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# New *Monascus* Metabolites with a Pyridine Structure in Red Fermented Rice

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*Monascus purpureus* produces several hitherto unknown compounds in addition to pigments and the mycotoxin citrinin. In red fermented rice (angkak, red koji) obtained as cultures of *M. purpureus* DSM1379 and DSM1603, we detected two compounds with identical UV absorption spectra and maxima at 306−307 nm. They were isolated by HPLC, and their structures were elucidated by intensive MS and NMR studies. Monascopyridine A (**3**) contains a $\gamma$-lactone, propenyl group, hexanoyl side chain, and a pyridine ring, whereas the more lipophilic compound, monascopyridine B, is a higher homologue of monascopyridine A with the more lipophilic octanoyl instead of the hexanoyl side chain. This is the first report of *Monascus* metabolites with a pyridine ring.

**KEYWORDS:** ***Monascus*; fungi; red fermented rice; metabolite; RP-HPLC; HR-MS; HPLC-MS/MS; NMR; chemical structure**

## INTRODUCTION

Strains of the genus *Monascus* are used for the production of red fermented rice, which is used as a natural food colorant and health food in East Asia (*1*). Production of red fermented rice, also known as "angkak" and "red koji", had reached 100 tons/year in Japan in 1977 (*2*). Its use is increasing in western countries because of the growing consumer interest in natural as opposed to synthetic food additives. However, "natural" is not a priori "safe". Therefore, characterization of the components of red fermented rice and their beneficial and possible adverse effects are essential for a risk versus benefit assessment. So far, the main pigments of red fermented rice and a few further components such as citrinin, monacolines, and monankarin have been identified (*3*−*6*), but knowledge of their pharmacological and toxicological effects is very limited (*6*−*8*). We have recently reported the isolation and chemical structure of monascodilone, a new metabolite of *Monascus purpureus* (*9*). We now report two additional new metabolites.

## MATERIALS AND METHODS

**Reagents.** Acetonitrile (HPLC grade) and phosphoric acid (analytical grade) were obtained from Merck (Darmstadt, 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 Bruker Avance DRX-600, Bruker Avance 500, and Varian Unity plus 600 spectrometers, operating at 600/500 MHz ($^1$H) and 150.9/125.7 MHz ($^{13}$C). Chemical shifts are given on the $\delta$-scale and were referenced to TMS. Pulse programs for the 1D and 2D NMR experiments were taken from the Bruker or Varian software library. $^1$H, $^{13}$C, DEPT-135, $^1$H,$^1$H-COSY, HMQC, HMBC, and $^1$H,$^1$H-NOESY spectra were recorded. Structure elucidation and NMR signal assignment were performed using widely accepted strategies (*10*, *11*).

**HPLC-ESI-MS/MS Analysis.** Mass spectrometry (HPLC-MS/MS) was performed with electrospray ionization (ESI) as previously reported (*9*). For HPLC, MeOH was used instead of acetonitrile.

**EI-HRMS and ESI-HRMS.** High-resolution mass spectra were measured on a MAT90 (Finnigan MAT, Bremen, Germany) instrument and a Quattro-LC instrument (Micromass, Manchester, UK).

**Circular Dichroism.** CD spectra were measured in acetonitrile solutions in a 1-cm cell on a Jasco J600 spectropolarimeter (Jasco, Gross-Umstadt, Germany).

**Organism, Preparation, and Extraction of Red Fermented Rice.** Two strains of *Monascus purpureus*, DSM1379 and DSM1603, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). They were cultured in Petri dishes on autoclaved ground rice or on a rich rice medium consisting of 20 g of ground rice, 8 g of sucrose, 2 g of yeast extract, 0.1 g of $KH_2PO_4$, 0.1 g of $CaCl_2$, and 0.5 g of $MgSO_4$ in 100 mL of water (*12*). Cultures were incubated at 30 °C for 3 weeks, dried at 40 °C, and pulverized. Aliquots of 0.1 g of the red fermented rice were extracted in a sonication waterbath with 2.0 mL of a mixture of 0.25 M phosphoric acid and acetonitrile (1:1) and subsequently centrifuged. The clear supernatant obtained was directly used for HPLC. Extracts for the preparative fractionation of red fermented rice and isolation of new compounds were prepared similarly but using acetonitrile only (see below).

