Aldehyde Dehydrogenase Inhibitors from the Mushroom *Clitocybe clavipes*

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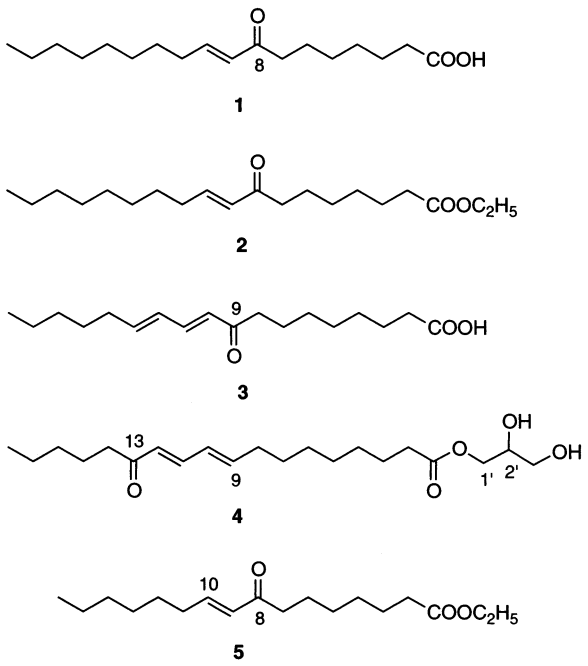
Five fatty acid derivatives including three novel compounds were isolated from the mushroom *Clitocybe clavipe*. Their structures were elucidated by spectral analyses. These compounds inhibited aldehyde dehydrogenase in vitro.

Clitocybe clavipes (Pers.: Fr.) Kummer (Hoteishimeji in Japanese) is widespread and common throughout temperate regions of the world. It is a delicious wild mushroom and popular in Japan. However, if ethanol is consumed with this mushroom, the person may experience one or more of the following symptoms: profound flushing, metallic taste, palpitations, hyperventilation, hypertension, tachycardia, nausea, vomiting, and occasionally collapse.[1,2] These symptoms are very similar to those caused by an aldehyde dehydrogenase inhibitor, coprine, isolated from the mushroom *Coprinus atramentarius*.[3−5] In addition, ingestion of the extract of *C. clavipes* in mice increased acetaldehyde concentration in their blood.[2] However, the toxic principles are still not known. This paper reports the isolation, structure determination, and biological activity of aldehyde dehydrogenase inhibitors from this mushroom.

Isolation was guided by aldehyde dehydrogenase inhibition activity of fractions. Fresh fruiting bodies of *C. clavipes* were extracted with 85% EtOH. The concentrated extract was partitioned between $CHCl_3$ and H_2O, and then ethyl acetate and H_2O. Since the $CHCl_3$-soluble fraction showed inhibitory activity, the fraction was separated by repeated silica gel chromatography and HPLC. As a result, compounds **1**−**5** were obtained as colorless oils.



The molecular formula of compound **1** was determined to be $C_{18}H_{32}O_3$ by HRFABMS. The NMR data of **1** agreed with those of a known compound, (*E*)-8-oxo-9-oactadecenoic acid.[6]

The molecular formula of **2** was determined as $C_{20}H_{36}O_3$ by HRFABMS. Its ^1H and ^{13}C NMR data were very similar to those of **1** and suggested that this compound was the ethyl ester of **1**. The position of the enone moiety was further confirmed by the HREIMS of mass fragments (Figure 1). Hydrolysis of **2** with lipase gave **1**.

Compound **3** had a molecular formula of $C_{18}H_{30}O_3$ by HRFABMS. Its ^{13}C NMR data were consistent with those of (*E*,*E*)-9-oxooctadeca-10,12-dienoic acid (Table 2).[7]

The molecular formula $C_{21}H_{36}O_5$ of **4** was suggested by HRFABMS. The ^1H NMR and ^{13}C NMR spectra of **4** indicated that this compound was a C18 fatty acid having an $\alpha,\beta,\gamma,\delta$-dienone moiety like **3**. Furthermore, this compound had a glycerol moiety [δ 4.17 (1H, dd, J = 11.6, 4.6 Hz), 4.12 (1H, dd, J = 11.6, 6.3), 3.90 (1H, m), 3.67 (1H, dd, J = 11.1, 3.4), 3.57 (1H, dd, J = 11.1, 5.5); δ 65.2, 70.2, 63.3]. The whole structure of **4** was determined by interpretation of COSY and HMBC correlations: COSY, H7/H8, H8/H9, H10/H11, H11/H12, H14/H15, H1′/H2′, H2′/H3′; HMBC, H1′/C1, H11/C13, H12/C13, H14/C13, H14/C15, H14/C16, H18/C16. The stereochemistry of C-9/C-10 was determined by the decoupling experiments of **4**; $J_{9,10}$ was 15.6 Hz. The absolute configuration at C-2′ remains undetermined.

