(+)- and (−)-*syn*-2-Isobutyl-4-methylazetidine-2,4-dicarboxylic Acids from the Extract of *Monascus pilosus*-Fermented Rice (Red-Mold Rice)

Toshihiro Akihisa,*[,†] Shoichi Mafune,[†] Motohiko Ukiya,[†] Yumiko Kimura,[‡] Ken Yasukawa,[‡] Takashi Suzuki,[‡] Harukuni Tokuda,[§] Nobukazu Tanabe,[⊥] and Tadahiko Fukuoka[⊥]

College of Science and Technology, Nihon University, 1-8 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8308, Japan, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi-shi, Chiba 274-8555, Japan, Department of Biochemistry, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan, and Research Center, Gunze, Co., Ishiburo 1, Ikurashinmachi, Ayabe-shi, Kyoto 623-8512, Japan

Received August 26, 2003

The structures of two enantiomeric azetidine-type amino acids isolated from the *n*-butanol-soluble fraction of the 70% ethanol extract of red-mold rice fermented with *Monascus pilosus* were established to be (+)-[**1**; (+)-monascumic acid] and (−)-*syn*-2-isobutyl-4-methylazetidine-2,4-dicarboxylic acids [**2**; (−)-monascumic acid] based on spectroscopic methods.

Species of the fungi *Monascus* (Eurotiaceae) have been utilized for making fermented food and preserving meat for hundreds of years. Red-mold rice fermented using *Monascus* spp. is effective in decreasing blood pressure[1] and lowering plasma cholesterol levels[2,3] and has antibacterial activity.[4] γ-Aminobutyric acid (GABA), which possesses anti-hypertensive effects in humans, has been isolated from red-mold rice.[5] Endo[3] discovered that *Monascus ruber* produces monacolin K (lovastatin; mevinolin), an active methylated form of compactin, in liquid fermentation. Monacolin K functions as an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the regulatory and rate-limiting enzyme of cholesterol biosynthesis.[6] The fact that red-mold rice can suppress the synthesis of cholesterol has also been confirmed.[7] In the course of our search for potential antitumor promoters from natural sources,[8] we were especially interested to undertake the investigation of red-mold rice constituents.[9] In this paper, we report the isolation and characterization of two enantiomeric azetidine (trimethyleneimine)-type amino acids, **1** and **2**, from the 70% ethanol extract of red-mold rice fermented with *Monascus pilosus*.

The molecular formula of compound **1** was determined as $C_{10}H_{17}NO_4$ from the HREIMS ([M]$^+$ *m*/*z* 215.1157) as well as from its ^{13}C NMR DEPT. The compound has two secondary methyls [δ_H 0.81 (d, J = 6.4 Hz), 0.88 (d, J = 6.6 Hz); solvent: DMSO-d_6], one tertiary methyl [δ_H 1.31 (s)], two methylenes [δ_C 43.7 (t), δ_H 1.36 (1H, dd, J = 2.9, 12.8 Hz) and 1.71 (1H, dd, J = 9.0, 12.8 Hz); and δ_C 45.0 (t), δ_H 1.99 (1H, d, J = 16.3 Hz) and 2.45 (1H, d, J = 16.3 Hz)], one methine [δ_C 23.7 (d), δ_H 1.66 (m)], two sp^3 quaternary carbons [δ_C 72.2 (t) and 77.6 (t)] adjacent to a secondary amine group [δ_H 1.91 (1H, s); ν_{max} 3422, 3290 cm^{-1}], and two carboxyls [ν_{max} 1717, 1681, 1239 cm^{-1}; δ_C 172.8 (s) and 173.6 (s); δ_H 7.94 (2H, s)]. These data, in combination with diagnostic MS fragment ions at *m*/*z* 170 [M − COOH]$^+$, 158 [M − C_4H_9 (isobutyl)]$^+$, and 154 [M − COOH − CH_3 − H]$^+$, suggested that **1** possesses a four-membered azetidine (trimethyleneimine) ring substituted with carboxyl and isobutyl groups at C-2 and carboxyl and methyl groups at C-4. The proposed structure of **1** was supported by the analysis of ^{13}C DEPT NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. The relative configuration of **1** was established by NOESY and difference NOE experiments. Compound **1** showed significant NOE correlations between H_a-6 (δ_H 1.36) and H_b-3 (δ_H 2.45)−H-5 (δ_H 1.31) (Figure 1), which suggested that the isobutyl group at C-2 and the methyl group at C-4 were oriented on the same face of the azetidine ring. We concluded that **1** is *syn*-2-isobutyl-4-methylazetidine-2,4-dicarboxylic acid, and since **1** exhibited positive specific optical rotation ([α]$^{25}_D$ +3.7°), we named it (+)-monascumic acid.

