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New *Monascus* Metabolite Isolated from Red Yeast Rice (Angkak, Red Koji)

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Red yeast rice (angkak, red koji) obtained as cultures of *Monascus purpureus* on rice was extracted and analyzed by HPLC. In addition to the known red, orange, and yellow pigments and the mycotoxin citrinin, a new *Monascus* metabolite was detected. It is present in the original red yeast rice and formed in higher amounts when red yeast rice is heated. High-resolution mass spectrometry indicated the molecular formula $C_{15}H_{12}O_4$. The chemical structure was elucidated by analysis of NMR data. The new compound, named monascodilone, is characterized by a propenyl group on a pyrone ring, an aromatic ring, and a γ-lactone group.

KEYWORDS: Monascus; fungi; red yeast rice; metabolite; RPHPLC; HRMS; HPLC−MS/MS; NMR; chemical structure

INTRODUCTION

Red yeast rice, also known as "red koji" or "angkak", obtained as cultures of 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 soybean cheese, meat, meat products, and fish. It is also used as a folk medicine (*1*). Several yellow, orange, and red pigments and some nonpigment compounds of *Monascus* have been isolated, and their chemical structures have been established (*2*, *4*−*7*). However, a great number of *Monascus* metabolites have not yet been characterized chemically, and the knowledge of pharmacological and toxicological properties of most *Monascus* metabolites is fragmentary (*2*, *3*, *8*−*11*). Only recently, the formation of the mycotoxin citrinin by *Monascus* has been reported (*12*). We are studying the constituents of red yeast rice and herein report a method for the analysis of soluble components of red yeast rice, and the isolation and chemical structure of a new metabolite of *Monascus purpureus*.

MATERIALS AND METHODS

Reagents. Acetonitrile (HPLC grade) and phosphoric acid (analytical grade) were obtained from Merck (Darmstadt, Germany), and citrinin was obtained from Sigma-Aldrich (Steinheim, Germany). HPLC-grade water was prepared using a Seralpur PRO90C (Seral-Elga, Ransbach-Baumbach, Germany).

NMR Spectroscopy. NMR spectra were recorded in $CDCl_3$ using a Bruker Avance DRX-600 or a DRX-500 spectrometer, operating at 600 or 500 MHz (^{1}H) and 150.9 MHz (^{13}C). Chemical shifts are given on the δ-scale and were referenced to TMS. Pulse programs for the 1D and 2D NMR experiments were taken from the Bruker software library.

For structure elucidation and NMR signal assignment ^{1}H, ^{13}C, H,H−COSY, H,C−HMQC, H,C−HMBC (optimized to $J_{C,H}$ = 5 Hz), and H,H−NOESY (mixing time = 800 ms) spectra were recorded.

HPLC−ESI−MS/MS Analysis. Mass spectrometry (HPLC−MS/MS) was performed on a Finnigan triple-stage quadrupole TSQ 7000 HPLC−MS/MS system (Finnigan MAT, Bremen, Germany) with electrospray ionization (ESI) as interface in the positive ion mode. For HPLC, an Applied Biosystems (Foster City, CA) model 140B pump was used. For ESI, the spray capillary voltage was set to 3.5 kV and the temperature of the heated inlet capillary was set to 200 °C. Nitrogen served both as sheath gas (482 MPa) and as auxiliary gas. HPLC was carried out on a 100 × 2.1 mm i.d., 3.5 μm, XTerra MS C18 column (Waters) with a linear gradient of water (0.05% TFA)/acetonitrile (0.05% TFA) at a flow rate of 0.2 mL/min from 10 to 100% acetonitrile in 7 min. Positive ions were detected scanning from *m*/*z* 200 to 600 with a total scan duration of 1.0 s. The MS/MS experiments were performed at a collision energy of 5−20 eV with argon (0.24 Pa) serving as collision gas, scanning a mass range from *m*/*z* 100 to 400.

EIMS and EIHRMS. Mass spectra were measured on a MAT90 (Finnigan MAT) instrument.

