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# CHEMICAL CONSTITUENTS ISOLATED FROM THE FUNGUS *Monascus* sp.

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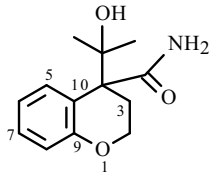
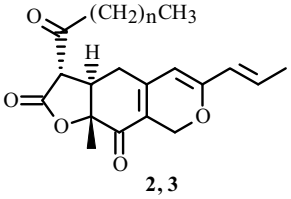
*Further study of a 95% EtOH extract of red mold rice fermented with the yellow mutant of the fungus* Monascus *sp. led to the isolation of one new chroman derivative, namely monascuskaochroman (**1**), together with nine known compounds. The structure of the new compound was determined as 4-(1-hydroxy-1-methyl-ethyl)-chroman-4-carboxylic acid amide. The known compounds were identified as monascin (**2**), ankaflavin (**3**), (3*R*,6*R*,7*E*)-(+)-3-hydroxy-4,7-megstigmadien-9-one (**4**), vanillin (**5**), syringic acid (**6**), 3,4,5-trimethoxybenzoic acid (7),* trans-*methyl* p-*coumarate (**8**), ferulic acid (**9**), and methyl* N-*methyl anthranylate (**10**).This is the first report of a naturally occurring chroman skeleton isolated from* Monascus *species. Compounds **4–10** 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 for structurally related compounds.*

**Keywords***: Monascus* sp., fungus, red mold rice, chroman, monascuskaochroman.

The filamentous fungi of the genera *Monascus* have been used for a long time as a meat colorant, meat preservative, health food, and in Chinese folk medicine [1, 2]. They comprise four representative species: *M. pilosus*, *M. purpureus*, *M. rubber*, and *M. anka*. These species belong to the class *Ascomycetes* and the family Monascaceae [1, 2]. 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 [3]. When the above-mentioned molds are grown on cooked rice, it can produce pigments and some bioactive metabolites. 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. *M. purpureus*, *M. anka*, and *M. pilosus* are representative natural colorants of the *Monascus* fungi traditionally used in East Asia as a source of pigments [2]. Red mold rice was a great discovery in ancient Chinese and is also used for medicinal purposes to aid digestion and blood circulation, strengthen the spleen, eliminate dampness, and remove blood stasis. *Monascus* can produce several secondary metabolites, such as six typical pigments, monacolins, γ-aminobutyric acid, and the mycotoxic cirinin [4]. These constituents are useful as food additives and pharmaceuticals, which has been reported for *Monascus* spp. [2].




**1**




**2, 3**

**2:** n = 4; **3:** n = 6

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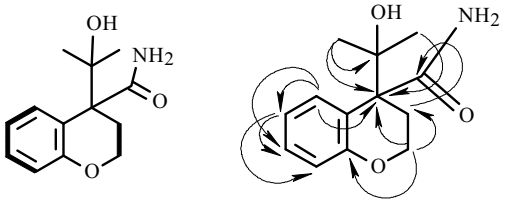


Fig. 1. Important COSY ( — ) and HMBC (H→C) correlations of **1**.

A variety of structurally diversified compounds, including polyketides [2], furanoisophthalides, amino acids [5, 6], azaphilones [3, 5–12], pyranoindole alkaloids [13], benzenoids [13], furans [13], steroids [13], and fatty acids [2] is widely distributed in the fungus *Monascus* species. Some major pigments of red mold rice and some secondary metabolites have been identified, but knowledge of their biological and toxicological effects is limited and partial. Some minor compounds like chroman derivatives or other types of compounds, except common pigments produced by *Monascus* sp., have received less attention. Consequently, characterization of the minor metabolites of red mold rice and their pharmacological mechanism or physiological effects is still obscure and deserving of further investigation. Careful examination of the EtOAc-soluble fraction of a 95% EtOH extract of red mold rice produced by *Monascus* sp. has resulted in the isolation of ten constituents, including a new chroman derivative, monascuskaochroman (**1**), together with nine known compounds. The structural elucidation of the new compound is described herein.

The EtOAc-soluble fraction of the 95% EtOH extract of red mold rice produced by *Monascus* sp. was fractionated by a combination of $SiO_2$, MPLC, as well as preparative TLC to afford 10 compounds (**1–10**), the structures of which were elucidated by 1D and 2D NMR spectra and comparison with literature data or authentic samples on a TLC plate. The new structure of **1** was identified according to the following spectroscopic evidence.