Compound **2**, which has the same molecular formula $C_{10}H_{17}NO_4$ (HREIMS [M]$^+$ *m*/*z* 215.1160; HRFABMS [M + H]$^+$ *m*/*z* 216.1235) as **1**, exhibited EIMS, IR, and ^{13}C and ^{1}H NMR spectral data, melting point data (Experimental Section), and NOE correlations (Figure 1) indistinguishable from those of **1**, suggesting that **2** was an enantiomer of **1** and possessed the structure *syn*-2-isobutyl-4-methylazetidine-2,4-dicarboxylic acid. We named **2** as (−)-monascumic acid since it exhibited an almost opposite specific rotation ([α]$^{25}_D$ −4.4°) of that for compound **1**.

The ^{13}C and ^{1}H NMR spectral data and the NOE correlations of compounds **1** and **2** determined in pyridine-d_5 were fully consistent with their proposed structures.

The two enantiomeric azetidine-type amino acids, (+)-(**1**) and (−)-monascumic acids (**2**), isolated from the 70% EtOH extract of red-mold rice fermented with *M. pilosus* in this study are the new naturally occurring compounds. The occurrence of the azetidine ring system in natural products is uncommon, and derivatives of this four-membered moiety have been isolated so far only from the roots and leaves of *Convallaria majalis* (lily-of-the-valley),[10] the culture broth of *Streptomyces cacaoi*,[11] the roots of barley,[12] and the Okinawan marine sponge *Penares* sp.[13] The former three azetidines[10−12] and monascumic acids (**1** and **2**), isolated in this study, are the α-amino acids or their derivatives possessing a secondary amine group.

Experimental Section

General Experimental Procedures. Crystallizations were performed in ethyl acetate (EtOAc), and melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter in acetone at 25 °C. IR spectra were recorded on a JASCO IR-300 spectrometer in KBr disks.

* To whom correspondence should be addressed. Fax: +81-3-3293-7572. E-mail: akihisa@chem.cst.nihon-u.ac.jp.
† College of Science and Technology, Nihon University.
‡ College of Pharmacy, Nihon University.
§ Kyoto Prefectural University of Medicine.
⊥ Gunze, Co.

10.1021/np030394i CCC: $27.50
Published on Web 01/22/2004

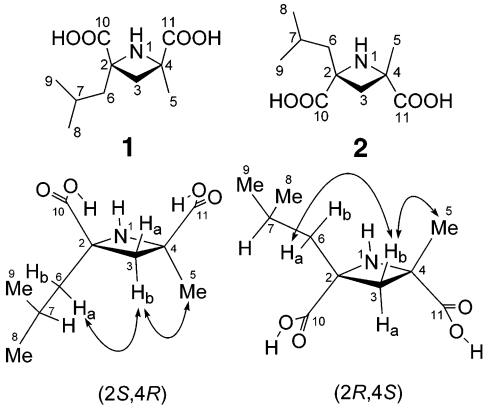

Figure 1. Structures and major NOE correlations (←→) for 1 [(2*S*,4*R*) or (2*R*,4*S*)] and 2 [(2*R*,4*S*) or (2*S*,4*R*)].