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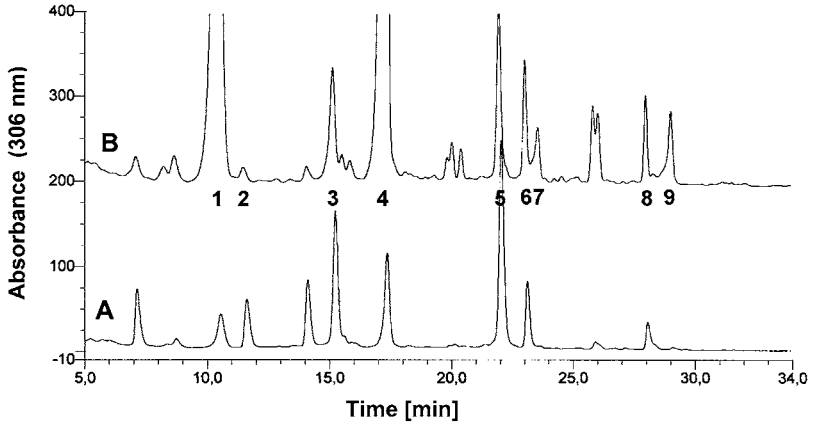


**Figure 1.** HPLC chromatograms of extracts of red fermented rice obtained from *M. purpureus* DSM1379 grown on rich rice medium (A) and from *M. purpureus* DSM1603 grown on ground rice (B): **1**, rubropunctamine; **2**, citrinin; **3**, monascopyridine A; **4**, monascorubramine; **5**, monascopyridine B; **6**, monascin; **7**, rubropunctatin; **8**, ankaflavin; **9**, monascorubrin. $\lambda = 306$ nm.

**HPLC Analysis.** The HPLC pump, sampler, diode array detector, and reverse-phase column have been described previously (*9*). A gradient of (A) 0.25 M phosphoric acid and acetonitrile (1:1) and (B) acetonitrile was used for analyses of red fermented rice: 0−5 min, 100% A; 5−28 min, 0−80% B; 28−34 min, 80% B; flow 1 mL/min. Chromatograms were recorded at the wavelengths 260, 306, 330, and 390 nm.

**Isolation of Monascopyridine A and Monascopyridine B.** The two compounds were obtained from the red fermented rice obtained with *M. purpureus* DSM1379 grown on rich rice medium or ground rice only. Aliquots of 1 g were extracted with 2.5 mL of acetonitrile by sonication and centrifugation. Extracts were fractionated by HPLC on a 300 mm × 7.8 mm i.d. TSKgel ODS-80TM column (TosoHaas, Stuttgart, Germany) using a linear gradient of (A) water and acetonitrile (40:60) and (B) acetonitrile: 0−5 min, 100% A; 5−28 min, 0−80% B; 28−34 min, 80% B; flow 3 mL/min. Fractions eluting at 12−13 and 18−19 min were collected and further fractionated on the same column by isocratic HPLC. Monascopyridine A eluted at 8.2 min with water/acetonitrile 30:70, and monascopyridine B eluted at 11.6 min with water/acetonitrile 25:75. Both compounds had absorption maxima at 306−307 nm. Upon removal of solvents and recrystallization from ethanol they were obtained as colorless needles.

