IDENTIFICATION OF MAJOR PIGMENTS CONTAINING D-AMINO ACID UNITS IN COMMERCIAL *MONASCUS* PIGMENTS

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Eight major pigments have been isolated from commercial *Monascus* pigments. The structural data on the pigments were obtained using NMR, MS, and semisyntheses. The pigments were characterized as alanine or aspartate derivatives of the orange pigments of *Monascus*, monascorubrin (**1**) and rubropunctatin (**2**), which have an azaphilone structure. The semisyntheses revealed that the amino acid units of the isolated compounds were the D-forms as well as the L-forms.

KEY WORDS monascorubrin; rubropunctatin; L- and D-alanine; L- and D-aspartic acid; *Monascus*

Species of the fungi *Monascus* have been utilized for making fermented food and preserving meat for hundreds of years. They are mainly utilized as sources of natural food colorants, monascus color and monascus yellow,[1] in Japan. It is known that the fungi *Monascus* produce six major pigments including **1** and **2**.[2] Since the six pigments are insoluble in water, the original monascus colors that contain these pigments have been restricted in use. Therefore, various water-soluble monascus colors have been developed.[3] In the present study, we investigated the major pigments of two commercial monascus colors. This is the first isolation of monascus pigments that contain the D-amino acid unit.

One commercial monascus color (color A, powdered, 5.5 g) was subjected to DIAION HP-20 column chromatography. The MeOH eluate was subjected to chromatography on Chromatorex ODS using H_2O–MeOH (60:40). Two crude red fractions from the column were subjected to medium-pressure liquid chromatography (MPLC) using H_2O–EtOH (78:22) to yield four red amorphous solids, **3** (13 mg), **4** (15 mg), **5** (10 mg), and **6** (17 mg). Compound **3**[4] gave a quasimolecular ion at m/z 454 $(M+H)^+$ from the positive FAB-MS and positive electrospray liquid chromatography (LC)-MS. The molecular formula of **3** was determined to be $C_{26}H_{31}N_1O_6$ [$(M+H)^+$ 454.2243, calcd 454.2230] based on high-resolution (HR) FAB-MS. The ^{1}H-NMR data of **3** were very similar to those of **1**,[5] except for the signals (δ 1.73 and δ.4.95) due to the isolated methylmethine protons. The ^{13}C-NMR data showed the existence of an additional carbonyl carbon as well as the isolated methylmethine carbons. All the above data suggested that the pyronoid oxygen of **1** was replaced with the nitrogen of alanine for the structure of **3**. Long-range correlations (Fig. 1) obtained by the HMBC technique confirmed the planar structure.

Compound **4**[6] gave quasimolecular ions at m/z 454 $(M+H)^+$ like **3** on the positive electrospray LC-MS. The ^{1}H-NMR data were very similar to those of **3**, except that the signals of H-3, -5, -7, -12, and -β were shifted to δ 6.52, δ 7.06, δ 6.71, δ 8.23, and δ 1.74, respectively. Compound **3** has two asymmetric carbons. One is C-9 and the other is Cα of the alanine unit. Considering these facts, it appears that **4** is a

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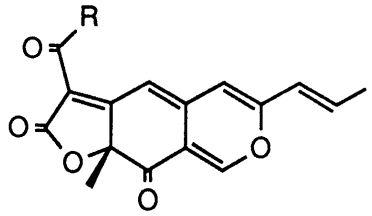
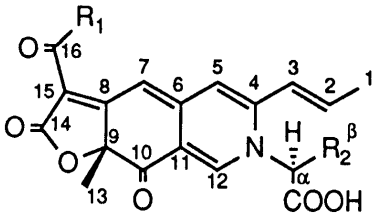
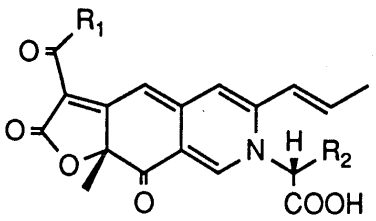