Compound **5** had a molecular formula of $C_{18}H_{32}O_3$ by HRFABMS. Its NMR data were very similar to those of **2**. Although all the other compounds were C18 fatty acid derivatives, this compound was an ethyl ester of a C16 fatty acid. The position of the enone was determined by interpretation of HMBC correlations: H11/C12, H12/C13, H12/C14, H16/C14.

Compounds **2** and **5** were not artifacts, because MeOH extracts of this mushroom also contained both compounds. Although the free acids of **2** and **4** have already been reported, compounds **2**, **4**, and **5** were novel compounds.[7,10−12] Compound **3** and the free acid of **4** inhibited acetyl CoA carboxylase.[7]

Compounds **1**, **2**, and **5** showed relatively strong inhibitory activity against aldehyde dehydrogenase: IC_{50} of 0.28, 0.43, and 0.17 mM, respectively. Compounds **3** and **4** exhibited very weak activity, and their inhibition ratios at 0.5 mM were less than 50%. Coprine inhibits aldehyde dehydrogenase by forming an adduct with a thiol group of a cysteine in the enzyme covalently.[8,9] Although the inhibition mechanism of the obtained compounds is unclear at

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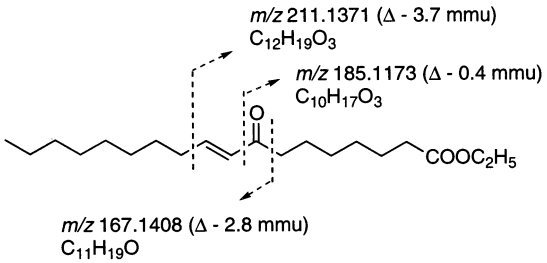

Figure 1. HREIMS of mass fragments of **2**.

the present time, these compounds may inhibit the enzyme by forming a Michael addition adduct between the enone and the thiol group in the enzyme.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR spectra were measured on a JEOL Lambda-500 spectrometer and are given in ppm (δ) downfield from internal TMS. Mass spectra were recorded on JEOL DX-303HF and DX-302 mass spectrometers. A JASCO grating infrared spectrophotometer was used to record the IR spectra. Optical rotation was measured by using a JASCO DIP-500 polarimeter. HPLC separations were performed with a JASCO Gulliver system using an ODS column (Wakopack Wakosil-II 5C18HG, 45 × 300 mm).

Fungus Materials. Mature fruiting bodies of *Clitocybe clavipes* (Pers.: Fr.) Kummer were collected at Narusawa Village, Yamanashi Prefecture in Japan, and identified by the first author. A voucher specimen of the organism (CC-99-09) is located in the department of the first author.

Extraction and Isolation. Fresh fruiting bodies of *C. clavipes* (8.0 kg) were extracted with 85% EtOH (15 L, 5 times). The solvent was concentrated under reduced pressure and partitioned between $CHCl_3$ and H_2O, and the H_2O layer was further partitioned between EtOAc and H_2O. The residue (43.1 g) obtained after removing $CHCl_3$ was fractionated by silica gel flash CC (100%, 90%, 70% $CHCl_3$/acetone, 80% $CHCl_3$/MeOH, MeOH, each 1 L) to obtain eight fractions. Fraction 5 (15.4 g) was further separated by silica gel flash CC (95%, 90%, 70% $CHCl_3$/acetone, each 350 mL) to afford nine fractions. Further purification of fraction 5-3 (980 mg) by reversed-phase HLPC (80% MeOH/H_2O) gave **1** (60.9 mg) and **5** (3.5 mg). Purification of fraction 5-4 (487 mg) by reversed-phase HLPC (80% MeOH/H_2O) afforded **2** (4.0 mg) and **4** (8.4 mg). Compound **3** (4.4 mg) was obtained from fraction 5-5 (4.9 g) by reversed-phase HLPC (99% MeOH/H_2O).

Hydrolysis of 2 to 1 with Lipase. Lipase from *Pseudomonus* sp. was obtained from Wako Pure Chemical Industries (Japan). The enzyme (2.0 mg) was suspended in 2.0 mL of 10 mM phosphate buffer (pH 7.4). To the suspension was added **2** (5.0 mg) in MeOH (1.0 mL), and the mixture was stirred slowly at 39 °C for 24 h. The reaction mixture was extracted with ethyl acetate, and the ethyl acetate-soluble fraction was passed down a silica flash column to afford **1** (3.1 mg, 68% yield).