Thin-Layer Chromatography. HPTLC was performed on 5 × 5 cm silica gel 60 plates (Merck, Darmstadt, Germany); chloroform/methanol (9:1) was used as solvent. Monascodilone revealed a blue fluorescence under black light (R_f 0.67).

Organism, Preparation, and Extraction of Red Yeast Rice. *Monascus purpureus* DSM1379 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braun-

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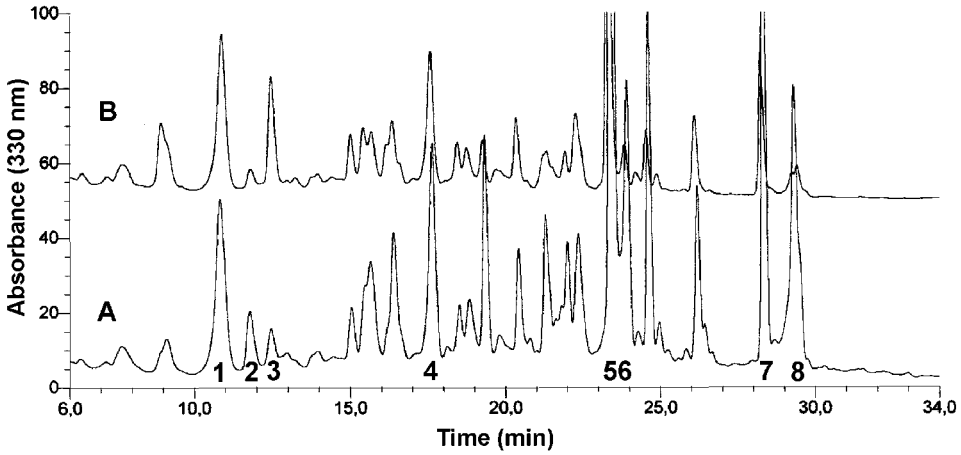

Figure 1. Chromatogram of two extracts of red yeast rice. Lower plot (A) extract of original red yeast rice; upper plot (B) extract of red yeast rice heated 30 min at 90 °C: **1**, rubropunctamin; **2**, citrinin; **3**, monascodilone; **4**, monascorubramin; **5**, monascin; **6**, rubropunctatin; **7**, ankaflavin; **8**, monascorubrin; $\lambda = 330$ nm.

schweig, Germany) and cultured in Petri dishes at 30 °C on autoclaved ground rice. Cultures were incubated for 3 weeks, dried at 40 °C, and pulverized. A 0.1-g aliquot of the red yeast rice so obtained was extracted for analytical purposes by sonication with 2.0 mL of a mixture of 0.25 M phosphoric acid and acetonitrile (1:1) and centrifugation to obtain a clear supernatant which was directly used for HPLC. Extracts for the preparative fractionation of red yeast rice and isolation of monascodilone were prepared similarly but using acetonitrile only.

HPLC Analysis. A Gynkotek M480 pump, sampler GINA50, and diode array detector, combined with Chromeleon software (Dionex Co., Idstein, Germany) were employed. Reverse-phase HPLC analyses were run on a 250 × 4.6 mm i.d. TSKgel ODS-80TM column (TosoHaas, Stuttgart, Germany) with a gradient of (A) 0.25 M phosphoric acid and acetonitrile (1:1), and (B) acetonitrile; 0−5 min, 100% A; 5−28 min, 0−80% B; 28−34 min, 80% B; and a flow of 1 mL/min. Chromatograms were recorded at the wavelengths 330, 390, 470, and 528 nm which correspond to characteristic absorption maxima of monascodilone and citrinin, and the yellow, orange, and red pigments, respectively. This allows differentiation of compounds even when their peaks overlap.