1 $R_1 = C_7H_{15}$
2 $R_1 = C_5H_{11}$



3 $R_1 = C_7H_{15}$, $R_2 = CH_3$
5 $R_1 = C_5H_{11}$, $R_2 = CH_3$
7 $R_1 = C_7H_{15}$, $R_2 = CH_2COOH$
9 $R_1 = C_5H_{11}$, $R_2 = CH_2COOH$



4 $R_1 = C_7H_{15}$, $R_2 = CH_3$
6 $R_1 = C_5H_{11}$, $R_2 = CH_3$
8 $R_1 = C_7H_{15}$, $R_2 = CH_2COOH$
10 $R_1 = C_5H_{11}$, $R_2 = CH_2COOH$

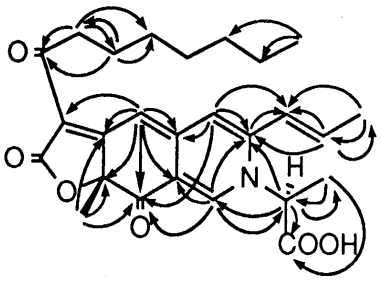


Fig. 1 Long-Range Correlation in HMBC Spectrum of **3**

diastereomer of **3**.

Compounds **5**[6] and **6**[6] gave quasimolecular ions at m/z 426 $(M+H)^+$ on the positive electrospray LC-MS. The ^{1}H-NMR spectra of **5** and **6** were quite similar to those of **3** and **4**, respectively, except that the intensity of the methylene protons at δ 1.30–1.34 of **5** and **6** were 4 H smaller than those of **3** and **4**. These facts suggested that the planar structures of **5** and **6** involved the insertion of the amino group of alanine in place of the pyronoid oxygen of **2**, and that **5** and **6** were diastereoisomers to each other.

Whalley et al. reported that the absolute configurations at C-9 in **1** and **2** were (R).[7] Considering the biosynthetic pathway of **3-6**, the absolute configuration at C-9 in **3-6** may be retained as (R). To clarify the absolute configurations of compounds **3-6**, a pigment mixture containing **1** and **2** was allowed to react with L- or D-alanine.[3,8] The HPLC analysis suggested that the reaction products of the pigment mixture and L-alanine contained two pigments. The pigment that eluted earlier had the same retention time as **5**, and the other had the same retention time as **3**. The ^{1}H-NMR (600 MHz) spectral data of the pigment with the shorter retention time were identical to those of **5**, and those of the other were identical to those of **3**. Thus, it was revealed that **3** and **5** contain L-alanine units. The reaction product of the pigment mixture and D-alanine also contained two pigments that were identified as **6** and **4** in a manner similar to that above. Thus, it was revealed that **4** and **6** contain D-alanine units.

Another commercial monascus color (color B, liquid, 15 ml) was subjected to chromatography on Chromatorex ODS with H_2O–MeOH (60:40). Two crude red fractions from the column were subjected to MPLC using H_2O–EtOH (95:5) to yield four red amorphous solids, **7**[9] (11 mg), **8**[9] (5 mg), **9**[9] (10 mg), and **10**[9] (6 mg). Their structures were determined in a manner similar to the cases of **3-6**. Compounds **7-10** were similar to **3–6** except that their amino acid units were L- or D-aspartic acid instead of L- or D-alanine.

While D-amino acids are quite common in nature as constituents of bacterial cell walls (D-alanine, D-glutamine) and of several antibiotics, pigments containing the D-amino acid unit are unique. Further studies are needed to clarify the origin of the D-amino acid unit, including inversion during the manufacturing process.