NMR spectra were recorded with a JEOL LA-400 spectrometer at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) in DMSO-d_6 or in pyridine-d_5 with tetramethylsilane as internal standard. Electron-impact mass spectra (EIMS; 70 eV) and high-resolution EIMS (HREIMS) were recorded on a JEOL JMS-BU20 spectrometer using a direct inlet system. HRFABMS were obtained with a JEOL JMS-BU20 spectrometer using glycerol as the matrix. Octadecyl silica (Chromatorex-ODS, 100−200 mesh; Fuji Silysia Chemical, Ltd., Aichi, Japan) was used for open column chromatography. Reversed-phase preparative HPLC was carried out on a 25 cm × 10 mm i.d. Pegasil ODS II (Senshu Scientific Co., Ltd., Tokyo, Japan) C_{18} silica column, at 25 °C with H_2O−CH_3CN−acetic acid (90:10:3, v/v/v) as mobile phase at 3 mL/min. A refractive index detector was used for reversed-phase HPLC.

Materials. Red-mold rice was prepared as follows.[5] Well-milled rice was immersed in H_2O for 12 h, removed from the H_2O, and autoclaved for 30 min at 121 °C. *M. pilosus* IFO 4520 obtained from the Institute for Fermentation (IFO), Osaka, Japan, was inoculated into the cooked paddy rice and cultured for 14 days at 30 °C under aerobic conditions. The rice was dried at 70 °C to about 8% moisture content.

Extraction and Fractionation. Red-mold rice (1.5 kg) was extracted with 12 L of 70% EtOH for 30 min under stirring. The mixture was suction-filtered, and the residue was washed with 3 L of 70% EtOH. The combined filtrate and wash were evaporated in vacuo at 50 °C to leave the 70% EtOH extract (152.9 g). The extract was suspended in 1 L of H_2O, and the suspension was extracted with EtOAc (5 times with 0.5 L each) and then with *n*-butanol (*n*-BuOH) (5 times with 0.5 L each). The EtOAc and *n*-BuOH solutions and the remaining H_2O phase were evaporated in vacuo at 50 °C to yield the EtOAc (26.1 g), *n*-BuOH (23.2 g), and H_2O (100.4 g) soluble fractions.

Isolation. A portion of the *n*-BuOH fraction (20.0 g) was subjected to chromatography on an octadecyl silica column (100 g). Stepwise gradient elution of the column with a mixture of solvents yielded the following 10 fractions with a descending order of polarity: fractions A [9.67 g; MeOH (M)−H_2O (H) (3:7, v/v) 1.5 L], B [1.76 g; M−H (3:7) 2.0 L], C [0.35 g; M−H (3:7) 4.0 L and M−H (7:3) 1.0 L], D [2.64 g; M−H (7:3) 3.0 L], E [0.56 g; M−H (7:3) 6.0 L and M−H (9:1) 1.0 L], F [0.46 g; M−H (9:1) 1.0 L], G [0.58 g; M−H (9:1) 2.0 L and M 0.5 L], H [0.59 g; M 2.5 L], I [0.44 g; M 2.5 L and M−EtOAc (E) (9:1) 3.5 L], and J [0.44 g; M−E (1:1) 1.5 L and E 2.0 L]. Preparative HPLC of a portion of fraction B (45 mg) yielded compounds **1** [10 mg; retention time (t_R) 30 min] and **2** (30 mg; t_R 26 min).