Compound **1** was obtained as a colorless optically inactive oil. $[\alpha]_D^{22}$ ±0° (*c* 0.05, $CHCl_3$). The HR-ESI-MS data determined the molecular formula to be $C_{13}H_{17}NO_3$ (*m/z* 258.1107 ($[M + Na]^+$; calcd 258.1106). Five indices of hydrogen deficiency (IHD) were determined from its $^{13}C$ NMR and DEPT spectra. UV absorption at 212 and 295 nm and IR absorption bands are due to the presence of hydroxyl (3400 $cm^{-1}$), amide amino (3300 $cm^{-1}$), a carbonyl (1695 $cm^{-1}$), and aromatic (1619, 1510) groups, suggesting the presence of a chroman nucleus [14, 15]. The presence of an amidocarbonyl (-$CONH_2$) group was revealed by IR absorption, along with a resonance signal in the $^{13}C$ NMR spectrum at δ 180.0.

The $^1H$ NMR spectrum of **1** showed four typical mutually coupling aromatic protons of chroman derivative at δ 6.85 (1H, dd, J = 8.4, 1.2 Hz, H-8), 7.04 (1H, td, J = 8.4, 1.2 Hz, H-6), 7.24 (1H, dd, J = 8.4, 1.2 Hz, H-7), and 7.30 (1H, dd, J = 8.4, 1.2 Hz, H-5), one $D_2O$ exchangeable $NH_2$ signal at δ 7.60 (2H, br.s, exchangeable with $D_2O$), two mutually coupled nonequivalent methylene H-atoms at δ 2.30 (1H, ddd, J = 14.8, 12.0, 2.4 Hz, $H_{ax}$-3), 2.67 (1H, ddd, J = 14.8, 9.0, 2.4 Hz, $H_{eq}$-3), 4.18 (1H, ddd, J = 14.4, 12.0, 2.4 Hz, $H_{ax}$-2), and 4.26 (1H, dd, J = 14.4, 9.0, 2.4 Hz, $H_{eq}$-2), and two methyl moieties attached to a 1-hydroxy-1-methyl-ethyl group observed at δ 1.04, 1.31 (each 3H, s, H-12, 13), indicating that **1** was probably a chroman derivative possessing a conjugated carbonyl group [14–18].

The $^{13}C$ NMR and DEPT spectrum showed that **1** had a total of 13 carbons for two Me, two $CH_2$, four aromatic CH, and five quaternary C atoms, including one C=O (δ 180.0). The carbon signals of **1** were assigned, from $^{13}C$ NMR, DEPT, and HSQC experiments, as two methyls at δ 23.5 (Me-13) and 24.2 (Me-12), two methylenes at δ 36.1 (C-3) and 64.4 (C-2), four aromatic olefinic carbons at δ 109.3 (C-8), 122.4 (C-6), 125.0 (C-5), and 128.5 (C-7), and four quaternary carbons at δ 70.2 (C-4), 84.8 (C-11), 135.2 (C-10), and 142.2 (C-9). The above data also point to a chroman derivative skeleton [14–18].

The above observation accompanied by the $^1H,^1H$-COSY, and HMBC (Fig. 1) spectra of **1** established the presence of the three partial substitutent fragments, **1a** (-$C(CH_3)_2(OH)$), **1b** (-$CONH_2$), and **1c** (chroman basic skeleton), for chroman derivative **1**. The entire skeleton of **1** was constructed with the aid of the HMBC spectrum (Fig. 1).

The $^1H$ and $^{13}C$ NMR long-range $^2J$ and $^3J$ HMBC correlations of the signal at $\delta_H$ 1.04 (Me-12) with the carbon signals at $\delta_C$ 84.8 (C-11) and 70.2 (C-4) helped to establish the connections of fragments **1a** at C-4. The significant correlations between $\delta_H$ 2.30/2.67 ($CH_2$-3) and $\delta_C$ 180.1 (C-14) helped to establish the connection of fragments **1b** and **1c** with C-4.

In addition, the key $^3J$ HMBC correlations (Fig. 1) from $\delta_H$ 7.30 (H-5) to $\delta_C$ 70.2 (C-4), $\delta_H$ 2.30/2.67 ($CH_2$-3) to $\delta_C$ 135.2 (C-10), and $\delta_H$ 4.18/4.26 ($CH_2$-2) to $\delta_C$ 142.2 (C-9) verify the junction of the dihydropyrano ring with the benzene moiety at C-9 and 10.