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REFERENCES AND NOTES

1) Sato K., Iwakami S., Goda Y., Okuyama E., Yoshihira K., Ichi T., Odake Y., Noguchi H., Sankawa U., *Heterocycles*, **34**, 2057 (1992).
2) Fielding B. C., Holker J. S. E., Jones D. F., Powell A. D. G., Richmond K. W., Robertson A., *J. Chem. Soc.*, **1961**, 4579.
3) Lin T. F., Yakushijin K., Büchi G. H., Demain A. L., *J. Industrial Microbiol.* **9**, 173 (1992).
4) $[\alpha]_D^{20}$ -2900° (c=0.0027, MeOH); IR (KBr) cm^{-1}: 1720, 1625, 1535, 1461; UV λ_{max} (MeOH) nm: 202, 248, 292, 420, 498; ^{1}H-NMR (600 MHz / CD_3OD) δ: 1.98 (3H, d, J=6.6 Hz, H-1), 6.59 (1H, qd, J=6.6, 15.3 Hz, H-2), 6.51 (1H, brd, J=15.3 Hz, H-3), 7.08 (1H, s, H-5), 6.72 (1H, s, H-7), 8.28 (1H, s, H-12), 1.67 (3H, s, H-13), 2.81 (2H, m, H-17), 1.60 (2H, quintet, J=7.2 Hz, H-18), 1.30-1.34 (8H, m, H-19, 20, 21, 22), 0.89 (3H, t, J=6.9 Hz, H-23), 4.95 (1H, q, J=7.2 Hz, Hα), 1.73 (3H, d, J=7.2 Hz, Hβ), ^{13}C-NMR (150 MHz / CD_3OD) δ: 19.16 (C-1), 141.26 (C-2), 123.71 (C-3), 152.98 (C-4), 119.42 (C-5), 153.51 (C-6), 98.84 (C-7), 173.68 (C-8), 86.39 (C-9), 196.35 (C-10), 119.61 (C-11), 141.59 (C-12), 30.73 (C-13), 173.89 (C-14), 102.05 (C-15), 198.73 (C-16), 41.27 (C-17), 26.45 (C-18), 30.65 (C-19), 30.33 (C-20), 32.97 (C-21), 23.71 (C-22), 14.44 (C-23), 65.48 (Cα), 174.66 (Cα-COOH), 18.71 (Cβ). Reduced pseudo-molecular ions $(M+2)^+$ and $(M+3)^+$ were observed during measurements of the FAB-MS spectra using a mixture of glycerol and 3-nitrobenzyl alcohol as a matrix. Thioglycerol (TG) adducts $(M+TG)^+$ and $(M+1)^+$ were observed instead of $(M+2)^+$ and $(M+3)^+$ when a mixture of glycerol, 3-nitrobenzyl alcohol, thioglycerol, and 10-camphorsulfonic acid was used as a matrix.
5) Sweeny,J. G., Estrada-Valdes M. C., Iacobucci G. A., Sato H., Sakamura S., *J. Agric. Food Chem.*, **29**, 1189 (1981).
6) **4**: $[\alpha]_D^{20}$ -3100° (c=0.0017, MeOH); IR (KBr) cm^{-1}: 1716, 1625, 1542, 1461; UV λ_{max} (MeOH) nm: 202, 248, 295, 420, 510; HR FAB-MS *m/z*: 454.2235 (calcd for $C_{26}H_{32}N_1O_6$: 454.2230). **5**:$[\alpha]_D^{20}$ -2500° (c=0.0025, MeOH); IR (KBr) cm^{-1}: 1718, 1627, 1540, 1459; UV λ_{max} (MeOH) nm: 202, 248, 292, 420, 498; HR FAB-MS *m/z*: 426.1916 (calcd. for $C_{24}H_{28}N_1O_6$: 426.1917). **6**: $[\alpha]_D^{20}$ -2800° (c=0.0028, MeOH); IR (KBr) cm^{-1}:1722, 1625, 1544, 1461; UV λ_{max} (MeOH) nm: 202, 248, 292, 420, 500.; HR FAB-MS *m/z*: 426.1927 (calcd for $C_{24}H_{28}N_1O_6$: 426.1917).
7) Whalley W. B., Ferguson G., Marsh W. C., Restivo R. J., *J. Chem. Soc. Perkin Trans .II*, **1976**, 1366.