**Monascopyridine A:** mp 161 °C; CD ($CH_3CN$) $\lambda_{ext}$ ($\Delta\epsilon$) 253 nm (−0.4), 279 nm (+4.4), 330 nm (−3.6); $^1$H NMR ($CDCl_3$, 600 MHz) $\delta$ 0.90 (3H, t, $J = 7.1$ Hz, H-19), 1.32 (4H, m, H-17, H-18), 1.48 (3H, s, H-10), 1.64 (2H, m, H-16), 2.02 (3H, dd, $J = 7.0$, 1.3 Hz, H-13), 2.65 (1H, dt, $J = 18.1$, 7.3 Hz, H-15), 3.00 (1H, dd, $J = 17.2$, 12.5 Hz, $H_{ax}$-9), 3.07 (1H, dt, $J = 18.1$, 7.5 Hz, H-15), 3.30 (1H, dd, $J = 17.2$, 4.4 Hz, $H_{eq}$-9), 3.36 (1H, ddd, $J = 12.9$, 12.5, 4.2 Hz, H-9a), 3.77 (1H, d, $J = 12.9$ Hz, H-1), 6.65 (1H, dq, $J = 15.4$, 1.3 Hz, H-11), 7.17 (1H, dq, $J = 15.4$, 7.0 Hz, H-12), 7.23 (1H, s, H-8), 9.15 (1H, s, H-5); $^{13}$C NMR ($CDCl_3$, 125.7 MHz) $\delta$ 201.7 (C-14), 188.8 (C-4), 168.3 (C-2), 156.5 (C-7), 153.5 (C-8a), 146.8 (C-5), 141.5 (C-12), 126.3 (C-11), 125.7 (C-4a), 121.7 (C-8), 83.1 (C-3a), 54.6 (C-1), 42.8 (C-15), 42.1 (C-9a), 31.1 (C-17), 30.1 (C-9), 22.8 (C-16), 22.4 (C-18), 19.0 (C-13), 16.8 (C-10), 13.9 (C-19). ESI-MS: *m/z* 356 (100) $[M + H]^+$, 388 (16) $[M + H + MeOH]^+$. ESI-MS/MS of *m/z* 356 (30 eV): *m/z* 213 (100), 312 (44), 356 (48). EI-HRMS: *m/z* 355.1784 (calculated for $C_{21}H_{25}NO_4$ 355.1784).

**Monascopyridine B:** mp 124 °C; CD ($CH_3CN$) $\lambda_{ext}$ ($\Delta\epsilon$) 253 nm (−1.1), 281 nm (+4.6), 330 nm (−2.4); $^1$H NMR ($CDCl_3$, 600 MHz) $\delta$ 0.88 (3H, t, $J = 7.2$ Hz, H-21), 1.25−1.32 (8H, m, H-17, H-18, H-19, H-20), 1.49 (3H, s, H-10), 1.61−1.66 (2H, m, H-16), 1.98 (3H, dd, $J = 7.0$, 1.5 Hz, H-13), 2.65 (1H, dt, $J = 18.3$, 7.2 Hz, H-15), 2.95 (1H, dd, $J = 17.7$, 12.3 Hz, $H_{ax}$-9), 3.07 (1H, dt, $J = 18.3$, 7.2 Hz, H-15), 3.22 (1H, dd, $J = 17.7$, 4.5 Hz, $H_{eq}$-9), 3.35 (1H, ddd, $J = 13.2$, 12.3, 4.5 Hz, H-9a), 3.74 (1H, d, $J = 13.2$ Hz, H-1), 6.52 (1H, dq, $J = 15.6$, 1.5 Hz, H-11), 7.00 (1H, dq, $J = 15.6$, 7.0 Hz, H-12), 7.06 (1H, s, H-8), 9.13 (1H, s, H-5); $^{13}$C NMR ($CDCl_3$,150.9 MHz) $\delta$ 14.1 (C-21), 16.9 (C-10), 18.7 (C-13), 22.6 (C-20), 23.1 (C-19), 28.9 (C-18), 29.0 (C-17), 29.6 (C-9), 31.6 (C-16), 42.3 (C-9a), 42.9 (C-15), 54.7 (C-1), 83.4 (C-3a), 120.3 (C-8), 124.3 (C-4a), 130.2 (C-11), 136.0 (C-12), 149.3 (C-8a), 150.7 (C-5), 160.1 (C-7), 168.9 (C-2), 190.7 (C-4), 201.9 (C-14). ESI-MS: *m/z* 384 (100) $[M + H]^+$, 416 (32) $[M + H + MeOH]^+$. ESI-MS/MS of *m/z* 384 (35 eV): *m/z* 213 (64), 340 (25), 384 (100). ESI-HRMS: *m/z* 384.2140 $[M + H]^+$ (calculated for $[M + H]^+$ $C_{23}H_{30}NO_4$ 384.2175).

