica gel), MPLC, and prep. TLC to obtain the pyrone derivative (**1**), azaphilones (**2–4**), triterpenoids (**5–6**), tocopheroid derivative (**7**), and benzenoids (**8–10**). The known isolates, monascin (**2**),[14] ankaflavin (**3**),[14] monasfluore B (**4**),[6] 3-*epi*-betulinic acid (**5**),[15] 3-*epi*-betulinic acid acetate (**6**),[15] α-tocospiro B (**7**),[16] methyl isovanillate (**8**),[17] *p*-dihydrocoumaric acid (**9**)[17] and methylparaben (**10**)[17] were readily identified by comparison of their spectral data (UV, IR, ^{1}H-NMR, MS) with the data from the corresponding values in the literature and by direct comparison with authentic samples on TLC plate. The new structure of **1** was identified based on the following evidences.

Compound **1** was obtained as colorless optically inactive oil. $[\alpha]^{22}_{D}$ = ±0 (*c* 0.08, $CHCl_3$). The molecular formula of compound **1** was determined as $C_{19}H_{32}O_5$ from its HR-ESI-MS data (*m/z* 363.2149 ($[M+Na]^+$; calc. 363.2147)) as well as from its ^{13}C-NMR and DEPT, requiring 4 degrees of unsaturation. UV absorption at 212, 242 and 285 nm and its IR absorption bands at 3400 cm^{-1} (OH), 1710 cm^{-1} (C=O), and 1641 (C=C), suggesting the presence of a pyran-2-one nucleus.[18-22] The presence of a conjugated carbonyl group was revealed by an IR absorption, along with a resonance signal in the ^{13}C-NMR spectrum at δ_C 165.0 (C-2).

The ^{1}H-NMR spectrum of **1** (Table 1) displayed an olefinic proton at δ_H 6.52 (1H, s), one methoxyl group at δ_H 3.93 (3H, s), two mutually coupled equivalent hydroxymeythyl ($-CH_2OH-$) at δ_H 3.05 (1H, br t, J = 6.0 Hz, D_2O exchangeable) and 4.54 (2H, br t, J = 6.0 Hz), were attributed to H-5, OCH_3-18, CH_2-19, and OH-19, respectively. Furthermore, one methyl at δ_H 1.50 (3H, s) being attached to a quaternary C-atom bearing an OH moiety (δ_H 2.44 (1H, br s, OH-7, D_2O exchangeable)), one terminal methyl group at δ_H 0.87 (1H, t, J = 6.8 Hz), and those for the remaining methylenes of the aliphatic chain at δ_H 1.11 (2H, br quin, J = 4.6 Hz), 1.23-1.38 (14H, br s), 1.70 (1H, ddd, J = 14.0, 12.4, 4.6 Hz) and 1.89 (1H, ddd, J = 14.0, 12.4, 4.6 Hz) due to CH_3-20, CH_3-17, CH_2-9, CH_2-8, CH_2-10~16 established the presence of one 2-hydroxydodecan-2-yl side chain (Table 1).

Table 1

NMR data ($CDCl_3$) for the new compound **1**. (δ in ppm, J in Hz)

No.	δ_H	$\delta_C{}^b$	DEPT
1	–	–	–
2	–	165.0	C (s)
3	–	103.6	C (s)
4	–	167.1	C (s)
5	6.52 (1H, s)	92.2	CH (d)
6	–	170.7	C (s)
7	–	74.0	C (s)
8	1.70 (1H, ddd, J = 14.0, 12.4, 4.6 Hz) 1.89 (1H, ddd, J = 14.0, 12.4, 4.6 Hz)	40.6	CH_2 (t)
9	1.11 (2H, br quin, J = 4.6 Hz)	23.5	CH_2 (t)
10	1.23-1.38 (br s)	29.3-29.6	CH_2 (t)
11	1.23-1.38 (br s)	29.3-29.6	CH_2 (t)
12	1.23-1.38 (br s)	29.3-29.6	CH_2 (t)

13	1.23-1.38 (br s)	29.3-29.6	CH_2 (t)
14	1.23-1.38 (br s)	29.3-29.6	CH_2 (t)
15	1.23-1.38 (br s)	31.8	CH_2 (t)
16	1.23-1.38 (br s)	22.6	CH_2 (t)
17	0.87 (1H, t, J = 6.8 Hz)	14.1	CH_3 (q)
OCH_3-18	3.93 (s)	56.6	CH_3 (q)
*CH_2*OH-19	4.54 (2H, dd, J = 6.0, 6.0 Hz, D_2O exchangeable)	54.6	CH_2 (t)
CH_3-20	1.50 (1H, s)	27.3	CH_3 (q)
OH-7	2.44 (br s, D_2O exchangeable)	–	–
CH_2*OH*-19	3.05 (1H, br t, J = 6.0 Hz, D_2O exchangeable)	–	–

[a] All spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C); assignment were aided by 2D-NMR, COSY, HSQC and HMBC experiments; [b] ^{13}C-NMR multiplicities were determined by DEPT experiment.

