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ARTICLE

Isolation and Structural Characterization of Two New Metabolites from *Monascus*

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Two new pale yellow metabolites have been isolated from commercially available Chinese food additive Red Monascus Pigment and from *Monascus ruber* culture broth. They were isolated by successive TLC and semipreparative HPLC. Their structural characterization was elucidated by a variety of spectroscopic techniques (UV, IR, NMR) and mass spectrometry. These two new metabolites present numerous similarities with monascorubrin and rubropunctatin, differing in their structure only by the absence of the lactone ring. High-resolution mass spectrometry indicated the molecular formulas $C_{20}H_{26}O_4$ and $C_{22}H_{30}O_4$. The new compounds, named monarubrin and rubropunctin, contain a propenyl group on a pyrone ring, an alkyl side chain, but no γ-lactone ring. The new metabolites have the property of producing strong blue fluorescence at 340 nm.

KEYWORDS: *Monascus*; metabolites; new pigments; Red Yeast Rice; monascorubrin; rubropunctatin

INTRODUCTION

Red yeast rice also known as koji, anka, and angkak was obtained by cultivation of *Monascus* species on rice grains. In certain region of Asia such as Taiwan, China, and Japan, anka has been used for hundreds of years as one of the starter cultures for brewing of red rice wine and as a natural food colorant (*1*). This food colorant is a source of various secondary metabolites of typical polyketide structures (*2*). Six well-known and structurally characterized metabolites were produced by this fungus and cover a range of colors: yellow, monascin **1** (*3*, *4*) and ankaflavin **2** (*5*); orange, rubropunctatin **3** and monascorubrin **4**; red-purple, rubropunctamine **5** and monascorubramine **6** (*6*) (**Figure 1**).

The red and yellow metabolites find applications as natural food additives. The orange metabolites possess a structure with a high affinity for primary amino groups, and the reaction with amino acids yields water-soluble red metabolites. Therefore, various water-soluble *Monascus* coloring agents have been developed (*7*−*11*).

In recent years, several new metabolites and pigments have been isolated from red yeast rice and, their chemical structures have been characterized as ankalactone (*12*), monascolidone (*13*), monascopyridine (*14*), monasfluore (*15*), monascusone (*16*), new red pigment (*17*), and monaspurpurone (*18*). Various biological activities as embryotoxicity, teratogenicity, immunosuppressive properties, antioxidant properties, and antibiotic and cytotoxic activities of oligoketide *Monascus* metabolites have been evaluated (*19*−*27*).

In this investigation we undertook the isolation of two new metabolites from the fungus *Monascus*, their purification, and their characterization using a variety of spectroscopic techniques and mass spectrometry.

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MATERIALS AND METHODS

Reagents. Acetonitrile [high-performance liquid chromatography (HPLC) grade] was obtained from Carlo Erba (Val de Rueil, France). All other chemicals were of analytical grade from Aldrich (Saint-Quentin Fallavier, France) and were not further purified.

Material, Microorganism, and Growth Conditions. The sample used was a commercial powder (Red Monascus Pigment) elaborated from a supplier food additive (Jiangmen Ke Lone Biotechn. CO, LTD, Guangdong, China). The strain used is a high-pigment-producing strain obtained from anka, *Monascus ruber* ATCC 96218. The fungus was grown in shake flasks and reactors with synthetic medium as described previously (*11*). The chemically defined fermentation medium contained (grams per liter of distilled water) glucose, 20; monosodium glutamate, 5; K_2HPO_4, 5; KH_2PO_4, 5; $CaCl_2$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and $MnSO_4 \cdot H_2O$, 0.03. The initial pH of the medium was adjusted to 6.5 with phosphoric acid (H_3PO_4). The stock culture was kept on potato dextrose agar (Difco). Spores were prepared by growth on potato agar slants for 10 days at 28 °C. The spores were washed with sterile water. A suspension of 10^8 spores was used to inoculate a 1 L baffled Erlenmeyer flask containing 200 mL of inoculum medium, which was incubated at 28 °C for 2 days. This inoculum was then transferred to a 2 L fermentor containing 1.6 L of synthetic medium. The culture was incubated at 30 °C at an aeration rate of 0.4 vol/vol/min, and the agitation speed was increased from 250 to 600 rpm.