Isolation of Monascodilone. Red yeast rice (1 g) was autoclaved (121 °C, 20 min), dried over silica gel, and extracted with 2.5 mL of acetonitrile by sonication and centrifugation. The supernatant was fractionated to obtain monascodilone by preparative HPLC on a 300 × 7.8 mm i.d. TSKgel ODS-80TM column (TosoHaas, Stuttgart, Germany) using a gradient corresponding to the one described above but using water/acetonitrile (40:60) instead of phosphoric acid/acetonitrile, and a flow of 3 mL/min. At these conditions, monascodilone eluted at 8.2 min with UV absorption maxima at 236 and 326 nm. After repeated purification by HPLC it formed colorless needles upon evaporation of acetonitrile and water: mp 45 °C; $[\alpha]_D^{25}$ +16 (c 0.2, MeOH).

^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.68 (3H, d, $J = 6.8$ Hz, H-10), 1.95 (3H, dd, $J = 7.0$, 1.7 Hz, H-13), 5.62 (1H, q, $J = 6.8$ Hz, H-1), 6.10 (1H, dq, $J = 15.4$, 1.7 Hz, H-11), 6.30 (1H, s, H-8), 6.76 (1H, dq, $J = 15.4$, 7.0 Hz, H-12), 7.36 (1H, s, H-9), 8.78 (1H, s, H-4).

^{13}C NMR ($CDCl_3$, 150.9 MHz) δ 168.5 (C-3), 160.8 (C-5), 155.8 (C-9a), 154.9 (C-7), 142.5 (C-8a), 134.8 (C-12), 128.9 ($^1J_{C,H} = 170$ Hz, C-4), 125.0 (C-3a), 122.8 (C-11), 121.7 (C-4a), 118.2 ($^1J_{C,H} = 163$ Hz, C-9), 102.7 (C-8), 77.2 ($^1J_{C,H} = 155$, C-1), 20.4 ($^1J_{C,H} = 128$ Hz, C-10), 18.5 ($^1J_{C,H} = 128$ Hz, C-13).

ESIMS, *m*/*z* (%) 257 (28), $[M + H]^+$, 298 (100), $[M + H +$ acetonitrile$]^+$; ESI MS/MS of *m*/*z* 298 (10 eV): *m*/*z* (%) 257 (100), $[M + H]^+$.

EIMS, *m*/*z* (%) 256 (100, M^+), 241 (18), 228 (35), 213 (24), 185 (28), 157 (5), 128 (10), 69 (9), 41 (6).

HRMS, *m*/*z* 256.0734 (calculated for $C_{15}H_{12}O_4$: 256.0736).

Reaction of Monascodilone with Ammonia. A total of 20 μg of monascodilone in 260 μL of acetonitrile was mixed at room temperature with 50 μL of 25% aqueous ammonia. After 30 min the product was isolated by isocratic HPLC (acetonitrile/water, 60:40; 15 min, 3 mL/min; retention time 4.7 min). Under the conditions of the phosphoric acid/acetonitrile gradient HPLC analysis described above, the product eluted at 5.9 min. ESI MS, *m*/*z* (%) 256 (35, $[M + H]^+$), 297 (100, $[M + H +$ acetonitrile$]^+$). ESI MS/MS of *m*/*z* 297 (−10 eV) *m*/*z* (%) 256 (100, $[M + H]^+$).

RESULTS AND DISCUSSION

Pigments produced by *Monascus* species have been isolated and their chemistry has been studied since 1926 (*13*); however, nonpigment compounds have received less attention. Because cultures of *Monascus*, in the form of red yeast rice, are traditionally added to foodstuffs in numerous Asian countries, and their use has been advocated in Europe and America as well, knowledge of all components of red yeast rice is of interest. Therefore we developed a HPLC technique using a gradient of phosphoric acid and acetonitrile which allows rapid qualitative and quantitative analysis of extracts of red yeast rice, and identification of new compounds. **Figure 1** shows a chromatogram of an extract of red yeast rice produced from *Monascus purpureus* DSM1379. The six main pigments, namely the red pigments rubropunctamin (**1**) and monascorubramin (**4**), the orange pigments rubropunctatin (**6**) and monascorubrin (**8**), and the yellow pigments monascin (**5**) and ankaflavin (**7**) (*1*,*6*) (**Figure 3**) were identified by comparison of retention times and UV−Vis absorption spectra with those of authentic reference compounds. The peak at 11.8 min was identified as the mycotoxin citrinin (**2**) also on the basis of retention time and UV absorption spectrum with $\lambda_{max} = 237$ and 334 nm. The peak area corresponded to a concentration of 0.19 mg/g red yeast rice. Citrinin, although known for 70 years (*14*), has only recently been detected in cultures of *Monascus* (*5*, *12*). The chromatogram of this particular culture also contains several peaks of as yet unidentified compounds.