The ^{13}C-NMR and DEPT spectrum showed that **1** had a total of 19 carbons for three Me, ten CH_2, one olefinic CH, and five quaternary C-atoms including one carbonyl group C=O (δ_C 165.0). The carbon atoms of the compound **1** were assigned from ^{13}C-NMR, DEPT, HSQC and HMBC experiments as shown in Table 1. These data also pointed to a pyrone derivative skeleton.[18-22]

The planar structure of **1** was deduced from 2D NOESY experiments (Figure 4). The pyran-2-one unit was established by HMBC correlations as shown in Figure 3. The ^{1}H- and ^{13}C-NMR chemical shifts of this moiety were in agreement with those reported previously.[18] Compared with common C=C bonds, C-6 at δ_C 170.7 and C-5 at δ_C 92.2 in the pyrone moiety were severely down- and upfield shifted, respectively, due to the enhanced effect from the O-atom at C-6 and the conjugated carbonyl C=O group at C-2.

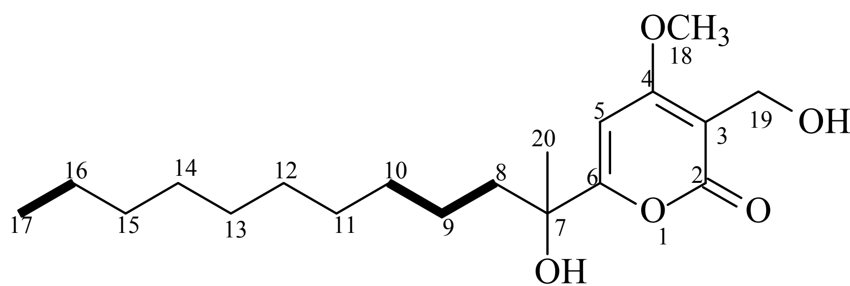

Fig. 2 – Important COSY (▬) contacts of **1**.

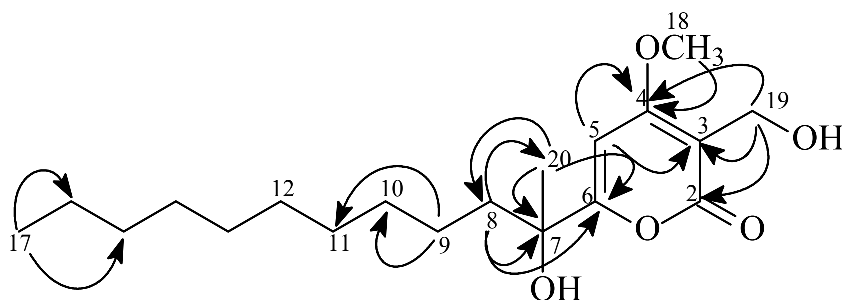

Fig. 3 – Key HMBC (H→C) correlations of **1**.

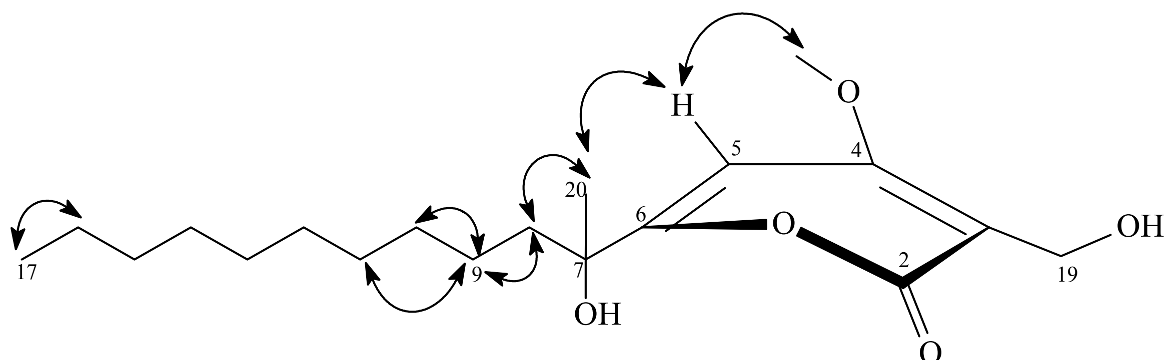

Fig. 4 – Major NOESY (H↔H) correlations of **1**.