Thin-Layer Chromatography (TLC). Analytical TLC was performed on 5 × 10 cm, 0.25 mm thick layer, silica gel 60 F254 plates (Merck, Darmstadt, Germany). Preparative TLC was performed on 20 × 20 cm, 2 mm thick layer, silica gel 60 F254 plates (Merck, Darmstadt, Germany). Chloroform/acetone (9/1) and petroleum ether/diethyl ether (5/5) were used successively as the solvent. Monarubrin and rubropunctin revealed a blue fluorescence under ultraviolet lamp.

Semipreparative HPLC. Semipreparative HPLC was performed using a pump (Waters 510), an automatic injector (ICS 508), and a fluorimetric detection system (ICS GROTON FD 500). Chromatographic separation was achieved at 25 °C using a 200 mm × 20 mm i.d., 5 μm Nucleosil C_{18} column from Macherey Nagel

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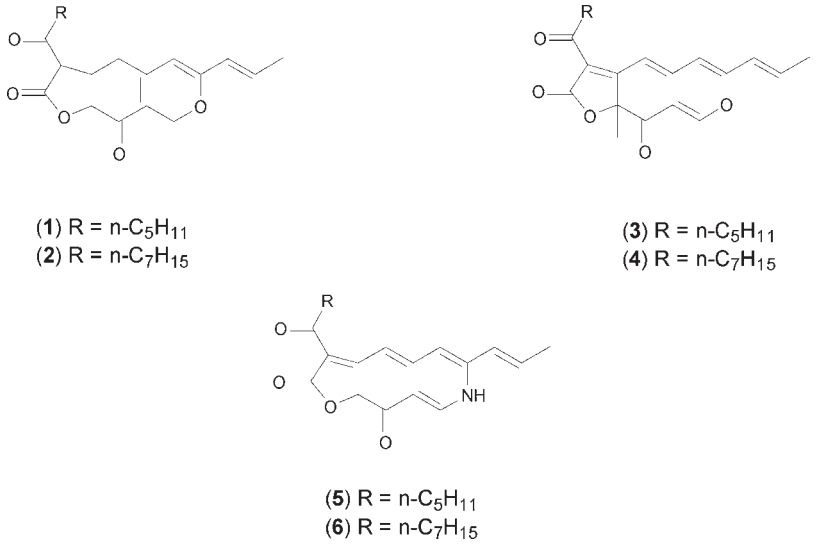


Figure 1. Structures of the yellow (monascin (**1**), ankaflavin (**2**)), orange (rubropunctatin (**3**), monascorubrin (**4**)), and red-purple (rubropunctamine (**5**), monascorubramine (**6**)) pigments of *Monascus*.

(Hoerd, France) with isocratic elution of acetonitrile/water (8/2) at a flow rate of 2 mL/min.

Isolation and Purification of Metabolites from a Commercial *Monascus* Coloring Powder. Ten grams of a commercial *Monascus* coloring powder was extracted in a shaken flask for 2 h with 500 mL of a mixture of chloroform/methanol (1/1). The mixture was suction-filtered and washed with 100 mL of chloroform/methanol (1/1). The extracts were concentrated using a vacuum rotary evaporator. The residue was suspended in a chloroform/acetone (9/1) mixture, subjected to a silica gel (130-270 mesh) column, and eluted with chloroform/acetone (9/1). The fractions with strong blue fluorescence were combined to give a main fraction according to TLC, and the solvent was removed under reduced pressure. The fraction was dissolved in a minimal amount of chloroform/acetone (9/1) mixture and applied to the preparative TLC plate as a thin layer horizontally. Mixtures of chloroform/acetone (9/1) and petroleum ether/diethyl ether (5/5) were used successively as eluents. The spots with strong blue fluorescence (Rf = 0.84 and Rf = 0.88) were scraped off and dissolved in a minimal amount of methanol. The silica gel was removed by filtration through filter paper, and the methanol was removed under reduced pressure. To further purify the two new metabolites, the methanol solution was fractionated to obtain monarubrin and rubropunctin by semipreparative HPLC. After evaporation of the eluent (acetonitrile/acetone), 12 mg of monarubrin and 8 mg of rubropunctin were obtained.

Isolation and Purification of Metabolites from Fermentation Broth (*10*). The mycelium was separated from the culture broth by filtration through a M14 membrane (0.8 μm pore size; Tec-sep, Bollene France), and then the filtrate was lyophilized. The crude extract was recovered with deionized water, and this solution was extracted with chloroform 3 × 100 mL. The extracts were concentrated using a vacuum rotary evaporator and treated exactly as described above.

