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Structural characterization of two new orange pigments with strong yellow fluorescence

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ABSTRACT

As a result of HPLC analysis of the methanol extract of red yeast rice, two new orange pigments with similar yellow fluorescence spectra (λ_{ex} = 472 nm, λ_{em} = 535 nm) and UV absorption spectra (λ_{max} = 470 nm) were found. When the rubropunctatin and monascorubrin were dissolved in methanol, two new yellow fluorescent compounds were also observed. The results indicated that the reaction of rubropunctatin and monascorubrin with methanol or d_4-methanol occurred. The yellow fluorescence pigments were isolated and purified from the red yeast rice. Structures were elucidated by ESI-MS, ESI-MS/MS, and NMR spectroscopy. High-resolution mass spectrometry indicated the molecular formulas $C_{22}H_{26}O_6$ and $C_{24}H_{30}O_6$. The structures of the two new yellow fluorescence pigments, named monasphilol-methoxy A and monasphilol-methoxy B, were similar to rubropunctatin and monascorubrin, respectively. However, the hydroxyl and methoxyl groups in monasphilol-methoxy A and monasphilol-methoxy B, respectively, were substituted for the conjugated ketone carbonyl in rubropunctatin and monascorubrin.

1. Introduction

Filamentous fungi of genus *Monascus* have been used for the production of food components, natural pigments and food supplements with positive effects on human health in Eastern Asia for several centuries (Patakova, 2013). Red yeast rice, also known as red mold rice, *Monascus*-fermented rice, koji, anka, angkak, and ben-koji, which is produced by growing *Monascus* spp. on rice to produce a red-colored product, has been reported to possess multiple bioactive secondary metabolites, including azaphilones, polyketides, pyranoindole alkaloids, γ-aminobutyric acid (GABA), dimerumic acid, steroids, furans, and benzenoids (Cheng et al., 2008, 2012a,b). Metabolites from red yeast rice have been applied to decrease blood pressure, lower plasma cholesterol levels and blood sugar, and remove blood stasis (Cheng et al., 2012a,b; Ma et al., 2000).

Monascus pigments are a group of fungal metabolites called azaphilones, and are usually composed of a 1*H*-isochromene skeleton connected with a propenyl side chain and an acyl chain from the acetyl to the decanoly unit (Gao et al., 2013). The six well-known *Monascus* pigments include red-purple (monascorubramine and rubropunctamine), orange (monascorubrin and rubropunctatin), and yellow (monascin and ankaflavin) (Patakova, 2013). Recently, additional azaphilone metabolites from *Monascus* spp. have been identified and characterized, including some fluorescent compounds (Gao et al., 2013; Chen et al., 2012; Hsu et al., 2010a,b). Several new yellow pigments with blue fluorescence were identified (Huang et al., 2008; Loret and Morel, 2010), and four new azaphilones with yellow fluorescence, monapilols A–D, were isolated from red mold dioscorea (Hsu et al., 2011, 2013). Many studies also revealed red, N-containing *Monascus* pigments that are derivatives of monascorubrin and rubropunctatin (Gao et al., 2013; Jung et al., 2003, 2005; Kim et al., 2006, 2007). However, no studies have been reported on the chemical reactions of *Monascus* orange pigments with methanol. The *Monascus* pigments usually exhibit biological activity manifested by the inhibition of different enzymes, leading to antioxidant, anti-inflammatory, anti-human immunodeficiency virus, antimicrobial, antitumor, or other characteristic activities (Gao et al., 2013; Su et al., 2005). Additionally, novel azaphilonoid derivatives from red mold rice have recently been reported to exert anti-inflammatory and anticancer effects on both human laryngeal carcinoma and

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colon adenocarcinoma cell lines (Hsu et al., 2010a,b, 2013; Li et al., 2010). Recently, the *Monascus* orange pigment, rubropunctatin, was purified from red yeast rice and its biological activities against human gastric adenocarcinoma BGC-823 cells were studied. The results shown that rubropunctatin has potential to be developed as a new natural anti-cancer agent (Zheng et al., 2010).