We isolated a new compound eluting at 12.5 min (**Figure 1**, peak 3) with UV absorption maxima at 236 and 326 nm. Red yeast rice samples produced in this laboratory contained about 0.3 mg/g of this compound. Upon heating red yeast rice with water (1:2, w/w) for 30 min at 90 °C several peaks changed: the citrinin (**2**), monascorubrin (**6**), and rubropunctatin (**8**) levels decreased significantly whereas the amount of the new compound increased greatly (**Figure 1**). Autoclaved samples of red

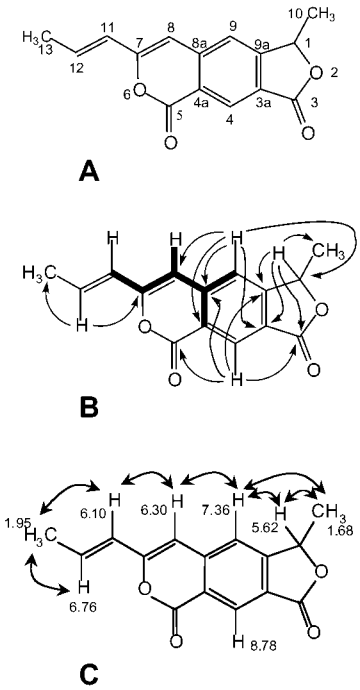

Figure 2. (A) Structure of monascodilone (**3**) and numbering system. (B) H,C–HMBC connectivities observed in monascodilone. The bold valences denote the connectivities of H-8, whereas the arrows show the observed cross-peaks among the other hydrogen and carbon atoms. (C) NOE responses indicating proximities in monascodilone.

yeast rice contained variable amounts of this compound, up to 5 mg/g. The origin of the new compound is not yet clear; heating of either citrinin or any of the 6 main pigments did not produce the compound (unpublished results).

The new compound, named monascodilone (**3**), was isolated and further purified by preparative HPLC. HPLC MS/MS measurements of monascodilone revealed a protonated molecular ion $[M + H]^+$ at *m*/*z* 257 and an acetonitrile adduct $[M + H + \text{acetonitrile}]^+$ at *m*/*z* 298. The high-resolution MS corresponded to the molecular formula $C_{15}H_{12}O_4$ indicating 10 double bond equivalents. For structure elucidation and NMR signal assignment ^1H, ^{13}C, H,H−COSY, H,C−HMQC, H,C−HMBC, and H,H−NOESY spectra were recorded. **Figure 2** illustrates the characteristic H,C−HMBC connectivities and the NOE responses of monascodilone (**3**) and the dilactone structure assigned to the new compound on the basis of these data.

This proposed structure has the propenyl group in common with the known *Monascus* pigments and with xanthomonasin A (**9**) (**Figure 3**) (*15*). In monascodilone (**3**) and in the pigments **5**−**8** the propenyl group is attached to a pyran ring which is, however, oxidized to an α-pyrone in the case of monascodilone. A further characteristic substructure is the $H_3C-CHR-O$ group; this group is also found in the *Monascus* metabolites citrinin (**2**) and in monankarin A (**10**) (**Figure 3**) (*8*); a similar $H_3C-CRR'-O$group is found in the *Monascus* pigments rubropunctamin (**1**), monascorubramin (**4**), monascin (**5**), rubropunctatin (**6**), ankaflavin (**7**), and monascorubrin (**8**) (**Figure 3**). The monascodilone structure has a γ-lactone attached to an aromatic ring, monascin and the other pigments have a related γ-lactone attached to a cyclohexenone ring. Altogether, the ring skeletons of monascodilone and the main pigments of *Monascus* are remarkably similar, a major difference being the absence of a $C_5H_{11}CO-$ or $C_7H_{15}CO-$ side chain in monascodilone. This results in a less lipophilic character of monascodilone (**3**) and