Nuclear Magnetic Resonance Spectroscopy (NMR). For NMR spectroscopy (^{1}H (400.13 Hz) and ^{13}C (100.612 Hz), a Bruker-ARX 400 spectrometer equipped with an ultrashim system was used. Samples were studied as solutions in $CDCl_3$ at 25 °C. Total assignments of the carbon and proton signals were made through COSY, HMQC, and HMBC experiments.

HPLC-Mass spectrometry. Mass spectra were recorded on an API 365 PESciex instrument. The mass detector was a triple quadripole equipped with an electrospray ionization interface and controlled by Analyst software.

High-Resolution Mass Spectrometry (HRMS). The mass spectra were recorded on a high-resolution Autospec Micromass apparatus with a triple sector double focusing instrument using a 20 keV cesium beam. Nitrobenzyl alcohol was used as matrix.

IR Spectra. The infrared absorption spectra were measured on a Perkin-Elmer model 1310 spectrophotometer. The dried crystal of the new red pigment was prepared using a KBr method.

UV Spectra. The maximum absorbance of the new red pigment was determined by UV, and the spectra were recorded with a Hewlett-Packard 8453 spectrophotometer.

Fluorescence Spectra. The fluorescence spectra were recorded with a SAFAS-FLX instrument.

Monarubrin (7): 1-[7,8-Dihydro-7-hydroxy-7-methyl-8-oxo-3-(1*E*)-1-propenyl-6*H*-2-benzopyran] Heptan-2-one. Electrospray LC/MS: *m*/*z* 331.2 [MH^+] $C_{20}H_{26}O_4$. HRMS: Anal. calcd for $C_{20}H_{26}O_4$: 331.1904 [MH^+]; found, 331.19021. $[\alpha]_D$ = +0.044 (*c* = 0.909 g/L MeOH). IR (KBr) cm^{-1}: 3470, 1710 (side chain ketonic carbonyl), 1666, 1620 (unsaturated ketone), 958. UV λ_{max} (MeOH) = 360 nm. Fluorescence: excitation, 340; emission, 490. TLC solvent systems: chloroform/acetone (9/1), Rf = 0.85; petroleum ether/diethyl ether (5/5), Rf = 0.37.

Rubropunctin (8): 1-[7,8-Dihydro-7-hydroxy-7-methyl-8-oxo-3-(1*E*)-1-propenyl-6*H*-2-benzopyran] Undecan-2-one. Electrospray LC /MS: *m*/*z* 359.2 [MH^+] $C_{22}H_{30}O_4$. HRMS: Anal. calcd for $C_{22}H_{30}O_4$: 359.22224 [MH^+]; found, 359.22177. $[\alpha]_D$ = +0.0272 (*c* = 6.36 g/L MeOH). IR (KBr) cm^{-1} = 3470, 1710, 1666, 1620, 958. UV λ_{max} (MeOH) = 360 nm. Fluorescence: excitation, 340; emission, 490. TLC solvent systems: chloroform/acetone (9/1), Rf = 0.88; petroleum ether/diethyl ether (5/5), Rf = 0.40.

RESULTS AND DISCUSSION

Isolation and Purification of the New Compounds. The two new oligoketides were isolated from a commercial *Monascus* coloring agent obtained from a Chinese manufacturer of food additives and also from supernatants of several cultures of *Monascus ruber* (ATCC 96218). The extraction of metabolites and chromatography of the crude extract on silica yielded a yellow extract that contained monascin, ankaflavin, and the two new compounds. The new compounds were separated from monascin and ankaflavin by preparative TLC and purified by semipreparative HPLC. They present a difference in retention time of about 3 min (**Figure 2**).