The ^{1}H-^{1}H COSY (Figure 2) and ^{1}H-^{1}H TOCSY spectra indicated H-^{1}H correlations between H-17/H-16, H-16/H-15/H-14/H-13/H-12/H-11/H-10, H-10/H-9, H-9/H-8, and H-8/CH_3-20, suggesting that the partial structure from C-8 to C-17 was a *n*-decyl group linked at C-7 due to the HMBC ^3J correlation between δ_H 1.50 (CH_3-20) and δ_C 40.6 (C-8), and between δ_H 1.70/1.89 (CH_2-8) and δ_C 27.3 (C-20), becoming one 2-hydroxydodecan-2-yl group. One methoxyl group (δ_H 3.93) was located at C-4 due to the HMBC ^3J correlation with C-4 (δ_C 167.1). The other hydroxymethyl moiety at δ_H 3.05 (CH_2-19) showed a ^2J correlation with δ_C 103.6 (C-3) in the HMBC spectrum, indicating the presence of a hydroxymethyl group at C-3.

The presence of a 2-hydroxydodecan-2-yl group was further established by HMBC correlations of CH_3-17 to C-16, and C-15, of H-9 to C-10, and C-11, and of H-8 to C-7, and CH_3-20, respectively. The connection between the pyrone and the 2-hydroxydodecan-2-yl units was established by the aid of HMBC analyses (Figure 3). The key ^3J HMBC correlation (Figure 3) from δ_H 6.52 (H-5) to δ_C 74.0 (C-7); δ_H 1.70/1.89 (H-8) to δ_C 170.7 (C-6), and δ_H 1.50 (CH_3-20) to δ_C 170.72 (C-6) verify the junction of the pyrone ring to the 2-hydroxydodecan-2-yl chain at C-6, and enabled us to draw the planar structure of **1**.

The relative stereochemistry of **1** was deduced from the nuclear Overhauser enhancement spectroscopy (NOESY) experiment (Figure 4). The attachment of the 2-hydroxydodecan-2-yl substituent at C-6 was confirmed by the correlation between CH_3-20 and H-5 in NOESY spectrum (Figure 4). The full assignment of this 2-hydroxydodecan-2-yl side-chain was further confirmed by COSY (Figure 2), HSQC, and HMBC (Figure 3) spectra. The correlations of H-5/OCH_3-18, and CH_3-20, and H-9/H-11 and 10, and CH_3-20/H-8 were also observed in the NOESY experiment (Figure 4) and further supported the position of each pyrone substitution. Because of the optical inactivity, **1** was concluded to be racemic.

On the basis of these data, the structure of compound **1** was, thus, deduced as 6-(2-hydroxydodecan-2-yl)-3-(hydroxymethyl)-4-methoxy-2*H*-pyran-2-one, and named monascuspyrone.

EXPERIMENTAL

General experimental procedure

All melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco P-1020 digital polarimeter, UV spectra were obtained on a Jasco UV-240 spectrophotometer in MeOH, and IR spectra (Neat) were taken on a Perkin-Elmer System 2000 FT-IR spectrometer. 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra using $CDCl_3$ as solvent were recorded on a Varian Unity Plus 400 and Varian Mercury-400 (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) spectrometer. The amount of 5 mg of compound **1** dissolved in 0.5ml $CDCl_3$ (0.029 M) was used to run all 2D-NMR spectra. Chemical shifts were internally referenced to the solvent signals in $CDCl_3$ (^{1}H, δ 7.26; ^{13}C, δ 77.0). Low-resolution ESI-MS spectra were obtained on an API 3000 (Applied Biosystems) and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer. Low-resolution EI-MS spectra were recorded on a Quattro GC/MS spectrometer having a direct inlet system. Silica gel (70-230, 230-400 mesh) (Merck) was used for column chromatography, and silica gel 60 F-254 (Merck) was used for TLC and prep. TLC.

Microorganism

Monascus pilosus BCRC 38072 was used throughout this study, and specimens deposited at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI).