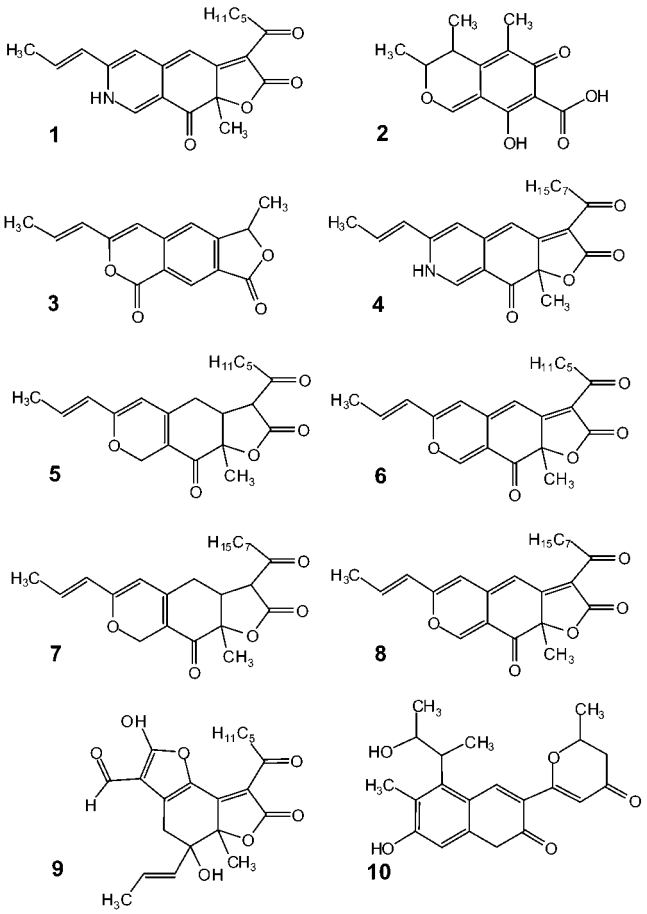

Figure 3. Structures of monascodilone (**3**), citrinin **(2),** and *Monascus* pigments: rubropunctamin (**1**); monascorubramin (**4**); monascin (**5**); rubropunctatin (**6**); ankaflavin (**7**); monascorubrin (**8**); xanthomonasin A (**9**); and monankarin A (**10**). Compound **10** was not found in our red yeast rice samples. A standard of compound **9** was not available.

is consistent with the shorter retention time in RPHPLC, similar to that of citrinin (**2**) (**Figure 1**).

Monascodilone (**3**) reacted readily with ammonia at room temperature to yield a more polar reaction product according to the retention time in the RPHPLC. The UV-absorption maxima (λ_{max} = 236 and 349 nm) revealed a red shift. The molecular weight being lower by 1 mass unit than that of monascodilone indicates a substitution of oxygen by NH, in agreement with the higher polarity and UV-absorption shift. The exchange probably takes place in the pyrone ring; this is analogous to the well-known reaction of rubropunctatin (**6**) with ammonia to form rubropunctamin (**1**) (*13, 16*).

As shown in **Figure 1**, monascodilone (**3**) was found not only in autoclaved, but also in our original laboratory-produced, red yeast rice. Furthermore, we detected monascodilone also in 6 of 12 commercial samples, in concentrations up to 0.4 mg/g (data not shown). Our samples of heated red yeast rice contained much higher levels of monascodilone, up to 5 mg/g. Because a steaming process is a common practice in the production of commercial red yeast rice, higher concentrations of monascodilone could be expected there as well. In summary, monascodilone (**3**) is a major nonpigment component of red yeast rice which appears to be formed from a yet unknown precursor preferentially at elevated temperatures. Pharmacological and toxicological properties of monascodilone are of interest in view of the uses of red yeast rice as a food additive and folk medicine.

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