The other significant HMBC plot (Fig. 1) was also revealed by the $^{1}H$ and $^{13}C$ NMR long-range correlations between the H atoms at $\delta_H$ 7.30 (H-5) and the C atoms at $\delta_C$ 122.4 (C-6), and 128.5 (C-7), between the H-atoms at $\delta_H$ 7.04 (H-6) and the C-atoms at $\delta_C$ 128.5 (C-7) and 109.3 (C-8), between the H-atoms at $\delta_H$ 4.18/4.26 ($CH_2$-2) and the C atoms at $\delta_C$ 36.1 (C-3) and 70.2 (C-4), between the H atom at $\delta_H$ 2.30/2.67 ($CH_2$-3) and the C-atoms at $\delta_H$ 70.2 (C-4) and 180.0 (C-14), and between the H atom at $\delta_H$ 1.31 (Me-13) and the C atoms at $\delta_H$ 84.8 (C-11) and 70.2 (C-4).

The relative stereochemistry of the chiral center (C-4) of **1** was deduced mainly from a nuclear Overhauser enhancement spectroscopy (NOESY) experiment. The results showed that H-5 ($\delta_H$ 7.30) was within the NOE distance from the methyl proton Me-12 ($\delta_H$ 1.04) and one methine $H_{ax}$-3 ($\delta_H$ 2.30), suggesting that the H-3 ($\delta_H$ 2.30) and the methyl proton (Me-12) were located at the axial position. The $\beta$-orientation of the 1-hydroxy-1-methyl-ethyl group [-$C(Me)_2$(OH)] attached at C-4 was further supported by the NOESY experiment, which showed the axial-axial interactions between Me-12 ($\delta_H$ 1.04) and both $H_{ax}$-3 ($\delta_H$ 2.30) and $H_{eq}$-3 (2.67). Accordingly, the -$CONH_2$ moiety was undoubtedly located at C-4 and is occupied an $\alpha$-orientation.

Other significant NOE correlations were also observed between H-8 ($\delta_H$ 6.85) and $H_{ax}$-2 ($\delta_H$ 4.18), between $H_{eq}$-2 ($\delta_H$ 4.26) and $H_{ax}$-3 ($\delta_H$ 2.30)/$H_{eq}$-3 ($\delta_H$ 2.67), between $H_{ax}$-2 ($\delta_H$ 4.18) and $H_{eq}$-3 ($\delta_H$ 2.67), and between Me-13 ($\delta_H$ 1.31) and both $H_{ax}$-3 ($\delta_H$ 2.30)/$H_{eq}$-3 ($\delta_H$ 2.67).

On the basis of the above data, the structure of compound **1** was thus deduced as (4*RS*)-4-(1-hydroxy-1-methyl-ethyl)-chroman-4-carboxylic acid amide and named monascuskaochroman.

The other known isolates, monascin (**2**) [19], ankaflavin (**3**) [19], (3*R*,6*R*,7*E*)-(+)-3-hydroxy-4,7-megstigmadien-9-one (**4**) [20], vanillin (**5**) [21], syringic acid (**6**) [21], 3,4,5-trimethoxybenzoic acid (**7**) [21], *trans*-methyl *p*-coumarate (**8**) [21], ferulic acid (**9**) [21], and methyl *N*-methyl anthranylate (**10**) [22] were readily identified by comparison of their spectral data (UV, IR, $^{1}H$ NMR, MS) with the corresponding data in the literature.

A review of the past literature regarding *Monascus* species [1, 3–13] reveals that azaphilone, furanoisophthalides, amino acid, and polyketides are the major metabolites. In our previous studies, we have reported over 15 metabolites, together with their DPPH free radical scavenging activity, from the mycelium of *M. pilosus* [13]. In the course of our search for potential nitric oxide (NO) inhibitors from natural sources, *Monascus* sp. has been found to be one of the active species. In this study, we focused on the minor secondary metabolites in the EtOAc-soluble fraction of a 95% EtOH extract of red mold rice produced by *Monascus* sp. The metabolite **1** found in this study is a novel, naturally occurring compound. It is interesting to note that this is the first report of a chroman derivative isolated from *Monascus* species. Among them, all known compounds, except **2** and **3**, were isolated for the first time from *Monascus* species. A comparison with the previous studies [1, 3–13] suggests that *Monascus* has distinct and diverse secondary metabolites, which arise under different fermentation conditions. It may be possible to find more novel constituents by cultivating *Monascus* under different cultivation conditions.

## EXPERIMENTAL

**General Experimental Procedures**. 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 (KBr or 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 (400 MHz for $^{1}H$ NMR, 100 MHz for $^{13}C$ NMR) spectrometer. Chemical shifts were internally referenced to the solvent signals in $CDCl_3$ ($^{1}H$, $\delta$ 7.26; $^{13}C$, $\delta$ 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 preparative TLC.