(+)-Monascumic Acid (1): colorless needles, mp 122−123 °C; $[\alpha]^{25}_D$ +3.7° (*c* 0.39, acetone); IR ν_{max} 3420 and 3290 (>NH), 1717, 1681, and 1239 (−COOH) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.95 (2H, s, 2 × COO*H*, s), 2.45 (1H, d, J = 16.3 Hz, H_b-3), 1.98 (1H, d, J = 16.3 Hz, H_a-3), 1.91 (1H, s, H-1), 1.70 (1H, dd, J = 8.8, 12.2 Hz, H_b-6), 1.66 (1H, m, H-7), 1.36 (1H, dd, J = 2.2, 12.2 Hz, H_a-6), 1.31 (3H, s, H-5), 0.87 (3H, d, J = 6.4 Hz, H-8 or H-9), 0.81 (3H, d, J = 6.1 Hz, H-9 or H-8); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.4 (C, C-11), 172.8 (C, C-10), 77.6 (C, C-4), 72.2 (C, C-2), 45.0 (CH_2, C-3), 43.7 (CH_2, C-6), 24.8 (CH_3, C-8 or C-9), 23.7 (CH, C-7), 21.9 (CH_3, C-9 or C-8), 21.7 (CH_3, C-5); ^{1}H NMR (pyridine-d_5, 400 MHz) δ 9.49 (2H, 2 × COO*H*, s), 2.94 (1H, d, J = 16.4 Hz, H_b-3), 2.76 (1H, d, J = 16.4 Hz, H_a-3), 2.34 (1H, dd, J = 8.3, 12.4 Hz, H_b-6), 2.11 (1H, m, H-7), 1.84 (3H, s, H-5), 1.76 (1H, br d, J = 12.4 Hz, H_a-6), 1.15 (3H, d, J = 6.6 Hz, H-8 or H-9), 1.01 (3H, d, J = 6.6 Hz, H-9 or H-8); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 174.8 (2C, CH_3, C-10 and C-11), 79.0 (C, C-4), 74.5 (C, C-2), 46.4 (CH_2, C-3), 45.1 (CH_2, C-6), 25.3 (CH, C-7), 25.1 (CH_3, C-8 or C-9), 22.5 (CH_3, C-9 or C-8), 22.3 (CH_3, C-5); NOESY and difference NOE experiments showed significant NOE correlations between H_a-6 (δ 1.36 in DMSO-d_6, 1.76 in pyridine-d_5) and H_b-3 (δ 2.45, 2.94)−H-5 (δ 1.31, 1.84); EIMS *m*/*z* 215 [M]$^+$ (6), 197 [M − H_2O]$^+$ (26), 172 [M − C_3H_7 (isopropyl)]$^+$ (41), 170 [M − COOH]$^+$ (16), 158 [M − C_4H_9 (isobutyl)]$^+$ (6), 154 [M − COOH − CH_3 − H]$^+$ (81), 141 (6), 130 (35), 112 [M − C_4H_9 − COOH − H]$^+$ (23), 88 (100), 85 (35), 70 (17), 58 (35); HREIMS *m*/*z* 215.1157 (calcd for $C_{10}H_{17}NO_4$, 215.1157).