Enzyme Inhibition Assay.[13] Aldehyde dehydrogenase from yeast was purchased from Sigma-Aldrich Fine Chemicals. The activity of the enzyme was assayed with a microplate reader at 22 °C by following the formation of NADH spectrophotometrically at 340 nm. Each faction or compound solution in MeOH (10 μL), 50 mM pyrophosphate buffer (pH 8.8, 180 μL), and 0.1 IU aldehyde dehydrogenase (10 μL) were added to each well and preincubated at 22 °C for 15 min. To the mixture were added NAD^+ (2.4 mM final concentration in the reaction mixture, 10 μL) and acetaldehyde (2.7 mM final concentration, 10 μL), and the entire plate was mixed prior to data acquisition. Data acquisition was initiated within 1 min of addition of substrate to the mixture in the microtiter wells. Inhibition was calculated by comparison of absorbance at 340 nm of each fraction or compound with that with no inhibitor. During chromatography, the data were obtained at 2−3 min after addition of substrate. For determining IC_{50}, the data were acquired at 10 min after addition of substrate.

Compound 1: colorless oil; IR (neat) ν_{max} 1702, 1687 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.80 (1H, dt, J = 15.9, 7.0 Hz, H-10), 6.06 (1H, d, J = 15.9 Hz, H-9), 2.50 (2H, t, J = 7.2 Hz, H-7), 2.32 (2H, t, J = 7.6 Hz, H-2), 2.18 (2H, dt, J = 7.0, 7.0 Hz, H-11), 1.60 (4H, m, H-3, H-6), 1.44 (2H, m, H-12), 1.23−1.31 (14H, m, H-4, H-5, H-13, H-14, H-15, H-16, H-17), 0.86 (3H, t, J = 6.7 Hz, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.7 (s, C-8), 177.2 (s, C-1), 147.5 (d, C-10), 130.3 (d, C-9), 39.9 (t, C-7), 33.8 (t, C-2), 32.5 (t, C-11), 31.8 (t, C-16), 29.3 (t, C-13*), 29.2 (t, C-5*), 29.2 (t, C-14*), 28.9 (t, C-4*), 28.8 (t, C-15*), 28.1 (t, C-12), 24.6 (t, C-3), 24.0 (t, C-6), 22.6 (t, C-17), 14.1 (q, C-18), *interchangeable; FABMS, m/z 297 $[M + H]^+$, 319 $[M + Na]^+$; HRFABMS, 297.2441 (calcd for $C_{18}H_{33}O_3$, 297.2429).

Compound 2: colorless oil; IR (neat) ν_{max} 1736, 1674 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.80 (1H, dt, J = 15.9, 7.0 Hz, H-10), 6.06 (1H, d, J = 15.9 Hz, H-9), 4.09 (2H, q, J = 7.0 Hz, H-1′), 2.50 (2H, t, J = 7.5 Hz, H-7), 2.25 (2H, t, J = 7.6 Hz, H-2), 2.18 (2H, dt, J = 7.0, 8.5 Hz, H-11), 1.59 (4H, m, H-3, H-6), 1.43 (2H, m, H-12), 1.23−1.31 (14H, m, H-4, H-5, H-13, H-14, H-15, H-16, H-17), 1.22 (3H, t, J = 7.0 Hz, H-2′), 0.85 (3H, t, J = 7.0 Hz, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.4 (s, C-8), 173.4 (s, C-1), 147.1 (d, C-10), 130.1 (d, C-9), 59.9 (t, C-1′), 39.7 (t, C-7), 34.0 (t, C-2), 32.2 (t, C-11), 31.6 (t, C-16), 29.1 (t, C-13*), 29.0 (t, C-14*), 29.0 (t, C-5*), 28.7 (t, C-15*), 28.7 (t, C-4*), 27.9 (t, C-12), 24.6 (t, C-3), 23.8 (t, C-6), 22.4 (t, C-17), 14.0 (q, C-2′), 13.9 (q, C-18), *interchangeable; FABMS, m/z 325 $[M + H]^+$, 347 $[M + Na]^+$; HRFABMS, 325.2740 (calcd for $C_{20}H_{37}O_3$, 325.2742).