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

**Cultivation and Preparation of Red Yeast Rice**. *Monascus* sp. was maintained on potato dextrose agar (PDA; Difco). The strain was cultured on PDA slants at 25°C for 6 days, and the spores were harvested using sterile water. The spores ($5 \times 10^5$) 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 obtained by mixing 100 mL mycelium broth and 100 mL RGY medium was inoculated into plastic boxes (25 cm × 30 cm) containing 1.2 kg sterile rice and cultivated at 25°C to produce 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 30 days of cultivation, the RMR was harvested and lyophilized for the extraction of metabolites.

**Extraction and Isolation**. Red mold rice of the *Monascus* sp. (1.5 kg) was 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, 3.4 g) and $H_2O$ (fraction B, 18.2 g) soluble fractions. The EtOAc-soluble fraction (3.4 g) was chromatographed over silica gel (70–230 mesh), eluting with *n*-hexane, and enriched with EtOAc to produce 7 fractions (A1–A7). Fraction A-2 (150 mg) was subjected to CC ($SiO_2$, 230–400 mesh; *n*-hexane–EtOAc, 8:1) to obtain (3*R*,6*R*,7*E*)-(+)-3-hydroxy-4,7-megstigmadien-9-one (**4**) (1.8 mg) and vanillin (**5**) (2.7 mg). Fraction A-2 (750 mg) was chromatographed on silica gel (230–400 mesh), employing *n*-hexane containing increasing amounts of acetone as elutent to produce 10 fractions (A-2-1–A-2-10). Fraction A-2-5 (130 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 5:1) to afford monascin (**2**) (5.3 mg) and monascuskaochroman (**1**) (5.0 mg). Fraction A-3 (1.4 g) was chromatographed over silica gel, eluting with $CH_2Cl_2$–MeOH (10:1) to obtain 16 fractions (A-3-1–A-3-16). Fraction A-3-3 (96 mg) was repeatedly purified by preparative TLC (*n*-hexane–acetone, 1:1) to afford ankaflavin (**3**) (6.8 mg), methyl *N*-methyl anthranylate (**10**) (2.1 mg), and 3,4,5-trimethoxybenzoic acid (**7**) (1.4 mg). Fraction A-3-15 (44 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 2:1) to give *trans*-methyl *p*-coumarate (**8**) (2.8 mg). Fraction A-6 (1.5 g) was resubjected to silica gel column chromatography ($CH_2Cl_2$–MeOH, 15:3) to afford 10 fractions (A-6-1–A-6-10). Fraction A-6-8 (74 mg) was repeatedly purified by preparative TLC ($CH_2Cl_2$–MeOH, 10:1) to afford ferulic acid (**9**) (1.9 mg). Fraction A-7 (850 mg) was resubjected to silica gel column chromatography ($CH_2Cl_2$–MeOH, 15:1) to afford 4 fractions (A-7-1–A-7-4). Fraction A-7-1 (114 mg), eluting with $CH_2Cl_2$–acetone 10:1, was further separated using preparative TLC (*n*-hexane–EtOAc, 2:1) to yield syringic acid (**6**) (3.2 mg).

**Monascuskaochroman (1)**. Colorless oil; $[\alpha]_D^{22}$ ±0° (*c* 0.05, $CHCl_3$). UV (MeOH, nm): 212 (4.25), 295 (3.60). IR (Neat, $cm^{-1}$): 3400 (OH), 3300 (NH), 1695 (C=O), 1619, 1510 (benzene ring). ESI-MS *m/z* 258 $[M + Na]^+$. HR-ESI-MS *m/z* 258.1107 $[M + Na]^+$ (calcd for $C_{13}H_{17}NO_3Na$, 258.1106). $^1H$ NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.04 (3H, s, H-12), 1.31 (3H, s, H-13), 2.30 (1H, ddd, J = 14.8, 12.0, 2.4, $H_{ax}$-3), 2.67 (1H, ddd, J = 14.8, 9.0, 2.4, $H_{eq}$-3), 4.18 (1H, ddd, J = 14.4, 12.0, 2.4, $H_{ax}$-2), 4.26 (1H, ddd, J = 14.4, 9.0, 2.4, $H_{eq}$-2), 6.85 (1H, dd, J = 8.4, 1.2, H-8), 7.04 (1H, td, J = 8.4, 1.2, H-6), 7.24 (1H, dd, J = 8.4, 1.2, H-7), 7.30 (1H, dd, J = 8.4, 1.2, H-5), 7.60 (2H, br.s, NH). $^{13}C$ NMR (100 MHz, $CDCl_3$, δ): 23.5 (C-13), 24.2 (C-12), 36.1 (C-3), 64.4 (C-2), 70.2 (C-4), 84.8 (C-11), 109.3 (C-8), 122.4 (C-6), 125.0 (C-5), 128.5 (C-7), 135.2 (C-10), 142.2 (C-9), 180.0 (C-14).

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