8) The pigment mixture was incubated in MeOH–100 mM phosphate buffer containing L-alanine at 30°C and shaken at 200 rpm for 2 h.
9) **7**: $[\alpha]_D^{20}$ -2000° (c=0.0027, MeOH); IR (KBr) cm^{-1}: 1708, 1629, 1546, 1465; UV λ_{max} (MeOH) nm: 204, 248, 278, 418, 490; ^{1}H-NMR (600 MHz / CD_3OD) δ: 1.98 (3H, dd, J=1.8, 6.6 Hz, H-1), 6.59 (1H, m, H-2), 6.56 (1H, m, H-3), 7.05 (1H, s, H-5), 6.70 (1H, s, H-7), 8.31 (1H, s, H-12), 1.63 (3H, s, H-13), 2.81 (2H, m, H-17), 1.60 (2H, quintet, J=7.2 Hz, H-18), 1.29-1.34 (8H, m, H-19, 20, 21, 22), 0.89 (3H, t, J=7.2 Hz, H-23), 5.33 (1H, dd, J=3.6, 10.8 Hz, Hα), 2.91 (1H, dd, J=10.8, 16.2 Hz, Hβ), 3.27 (1H, dd, J=3.6, 16.2 Hz, Hβ'), ^{13}C-NMR (150 MHz / CD_3OD) δ: 19.15 (C-1), 140.76 (C-2), 124.45 (C-3), 153.69 (C-4), 119.47 (C-5), 153.89 (C-6), 98.84 (C-7), 173.35 (C-8), 86.90 (C-9), 196.53 (C-10), 119.56 (C-11), 142.19 (C-12), 30.64 (C-13), 174.00 (C-14), 101.51 (C-15), 198.70 (C-16), 41.18 (C-17), 26.52 (C-18), 30.66 (C-19), 30.30 (C-20), 32.96 (C-21), 23.69 (C-22), 14.43 (C-23), 68.08 (Cα), 176.94 (Cα-COOH), 41.83 (Cβ), 173.85 (Cβ-COOH). The signals of C-12 and Cα were too small to be detected by 1D ^{13}C NMR but observed as a cross-peak of HMBC. HR FAB-MS m/z: 498.2145, (calcd for $C_{27}H_{32}N_1O_8$: 498.2128). **8**: $[\alpha]_D^{20}$ -2600° (c=0.0027, MeOH); IR (KBr) cm^{-1}: 1710, 1625, 1546, 1463; UV λ_{max} (MeOH) nm: 204, 250, 295, 418, 505; ^{1}H-NMR data of **8** were similar to those of **7**, except that the signals of H-3, -12, -13, and Hβ were shifted to δ 6.78 (1H, m), δ 8.23 (1H, s), δ 1.66 (3H, s), and δ 2.95 (1H, dd, J =11.3, 16.8 Hz); electrospray LC-MS: m/z 498 $(M+H)^+$; HR FAB-MS m/z: 498.2126 (calcd for $C_{27}H_{32}N_1O_8$: 498.2128). **9**: $[\alpha]_D^{20}$ -2200° (c=0.0026, MeOH); IR (KBr) cm^{-1}: 1722, 1625, 1535, 1461; UV λ_{max} (MeOH) nm: 204, 248, 294, 418, 500; ^{1}H-NMR data of **9** were similar to those of **7**, except that intensity of the methylene proton at 1.33-1.36 of **9** was 4H smaller than that (at 1.29–1.34) of **7**, and H-21 was changed to δ 0.91 (3H, t, J=6.9 Hz); electrospray LC-MS: m/z 470 $(M+H)^+$; HR FAB-MS m/z: 470.1810 (calcd for $C_{25}H_{28}N_1O_8$: 470.1815). **10**: $[\alpha]_D^{20}$ -1400° (c=0.0025, MeOH); IR (KBr) cm^{-1}: 1714, 1625, 1546, 1463; UV λ_{max} (MeOH) nm: 204, 248, 280, 418, 500; ^{1}H-NMR data of **10** were similar to those of **9**, except that the signals of H-3, -12, -13, and Hβ were shifted to δ 6.78 (1H, m), δ 8.28 (1H, s), δ 1.66 (3H, s), and δ 2.95 (1H, dd, J=11.3, 16.8 Hz); electrospray LC-MS: m/z 470 $(M+H)^+$; HR FAB-MS m/z: 470.1820 (calcd for $C_{25}H_{28}N_1O_8$: 470.1815).

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