## RESULTS AND DISCUSSION

In the course of HPLC analyses of extracts of red fermented rice obtained with *Monascus* species, we detected two peaks with identical UV absorption spectra and absorption maxima at 306−307 nm. These peaks, numbered **3** and **5** in **Figure 1**, with retention times of 15.2 and 22.0 min, were seen in samples of red fermented rice obtained with the strains DSM1379 and DSM1603 of *M. purpureus* on a rich medium containing rice, sucrose, yeast extract, and salts and likewise on a medium consisting of ground rice only. On ground rice, both strains formed additionally citrinin (**2**), the two red main pigments rubropunctamine (**1**) and monascorubramine (**4**), the yellow pigments monascin (**6**) and ankaflavin (**8**), and the orange pigments rubropunctatin (**7**) and monascorubrin (**9**). On the rich rice medium, both strains produced comparatively small amounts of red and yellow pigments and only traces of the orange pigments. These compounds were identified by comparison of retention times and UV−vis absorption spectra with those of authentic reference compounds.

Compound **3**, later named monascopyridine A, was isolated by preparative HPLC. The high-resolution mass spectrum of **3** corresponded to a molecular formula $C_{21}H_{25}O_4N$ indicating 10 double bond equivalents. In the $^{13}$C NMR spectrum the signals at 201.7, 188.8, and 168.3 are characteristic for ketone C═O, conjugated ketone, and ester (lactone) groups, respectively. The peaks at 146.8, 141.5, 126.3, and 121.7 can be assigned to ═CH, and the signals at 156.5, 153.5, and 125.7 assigned to quaternary $sp^2$ carbon atoms. In the $sp^3$ range of the $^{13}$C and DEPT-135 spectra the peaks revealed the presence of three $CH_3$, five $CH_2$, two CH, and one quaternary carbon atoms. The latter carbon atom is bonded to oxygen.

In the $^1$H NMR spectrum, the methyl signals at $\delta = 1.48$, 2.02, and 0.90 showed singlet, doublet, and triplet multiplicities, respectively, in accord with the presence of quaternary C, CH, and $CH_2$ neighbors. Utilizing the $^1$H,$^1$H-COSY experiment, the correlations starting from the $CH_3$ signal at $\delta = 0.90$ allowed the identification of a $CH_3(CH_2)_4$ moiety. The methyl group at $\delta = 2.02$ formed also a separate spin system together with the

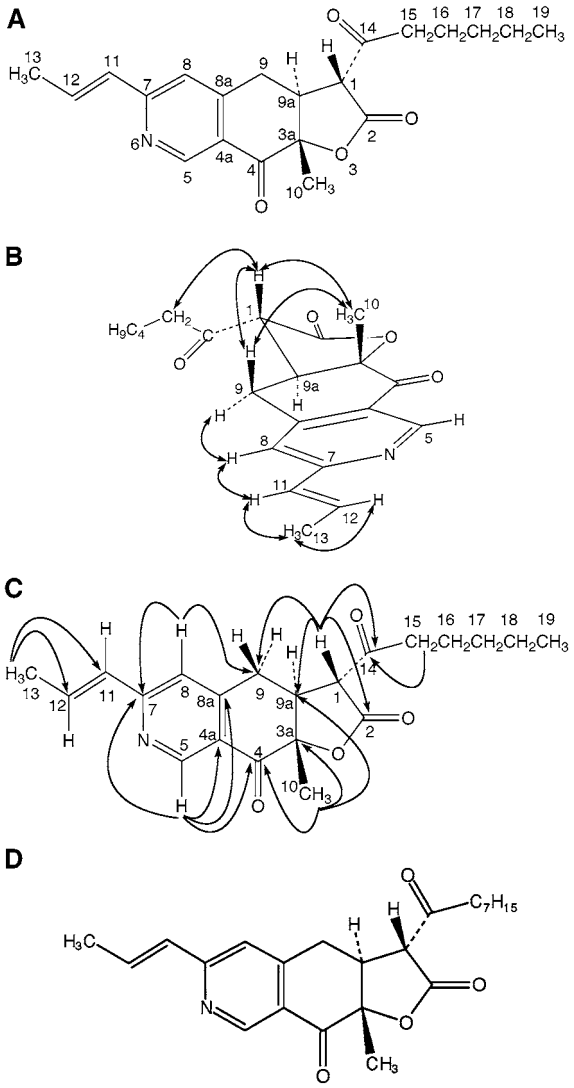


**Figure 2.** (A) Structure of monascopyridine A (**3**) and numbering system. (B) NOESY responses indicating proximities in monascopyridine A. (C) HMBC connectivities in monascopyridine A. (D) Structure of monascopyridine B (**5**).