In this study, we undertook the isolation two new yellow fluorescent compounds, monasphilol-methoxy A (MMA) and monasphilol-methoxy B (MMB), from the red yeast rice with methanol as extraction solvent or the reaction of rubropunctatin and monascorubrin with methanol and methanol-d_4 (CD_3OD), respectively. The structures of these compounds were established by mass spectrometry and NMR spectroscopy using a combination of ^{1}D NMR methods (^{1}H NMR and ^{13}C NMR) and 2D shift-correlated NMR techniques, for the complete ^{1}H and ^{13}C signal assignments.

2. Results and discussion

2.1. Isolation and purification of orange pigments

The two new orange pigments with strong yellow fluorescence were isolated from the reaction of rubropunctatin and monascorubrin with methanol or CD_3OD, and also isolated and purified from the red yeast rice. In addition to methanol, four other organic solvents, including acetone, ethyl acetate, dichloromethane and n-hexane were utilized for extracting and re-dissolving red yeast rice. MMA and MMB were obtained from red yeast rice only after methanol extraction. The results suggest that MMA and MMB were obtained from the reactions of extraction components (rubropunctatin and monascorubrin) with methanol. To isolate the orange pigments, it was necessary that the methanol extract be chromatographed over silica gel. The adsorption column was eluted with various ratios of hexane and ethyl acetate to elute less-polar products containing free pigments, such as rubropunctatin and monascorubrin. MMA and MMB were eluted with a hexane/ethyl acetate solution (7/3, v/v). The fractions containing MMA, rubropunctatin, MMB and monascorubrin were further purified by semi-preparative HPLC.

2.2. TLC and LC-Q-TOF-MS analysis of MMA and MMB

For TLC analysis, two new compounds were colored bright orange and demonstrated yellow fluorescence under UV-light irradiation (λ_{max} = 365 nm), with an R_f value of approximately 0.60–0.73. MMA and MMB remained bright orange and demonstrated yellow fluorescence under UV-light irradiation (λ_{max} = 365 nm) (Fig. 1A), and both exhibited similar UV absorption spectra (λ_{max} = 470 nm) (Supplementary Fig. S1) and fluorescence spectra, with maximum excitation and emission at 472 nm and 535 nm, respectively (Fig. 1B). The fluorescence spectra of MMA and MMB were similar to those of monapilols A–D, four recently isolated and characterized bioactive azaphilonoidal pigments from *Monascus*-fermented dioscorea (Hsu et al., 2011). However, unlike MMA and MMB, the compounds rubropunctatin and monascorubrin showed no visible fluorescence, probably resulting from the substitution of a ketone for the hydroxyl group in monapilols A–D (Hsu et al., 2011). HRESIMS established the masses of the molecular ions $[M+H]^+$ of MMA and MMB as *m/z* 387.1810, and 415.2121, corresponding to molecular formulas $C_{22}H_{26}O_6$ and $C_{24}H_{30}O_6$, respectively (Fig. S2). For ESI-Q-TOF-MS/MS analysis, collision energies ramped from 10 to 30 eV was used for MS/MS experiments. As collision energies increased, the mass spectra became more complex and produced an array of low-abundance ions which were difficult to interpret. Fragment ions at *m/z* 257, 229, and 213 were found in MS/MS spectra of both rubropunctatin and monascorubrin. Compared to the spectra of rubropunctatin and monascorubrin, different fragment ions at *m/z* 289, 247 and 192 were observed for both MMA and MMB. These results also suggest that the cleavage pathways of MMA and MMB are different from those of rubropunctatin and monascorubrin, respectively.