Structural Analysis of the New Compounds. The new compounds were analyzed by high-performance liquid chromatography coupled with a mass spectrometer (LC/MS) and found to have a molecular weight of 331 for **7** and 359 for **8**. The IR spectra of metabolites **7** and **8** were similar and indicated the presence of an alcohol at 3470 cm^{-1}, a saturated ketone at 1710 cm^{-1}, a cyclic cyclohexenone carbonyl vibration at 1675 cm^{-1}, a conjugated diene at 1620 cm^{-1}, and a *trans* double bond at 958 cm^{-1}. These results revealed that the two novel metabolites shared a similar chemical structure and that they were significantly different from

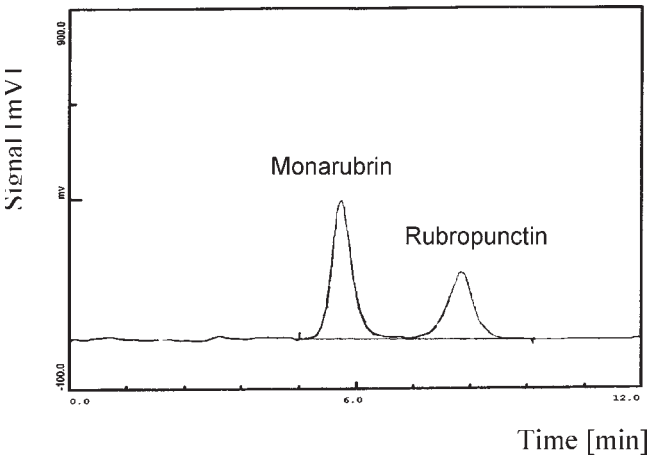


Figure 2. HPLC chromatogram of monarubrin (**7**) and rubropunctin (**8**) detected via a fluorophotometric detector.

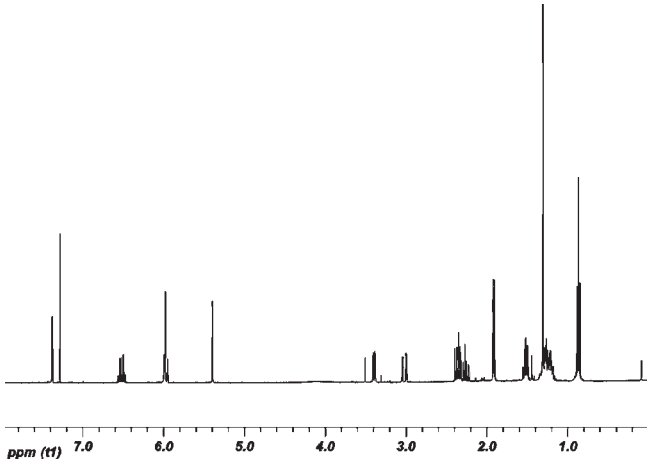


Figure 3. ^{1}H NMR spectrum of the new *Monascus* red pigment: monarubrin (**7**).

both monascin and ankaflavin, since no band corresponding to the lactone carbonyl vibration at 1760 cm^{-1} was obtained, indicating the absence of the lactone ring.

The absence of the lactone ring was also confirmed for both compounds by the absence of the signal corresponding to the carbonyl group of the lactone by ^{13}C NMR at 171 ppm. The molecular formulas of the compounds were determined by high-resolution mass spectrometry to be $C_{20}H_{26}O_4$ and $C_{22}H_{30}O_4$, respectively. The difference of 28 amu is due to the presence of two methylene units in the alkyl chain as confirmed by NMR analysis in which two carbons with chemical shifts of 29.25 and 29.29 ppm were observed.

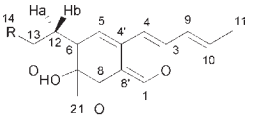
The complete structure of monascorubrin and rubropunctin was established by analysis of ^{1}H, ^{13}C, DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC NMR spectra. In the ^{1}H NMR spectrum (**Figure 3**), the methyl signals at δ 0.86, 1.31, and 1.91 ppm showed triplet, singlet, and doublet multiplicities, respectively, in accordance with the presence of CH_2, quaternary C, and CH neighbors. Using the ^{1}H−^{1}H COSY experiment, the correlations starting from the CH_3 signal at δ 0.86 ppm allowed for the identification of a $(CH_2)_4CH_3$ moiety. The methyl group at δ 1.91 ppm formed a separate spin system together with the protons of a *trans*-(*E*)-disubstituted carbon−carbon double bond, and this allowed the identification of a propenyl group.

The ^{13}C NMR spectrum of monarubrin showed the presence of 20 carbon atoms. The signals at δ 210.09 and 198.56 ppm were characteristic for ketone C=O and conjugated ketone, respectively. The peaks at 146.19, 134.55, 123.29, 107.10, and 103.92 ppm were assigned to =CH, the peak at 40.68 ppm was assigned to CH (C6), the signals at 155.66, 146.19, and 120.26 ppm were assigned to quaternary sp^2 carbons atoms, and the signal at 73.30 ppm to quaternary sp^3 carbon atoms.