Compound 3: colorless oil; IR (neat) ν_{max} 1704, 1689 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.11 (1H, m, H-11), 6.15 (2H, m, H12, H-13), 6.05 (1H, d, J = 15.3 Hz, H-10), 2.51 (2H, t, J = 7.3 Hz, H-8), 2.32 (2H, t, J = 6.7 Hz, H-2), 2.16 (2H, m, H-14), 1.60 (4H, m, H-3, H-7), 1.42 (2H, m, H-15), 1.23−1.31 (10H, m, H-4, H-5, H-6, H-16, H-17) 0.87 (3H, t, J = 6.9 Hz, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.1 (s, C-9), 178.7 (s, C-1), 145.8 (d, C-13), 143.0 (d, C-11), 128.8 (d, C-12), 127.8 (d, C-10), 40.4 (t, C-8), 33.9 (t, C-2), 33.1 (t, C-14), 31.4 (t, C-16), 29.1 (t, C-6*), 29.0 (t, C-5*), 28.9 (t, C-4*), 28.4 (t, C-15), 24.8 (t, C-3), 24.3 (t, C-7), 22.5 (t, C-17), 14.0 (q, C-18), *interchangeable; FABMS, m/z 295 $[M + H]^+$; HRFABMS, 295.2281 (calcd for $C_{18}H_{31}O_3$, 295.2273).

Compound 4: colorless oil; IR (neat) ν_{max} 1704, 1689 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.11 (1H, m, H-11), 6.14 (2H, m, H-9, H-10), 6.06 (1H, d, J = 15.3 Hz, H-12), 4.17 (1H, dd, J = 11.6, 4.6 Hz, H-1′a), 4.12 (1H, dd, J = 11.6, 6.3 Hz, H-1′b) 3.90 (1H, m, H-2′), 3.67 (1H, dd, J = 11.1, 3.4 Hz, H-3′a), 3.57 (1H, dd, J = 11.1, 5.5 Hz, H-3′b), 2.51 (2H, t, J = 7.5 Hz, H-14), 2.32 (2H, t, J = 7.6 Hz, H-2), 2.15 (2H, m, H-8), 1.60 (4H, m, H-3, H-15), 1.41 (2H, m, H-7), 1.23−1.31 (10H, m, H-4, H-5, H-6, H-16, H-17), 0.87 (3H, t, J = 6.9 Hz, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.3 (s, C-13), 174.2 (s, C-1), 145.4 (d, C-9), 142.9 (d, C-11), 129.0 (d, C-10), 127.9 (d, C-12), 70.2 (d, C-2′), 65.2 (t, C-1′), 63.3 (t, C-3′), 40.5 (t, C-14), 34.1 (t, C-2), 33.0 (t, C-8), 31.5 (t, C-16), 29.0 (t, C-4*), 29.0 (t, C-5*), 28.9 (t, C-6*), 28.6 (t, C-7), 24.8 (t, C-3), 24.1 (t, C-15), 22.5 (t, C-17), 13.9 (q, C-18). *interchangeable; FABMS, m/z 369 $[M + H]^+$; HRFABMS, 369.2325 (calcd for $C_{21}H_{37}O_5$, 369.2641); $[\alpha]^{20}_D$ −23° (c 0.10, $CHCl_3$).

Compound 5: colorless oil; IR (neat) ν_{max} 1735, 1673 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.79 (1H, dt, J = 15.9, 7.0 Hz, H-10), 6.06 (1H, d, J = 15.9 Hz, H-9), 4.09 (2H, q, J = 7.0 Hz, H-1′), 2.50 (2H, t, J = 7.3 Hz, H-7), 2.25 (2H, t, J = 7.5 Hz, H-2), 2.18 (2H, dt, J = 7.0. 7.3 Hz, H-11), 1.59 (4H, m, H-3, H-6), 1.43 (2H, m, H-12), 1.23−1.31 (10H, m, H-4, H-5, H-13, H-14, H-15), 1.23 (3H, t, J = 7.0 Hz, H-2′), 0.86 (3H, t, J = 6.7 Hz, H-16); ^{13}C NMR, ($CDCl_3$, 100 MHz), δ 200.8 (s, C-8), 173.8 (s, C-1), 147.4 (d, C-10), 130.3 (d, C-9), 60.2 (t, C-1′), 39.9 (t, C-7), 34.3 (t, C-2), 32.4 (t, C-11), 31.6 (t, C-14), 28.9 (t, C-13*), 28.9 (t, C-5*), 28.8 (t, C-4*), 28.1 (t, C-12), 24.8 (t, C-3), 24.1 (t, C-6), 22.5 (t, C-15), 14.2 (q, C-2′), 14.0 (q, C-16), *interchangeable; FABMS, m/z 297 $[M + H]^+$; HRFABMS, 297.4525 (calcd for $C_{18}H_{32}O_3$, 297.4527).

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