(−)-Monascumic Acid (2): colorless needles, mp 122−123 °C; $[\alpha]^{25}_D$ −4.4° (*c* 0.36, acetone); IR ν_{max} 3422 and 3290 (>NH), 1718, 1682, and 1239 (−COOH) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.95 (2H, s, 2 × COO*H*, s), 2.45 (1H, d, J = 16.3 Hz, H_b-3), 1.99 (1H, d, J = 16.3 Hz, H_a-3), 1.91 (1H, s, H-1), 1.71 (1H, dd, J = 9.0, 12.8 Hz, H_b-6), 1.66 (1H, m, H-7), 1.36 (1H, dd, J = 2.9, 12.8 Hz, H_a-6), 1.31 (3H, s, H-5), 0.88 (3H, d, J = 6.6 Hz, H-8 or H-9), 0.81 (3H, d, J = 6.4 Hz, H-9 or H-8); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.6 (C, C-11), 172.8 (C, C-10), 77.6 (C, C-4), 72.2 (C, C-2), 45.0 (CH_2, C-3), 43.7 (CH_2, C-6), 24.8 (CH_3, C-8 or C-9), 23.7 (CH, C-7), 21.9 (CH_3, C-9 or C-8), 21.6 (CH_3, C-5); ^{1}H NMR (pyridine-d_5, 400 MHz) δ 9.40 (2H, 2 × COO*H*, s), 2.93 (1H, d, J = 16.0 Hz, H_b-3), 2.76 (1H, d, J = 16.0 Hz, H_a-3), 2.33 (1H, dd, J = 8.0, 12.8 Hz, H_b-6), 2.10 (1H, m, H-7), 1.84 (3H, s, H-5), 1.74 (1H, br d, J = 12.8 Hz, H_a-6), 1.13 (3H, d, J = 6.6 Hz, H-8 or H-9), 1.00 (3H, d, J = 6.6 Hz, H-9 or H-8); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 174.9 (2C, CH_3, C-10 and C-11), 79.0 (C, C-4), 74.5 (C, C-2), 46.4 (CH_2, C-3), 45.1 (CH_2, C-6), 25.3 (CH, C-7), 25.1 (CH_3, C-8 or C-9), 22.6 (CH_3, C-9 or C-8), 22.3 (CH_3, C-5); NOESY and difference NOE experiments showed significant NOE correlations between H_a-6 (δ 1.36 in DMSO-d_6, 1.74 in pyridine-d_5) and H_b-3 (δ 2.45, 2.93)−H-5 (δ 1.31, 1.84); EIMS *m*/*z* 215 [M]$^+$ (16), 197 (67), 172 (80), 170 (45), 158 (16), 154 (100), 141 (13), 130 (70), 112 (42), 88 (97), 85 (61), 70 (25), 58 (52); HREIMS *m*/*z* 215.1160 (calcd for $C_{10}H_{17}NO_4$, 215.1157); HRFABMS *m*/*z* 216.1236 (calcd for $C_{10}H_{18}NO_4$, 216.1235).

Acknowledgment. This work was supported, in part, by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, to promote multidisciplinary research projects, and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References and Notes

(1) Tsuji, K.; Ichikawa, T.; Tanabe, N.; Abe, S.; Tarui, S.; Nakagawa, Y. *Nippon Nogeikagaku Kaishi* **1992**, *66*, 1241−1246.
(2) Endo, A. *J. Antibiot.* **1979**, *32*, 852−854.
(3) Endo, A. *J. Med. Chem.* **1985**, *28*, 401−405.
(4) Wong, H. C.; Bau, Y. S. *Plant Physiol.* **1977**, *60*, 578−581.
(5) Kohama, Y.; Matsumoto, S.; Mimura, T.; Tanabe, N.; Inada, A.; Nakanishi, T. *Chem. Pharm. Bull.* **1987**, *35*, 2484−2489.
(6) Albert, A. W. *Am. J. Cardiol.* **1988**, *62*, 10J−15J.
(7) Martinokova, L.; Juzlova, P.; Vesely D. *J. Appl. Bacteriol.* **1995**, *79*, 609−616.
(8) Akihisa, T.; Yasukawa, K. In Studies in *Natural Products Chemistry, Vol. 25. Bioactive Natural Products (Part F)*; Atta-ur-Rahman, Ed.; Elsevier Science B.V.: Amsterdam, 2001; pp 43−87.
(9) Ukiya, M.; Akihisa, T.; Tokuda, H.; Mafune, S.; Tanabe, N.; Fukuoka, T.; Nishino, H. *Abstracts for the Papers presented at the 7th Annual Meeting of the Japanese Society for Food Factors (JSoFF)*; 2002; p 41.
(10) Cromwell, N. H.; Phillips, B. *Chem. Rev.* **1979**, *79*, 331−358.
(11) Isono, K.; Asahi, K.; Suzuki, S. *J. Am. Chem. Soc.* **1969**, *91*, 7490−7505.
(12) Nomoto, K.; Mino, Y.; Ishida, T.; Yoshioka, H.; Ota, N.; Inoue, M.; Takagai, S.; Takemoto, T. *J. Chem. Soc., Chem. Commun.* **1981**, 338−339.
(13) Kobayashi, J.; Cheng, J.; Ishibashi, M.; Wälchli, M. R.; Yamamura, S.; Ohizumi, Y. *J. Chem. Soc., Perkin Trans. 1* **1991**, 1135−1137.

NP030394I