2.3. NMR analysis of MMA and MMB

For MMB structural elucidation, monascorubrin was dissolved in $CDCl_3$ and CD_3OD, and NMR spectra, including ^{1}H NMR, ^{13}C NMR, DEPT-135, HSQC, HMBC, COSY, NOESY and TOCSY, were recorded. When monascorubrin was dissolved in $CDCl_3$, NMR data were similar to the reference values for monascorubrin as previously reported (Kumasaki et al., 1962; Ogihara et al., 2000). When monascorubrin was dissolved in CD_3OD, ^{1}H NMR and ^{13}C NMR spectra were changed as compared to $CDCl_3$ (Fig. S3), and the chemical shift of protons placed H-8, H-5 and H-10 in a lower field (Fig. S4). As shown in Fig. S5, as the standing time of monascorubrin in CD_3OD solution increased, new signals at δ 7.69, 6.60, 6.37, 6.25 and 1.44 were observed in the ^{1}H NMR spectra. These results indicated that a chemical reaction had occurred between monascorubrin and CD_3OD. Additionally, when monascorubrin was dissolved in CD_3OD, there were double signals in the ^{1}H NMR (Fig. S4) and DEPT-135 (Fig. S6) spectra as compared with $CDCl_3$. This suggested the presence of an additional compound with a structure similar to that of monascorubrin. NMR signals representing MMB were deduced from comparisons of the NMR spectra of monascorubrin in $CDCl_3$ and in CD_3OD (Figs. S4–S9).

In the ^{1}H NMR spectrum, there were five olefinic, five methylene and three methyl signals. The methyl signals at δ 0.89–0.92, 1.44 and 1.93–1.96 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 correlation of the CH_3 signal at δ 0.89–0.92 allowed the identification of a $CH_3(CH_2)_6$ moiety. The methyl group at δ 1.93–1.96 allowed the identification of a propenyl substitutent. One conspicuous signal of the ^{1}H NMR spectrum was the singlet at δ 7.69 (H-8). This extreme chemical shift can be explained by the strong deshielding effect of a coplanar, *per*-positioned hydroxyl group. In the ^{13}C NMR spectrum of MMB the signals at δ 198.70 and 171.68 were characteristic for ketone C=O and ester (lactone) groups, respectively (Fig. S3). Moreover, the new carbon signal at 98.34 ppm was confirmed for the C-9. As shown in Fig. S7, MMB was significantly different from monascorubrin; no carbon signal corresponding to the conjugated ketone at 192 ppm was obtained, indicating the absence of the conjugated ketone. The peaks at δ 149.69, 136.44, 123.93, 110.14 and 103.72 were assigned to =CH, and the signals at δ 174.68, 158.86, 145.05, 117.71 and 111.09 were assigned to quaternary sp^2 carbon atoms. In the ^{13}C NMR and DEPT-135 spectra the peaks revealed the presence of three CH_3, six CH_2, six CH and eight quaternary carbon atoms (Fig. S6). However, the carbon signal for $-OCD_3$ was not found in the ^{13}C NMR and DEPT-135 spectra. The HSQC spectra revealed directly bonded carbon–proton pairs, and the results showed that H–C signals correlated with 7.69/149.69, 6.63/103.72, 6.60/136.44, 6.37/110.14, 6.20–6.21/123.93, 2.86–2.91/42.29, 1.93–1.96/18.72, 1.59–1.61/25.27, 1.44/23.1, and 0.89–0.92/14.49 (Fig. S9).

The HMBC data made it possible to establish full connectivity within the molecule. NMR data, together with data in the literature reported for related systems (Hsu et al., 2011), and HMBC spectral cross peaks of H-8/C-8a, C-4a, C-6, C-9; H-4/C-9a, C-8a, C-4a, C-3a, C-3, C-5; H-11/C-12, C-6; H-5/C-8a, C-4a, C-4, C-10, C-6; H-10/C-12, C-5, C-6, C-11; H-15/C-16, C-17, C-14; H-16/C14, C-15, C-17; H-11/C-6, C-12; and H-12/C-6, C-10, and C-11 (Fig. S7) suggested the presence of an azaphilone bearing a five-membered unsaturated lactone ring. This was confirmed as 9-hydroxy-9a-methyl-9,9a-dihydro-2H-furo[3,2-g]isochromen-2-one (Hsu et al., 2011). From