Cultivation and preparation of red mold rice

Monascus pilosus BCRC 38072 was maintained on potato dextrose agar (PDA; Difco). The strain was cultured on PDA

slants at 25°C for 6 days and then the spores were harvested by sterile water. The spores (5×10^5) were seeded into 300 ml shake flasks containing 50 ml RGY medium (3% rice starch, 7% glycerol, 1.1% polypeptone, 3.2% soybean powder, 0.2% $MgSO_4$, 0.2% $NaNO_3$), and cultivated with shaking (150 rpm) at 25 °C for 3 days. After the mycelium enrichment step, an inoculum mixing 100 ml mycelium broth and 100 ml RGY medium was inoculated into plastic boxes (25 cm × 30 cm) containing 1 kg sterile rice and cultivated at 25°C for producing red mold rice (RMR; also called *beni-koji* in Japan). At day 7, 150 ml RGY medium was added for maintaining the growth of cells. After 28 days of cultivation, the RMR was harvested and lyophilized for the extraction of metabolites.

Extraction and Isolation

Red mold rice of the *M. pilosus* BCRC 38072 (1.0 kg) were extracted three times with 95% EtOH at room temperature. The ethanol syrup extract was partitioned between EtOAc and H_2O (1:1) to afford EtOAc (fraction A, 2.0 g) and H_2O soluble fractions. The EtOAc-soluble fraction (2.0 g) was submitted to silica gel (SiO_2, 70–230 mesh), eluting with *n*-hexane and enriched with acetone to produce 15 subfractions (A1—A15). Fraction A-3 (35 mg) was subjected to CC (SiO_2, 230-400 mesh; *n*-hexane–EtOAc, 8:1) to obtain monascin (**2**) (2.3 mg), ankaflavin (**3**) (6.8 mg), and methylparaben (**10**) (0.8 mg). Fr. A-4 (164 mg) was chromatographed on silica gel (230-400 mesh), employing *n*-hexane containing increasing amounts of acetone as elute to produce 10 subfractions (A-4-1–A-4-10). Fr. A-4-8 (30 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 1:1) to afford monascuspyrone (**1**) (5.1 mg). Fraction A-6 (80 mg) was chromatographed over silica gel, eluting with CH_2Cl_2-acetone (10:1) to obtain 16 subfractions (A-6-1–A-6-20). Fraction A-6-11 (18 mg) was repeatedly purified by preparative TLC (*n*-hexane–acetone, 1:1) to afford 3-*epi*-betulinic acid (**5**) (1.7 mg) and 3-*epi*-betulinic acid acetate (**6**) (1.5 mg). Fraction A-6-12 (44 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 2:1) to give α-tocospiro B (**7**) (1.1 mg). Fraction A-11 (152 mg) was resubjected to silica gel column chromatography (CH_2Cl_2-MeOH, 25:1) to afford 10 subfractions (A-11-1–A-11-10). Fraction A-11-3 (65 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–MeOH, 10:1) to afford monasfluore B (**4**) (2.5 mg) and methyl isovanillate (**8**) (1.9 mg). Fraction A-12 (350 mg) was resubjected to silica gel column chromatography (CH_2Cl_2-MeOH, 15:1) to afford 10 subfractions (A-12-1–A-12-10). Fraction A-12-8 (114 mg), eluting with CH_2Cl_2–acetone 10:1, was further separated using preparative TLC (CH_2Cl_2–EtOAc, 15:1) to yield *p*-dihydrocoumaric acid (**9**) (2.7 mg).

Spectral data

Monascuspyrone (**1**). Colorless oil; ($[\alpha]_D^{22}$ = ±0 (*c* 0.08, $CHCl_3$); UV (MeOH): 210 (4.25), 242 (3.85), 285 (3.85) nm; IR (Neat): 3400 (OH), 1710 (C=O), 1641 (C=C) cm^{-1}; ESI-MS *m/z* 363 $[M+Na]^+$; HR-ESI-MS *m/z* 363.2149 $[M+Na]^+$ (calcd for $C_{19}H_{32}O_5Na$, 363.2147); ^1H- and ^{13}C-NMR spectra are shown in **Table 1**.

Authentic samples

The source of these authentic samples has isolated from other *Monascus* sp. by our research group.

CONCLUSION

In summary, furanoisophthalides, azaphilone, amino acid, and polyketides are the major metabolites in *Monascus* species[1-13] according to the past literatures. In our previous studies, we have reported over fifteen constituents, together with their biological activity from the mycelium of *M. pilosus*.[5] In the course of our continuous searching for potential diverse secondary metabolites from natural sources, we were intended to undertake the investigation of red mold rice metabolites, and *M. pilosus* BCRC 38072 has been found to have more metabolites that were detected by using TLC profile analyses.