protons of a *trans*-(*E*)-disubstituted carbon−carbon double bond, and this allowed the identification of a propenyl substituent. The remaining two methine and two methylene protons ($\delta$ = 3.77 d, 3.36 ddd, and 3.30 dd/3.00 dd) formed the third spin system. The HMQC spectrum enabled assignment of the directly bonded C−H moieties. The two- and three-bond proton−carbon connectivities of the building elements of the molecule were detected by the HMBC, and the steric proximities of the protons by the NOESY experiment. **Figure 2** illustrates the molecular structure derived from the characteristic HMBC connectivities and NOESY responses of the new compound monascopyridine A (**3**).

The 7.23/30.1 cross-peak proves that the $H_2C$-9 methylene is connected to the C-8a atom of the pyridine ring, and the 9.15/188.8 correlation shows that the C-4 keto group is attached to C-4a in the same ring. From the HMBC response between the singlet methyl at $\delta$ = 1.48 and the C-4 signal we can conclude that these protons are located three bonds distant from C-4. The $H_3C$-10 protons correlate with one methine carbon atom, and in this way the latter can be assigned to C-9a. The doublet at $\delta$ = 3.77 (H-1) correlates with the carbon atoms C-9, C-9a, and with the lactone carbonyl C-2. This allows the determination

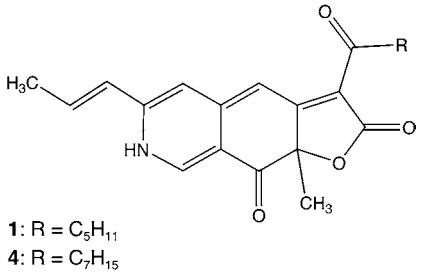


**Figure 3.** Structure of the red pigments rubropunctamine (**1**) and monascorubramine (**4**).

of the position of the lactone ring. On the basis of the cross-peaks between H-1/C(14)=O the position of the $C_5H_{11}C$=O moiety is also obvious.

The strong NOESY responses $H_3$-10/H-1, $H_3$-10/$H_{ax}$-9, and H-1/$H_{ax}$-9 revealed the *cis* arrangement of these protons. In addition the values of $^3J$(H-1,H-9a) ≈ $^3J$(H-9a, $H_{ax}$-9) ≈ 12.5 Hz proved the *trans*-antiperiplanar positions of these protons. The H-8/H-11 NOESY cross-peak reports on a preferred conformation of the propenyl group around the C(7)−C(11) bond. The observed small broadening of the signals of the pyridine and propenyl moieties is in accord with an intermediate rate of rotation around the C(7)−C(11) bond. At raised temperature these signals become sharp.

One rather conspicuous signal of the $^1$H NMR spectrum was the singulet at $\delta$ = 9.15 (H-5). This extreme chemical shift can be well explained by the strong deshielding effect of the coplanar, *peri*-positioned carbonyl group. In the HMBC spectrum the one-bond $^1J$(C,H) correlation of the C-5 carbon breaks through, and its value of 187 Hz is in good accordance with an α-positioned =CH in a pyridine ring *(11)*, supporting further the suggested structure of monascopyridine A (**3**).

Compound **5** (**Figures 1** and **2**), an analogue of monascopyridine A, revealed a molecular weight of 383. This is 28 units higher than that of monascopyridine A, suggesting two additional methylene groups. The identity of the UV spectrum with that of monascopyridine A indicates the presence of the same chromophore. The NMR data confirmed that compound **5** is a higher homologue of monascopyridine A with the more lipophilic octanoyl instead of the hexanoyl side chain, and thus this compound was named monascopyridine B. The pairwise formation of metabolites with a hexanoyl or a octanoyl substituent is well-known from the pigments of *Monascus*.

The monascopyridines have the N-containing ring skeleton in common with the red *Monascus* pigments rubropunctamine (**1**) and monascorubramine (**4**) (**Figure 3**). They are formally dihydro derivatives of these pigments, and their biosynthesis is very likely coupled with the synthesis of the pigments. The monascopyridines are, however, distinguished by the aromatic pyridine ring which has previously not been found in metabolites of *Monascus*. In view of this unique structure, it is conceivable that the pharmacological and toxicological effects of the monascopyridines also differ significantly from those of the pigments.