The HMQC spectroscopic data of monarubrin were used to assign protons attached to their corresponding carbons (**Table 1**). The complete structure was established by the HMBC spectrum, which shows correlation between protons and carbons for two or three bonds. On the basis of these spectroscopic data, the

Table 1. NMR Analysis of 7 and 8: ^{1}H (400 MHz) and ^{13}C (100 MHz)

	7		8	
carbon	δ_C	δ_H	δ_C	δ_H
1	146.03	7.38 s	146	7.38 s
3	155.66		155	
4	107.10	5.97, s	107	5.97, s
4′	120.26		146	
5	103.92	5.39, s	103	5.39
6	40.68	3.38 ($J_{H12a-H6}$ = 3 Hz, $J_{H12b-H6}$ = 10 Hz), dd	40.7	3.38 ($J_{H12a-H6}$ = 3 Hz, $J_{H12b-H6}$ = 10 Hz), dd
7	73.30		73	
8	198.56		198	
8′	146.19		146	
9	123.29	5.97 (J_{H9-H11} = 1.7 Hz, J_{H9-H10} = 15.4 Hz), dq	123	5.97 (J_{H9-H11} = 1.7 Hz, J_{H9-H10} = 15.4 Hz), dq
10	134.55	6.51 ($J_{H10-H11}$ = 7 Hz, J_{H9-H10} = 15.4 Hz), dq	134	6.51 ($J_{H10-H11}$ = 7 Hz, J_{H9-H10} = 15.4 Hz), dq
11	18.70	1.91 ($J_{H10-H11}$ = 7 Hz, J_{H9-H11} = 1.7 Hz), dd	18.69	1.91 ($J_{H10-H11}$ = 7, J_{H9-H11} = 1.7 Hz), d
12a	40.72	2.35 (Ha), dd ($J_{H12a-H12b}$ = 18 Hz, $J_{H12a-H6}$ = 10 Hz)	40.7	2.35 (Ha), dd ($J_{H12a-H12b}$ = 18 Hz, $J_{H12a-H6}$ = 10 Hz)
12b	40.72	3 (Hb) ($J_{H12a-H12b}$ = 18 Hz, $J_{H12b-H6}$ = 3 Hz)	40.7	3 (Hb) ($J_{H12a-H12b}$ = 18 Hz, $J_{H12b-H6}$ = 3 Hz)
13	210.09		210	
14	43.68	2.39, t	43.71	2.39, t
15	23.64	1.51, q	23.00	1.51, q
16	31.51	1.25, m	29.25	1.25, m
17	22.62	1.26, m	29.29	1.23, m
18	14.09	0.86, t	31.85	1.26, m
19			22.72	1.23, m
20			14.27	0.87, t
21	27.03	1.31, s	27.03	1.31, s
OH		8.7, s		8.2, s



(**7**) Monarubrin R = n-C_5H_{11}
(**8**) Rubropunctin R = n-C_7H_{15}

Figure 4. Structure and atom numbering of the two new metabolites: monarubrin (**7**) and rubropunctin (**8**).

structure of monarubrin (**7**) was therefore identified as 1-[7, 8-dihydro-7-hydroxy-7-methyl-8-oxo-3-(1*E*)-1-propenyl-6*H*-2-benzopyran] heptan-2-one (**Figure 4**). Rubropunctin (**8**), an analogue of monarubrin with 28 units less on the side chain, showed identical UV and fluorescence spectra that indicated the presence of the same chromophore. The NMR data confirmed that rubropunctin is a lower homologue of monarubrin, with less lipophilic pentyl instead of heptyl side chains. The presence of metabolites with a pentyl or heptyl side chain is well-known from the pigments of *Monascus*.

This is the first report of metabolites with such chemical structures from a *Monascus* species. The already large range of metabolites produced by this fungi can therefore be even more diverse than was previously thought. We suppose that these new metabolites are new pigments or intermediates in the biodegradation of the known pigments (monascorubrin and rubropunctatin). The two new molecules with polyketide structures were shown to be present in commercially available food additives prepared from *Monascus* as well as in culture supernatants from this fungus. In the light of the known biological activities of some of *Monascus* metabolites in red yeast rice (angkak), it will be interesting to determine whether these novel metabolites also possess biological characteristics susceptible to provoke health hazards or benefits.

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