In this successive study, we focused on the minor secondary metabolites appearing in the EtOAc-soluble fraction of a 95% EtOH extract of the red mold rice produced by *M. pilosus* BCRC 38072. The new natural metabolite **1**, found in this study is first, naturally occurring compound. It is worthy to mention that this is the first report of a pyrone derivative isolated from *Monascus* species. Among them, all known compounds except **2–4** and **10**, were isolated for the first time from *Monascus* species. Comparison with the previous studies, the results suggest that *Monascus* has distinct and diverse secondary metabolites, which arise under different fermentation conditions. It may therefore be possible to find more new bioactive natural products by cultivating *Monascus* under different conditions.

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REFERENCES

1. J. Ma, Y. Li, Q. Ye, J. Li, Y. Hua, D. Ju, D. Zhang, R. Cooper and M. Chang, *J. Agric. Food Chem.*, **2000**, *48*, 5220-5225.
2. Z. Huang, Y. Xu, L. Li and Y. Li, *J. Agric. Food Chem.*, **2008**, *56*, 112-118.
3. T. Akihisa, S. Mafune, M. Ukiya, Y. Kimura, K. Yasukawa, T. Suzuki, H. Tokuda, N. Tanabe and T. Fukuoka, *J. Nat. Prod.*, **2004**, *67*, 479-480.
4. T. Akihisa, H. Tokuda, K. Yasukawa, M. Ukiya, A. Kiyota, N. Sakamoto, T. Suzuki, N. Tanabe and H. Nishino, *J. Agric. Food Chem.*, **2005**, *53*, 562-565.
5. P. J. Blanc, M. O. Loret and G. Goma, *Biotech. Lett.*, **1995**, *17*, 291-294.
6. M. J. Cheng, M. D. Wu, I. S. Chen and G. F. Yuan, *Chem. Pharm. Bull.*, **2008**, *56*, 394-397.
7. P. Juzlová, L. Martínková and V. Kren, *J. Ind. Microbiol.*, **1996**, *16*, 163-170.

8. P. Juzlová, T. Rezanka, L. Martínková and V. Kren, *Phytochemistry*, **1996**, *43*, 151-153.
9. K. Sato, Y. Goda, S. S. Sakamoto, H. Shibata, T. Maitani and T. Yamada, *Chem. Pharm. Bull.*, **1997**, *45*, 227-229.
10. H. Nozaki, S. Date, H. Kondo, H. Kiyohara, D. Takaoka, T. Tada and M. Nakayama, *Agric. Biol. Chem.*, **1991**, *55*, 899-900.
11. S. Jongrungruangchok, P. Kittakoop, B. Yongsmith, R. Bavovada, S. Tanasupawat, N. Lartpornmatulee and Y. Thebtaranonth, *Phytochemistry*, **2004**, *65*, 2569-2575.
12. D. Wild, G. Tóth and H. U. Humpf, *J. Agric. Food Chem.*, **2002**, *50*, 3999-4002.
13. D. Wild, G. Tóth and H. U. Humpf, *J. Agric. Food Chem.*, **2003**, *51*, 5493-5496.
14. L. L. Li, J. P. Chen and L. Y. Kong, *Zhong Guo Tian Ran Yao Wu*, **2006**, *4*, 32-35.
15. W. Herz, P. S. Santhanam, and I. Wahlberg, *Phytochemistry*, **1972**, *11*, 3061-3063.
16. Y. M. Chiang and Y. H. Kuo, *Tetrahedron Lett.*, **2003**, *44*, 5125-5128.
17. C. Y. Chen, F. R. Chang, C. M. Teng, Y. C. Wu, *J. Chin. Chem. Soc.*, **1999**, *46*, 77-86.
18. D. N. Quang, T. Hashimoto, M. Tanaka, M. Baumgartner, M. Stadler and Y. Asakawa, *J. Nat. Prod.*, **2002**, *65*, 1869-1874.
19. V. Rukachaisirikul, J. Kaeobamrung, W. Panwiriyarat, P. Saitai, Y. Sukpondma, S. Phongpaichit and J. Sakayaroj, *Chem. Pharm. Bull.*, **2007**, *55*, 1383-1384.
20. Y. Qiu, Y. Chen, Y. Pei, H. Matsuda and M. Yoshikawa, *Chem. Pharm. Bull.*, **2002**, *50*, 1507-1510.
21. V. Rukachaisirikul, J. Kaeobamrung, W. Panwiriyarat, P. Saitai, Y. Sukpondma, S. Phongpaichit and J. Sakayaroj, *Chem. Pharm. Bull.*, **2007**, *55*, 1383-1384.
22. X. Li, M. K. Kim, U. Lee, S. K. Kim, J. S. Kang, H. D. Choi and B. W. Son, *Chem. Pharm. Bull.*, **2005**, *53*, 453-455.