The red fermented rice sample shown in **Figure 1** obtained with *M. purpureus* DSM1379 on rich rice medium contained 0.8 mg/g monascopyridine A and 1.7 mg/g monascopyridine B; the corresponding values for the product obtained with *M. purpureus* DSM1603 on rice were 1.0 mg/g and 1.3 mg/g. The amounts of these compounds found in red fermented rice were variable, maximum values found being 6 mg monascopyridine A and similar amounts of monascopyridine B per gram. Monascopyridines were found also in a red fermented rice that

had been produced by inoculation of rich rice medium with a *Monascus* sp. isolated from a commercial red fermented rice. It can therefore be assumed that various *Monascus* species and strains can produce these hitherto unknown compounds.

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## LITERATURE CITED

(1) Ma, J.; Li, Y.; Ye, Q.; Li, J.; Hua, Y.; Ju, D.; Zhang, D.; Cooper, R.; Chang, M. Constituents of red yeast rice, a traditional food and medicine. *J. Agric. Food Chem.* **2000**, *48*, 5220−5225.

(2) Sweeny, J. G.; Estrada-Valdes, M.; Iacobucci, G. A.; Sato, H.; Sakamura, S. Photoprotection of the red pigments of *Monascus anka* in aqueous media by 1,4,6-trihydroxynaphthalene. *J. Agric. Food Chem.* **1981**, *29*, 1189−1193.

(3) Juzlová, P.; Martinková, L.; Kren, V. Secondary metabolites of the fungus *Monascus*—a review. *J. Ind. Microbiol.* **1996**, *16*, 163−170.

(4) Blanc, P. J.; Laussac, J. P.; Le Bars, J.; Le Bars, P.; Loret, M. O.; Pareilleux, A.; Prome, D.; Prome, J. C.; Santerre, A. L.; Goma, G. Characterization of monascidin A from *Monascus* as citrinin. *Int. J. Food Microbiol.* **1995**, *27*, 201−213.

(5) Endo, A.; Komagata, D.; Shimada, H. Monacolin M, a new inhibitor of cholesterol biosynthesis. *J. Antibiot.* **1986**, *39*, 1670−1673.

(6) Hossain, C. F.; Okuyama, E.; Yamazaki, M. A new series of coumarin derivatives having monoamine oxidase inhibitory activity from *Monascus anka*. *Chem. Pharm. Bull.* **1996**, *44*, 1535−1539.

(7) Martinková, L.; Patáková-Juzlová, P.; Kren, V.; Kucerová, Z.; Havlicek, V.; Olsovsky, P.; Hovorka, O.; Rihová, B.; Vesely, D.; Veselá, D.; Ulrichová, J.; Prikrylová, V. Biological activities of oligoketide pigments of *Monascus purpureus*. *Food Addit. Contam.* **1999**, *16*, 15−24.

(8) Wang, I. K.; Lin-Shiau, S. Y.; Chen, P. C.; Lin, J. K. Hypotriglyceridemic effect of Anka (a fermented rice product of *Monascus* sp.) in rats. *J. Agric. Food Chem.* **2000**, *48*, 3183−3189.

(9) Wild, D.; Tóth, G.; Humpf, H.-U. New *Monascus* metabolite isolated from red yeast rice (angkak, red koji). *J. Agric. Food Chem.* **2002**, *50*, 3999−4002.

(10) Duddeck, H.; Dietrich, W.; Tóth, G. *Structure Elucidation by Modern NMR. A Workbook*; Springer-Steinkopff: Darmstadt, 1998. [Duddeck, H.; Dietrich, W.; Tóth, G. *Elucidación estructural mediante RMN. Ejercicios y problemas*; Springer Ibérica: Barcelona, 2000].

(11) Pretsch, E.; Tóth, G.; Munk, M. E.; Badertscher, M. *Computer-Aided Structure Elucidation. Spectra Interpretation and Structure Generation*; Wiley-VCH: Weinheim, 2002.

(12) Fink-Gremmels, J.; Dresel, J.; Leistner, L. Use of *Monascus* extracts as an alternative to nitrite in meat products. *Fleischwirtschaft* **1991**, *71*, 1184−1186.

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