A New Inhibitor of 5′-Hydroxyaverantin Dehydrogenase, an Enzyme Involved in Aflatoxin Biosynthesis, from *Trichoderma hamatum*

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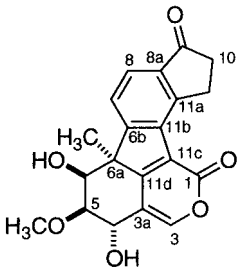
Screening for inhibitors of 5′-hydroxyaverantin dehydrogenase, an enzyme involved in aflatoxin biosynthesis, resulted in the isolation of a new metabolite (**1**) from *Trichoderma hamatum*. On the basis of spectroscopic data, **1** was determined to be 4,6-dihydroxy-5-methoxy-6a-methylcyclohexa[*de*]indano-[7,6-*e*]cyclopenta[*c*]2*H*-pyran-1,9-dione.

Aflatoxins are toxic, carcinogenic secondary metabolites produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*,[1] and contamination of foodstuffs by these toxins, which may seriously affect the health of animals and humans, is an important problem to be solved.[2] Inhibition of aflatoxin biosynthesis could be an effective way to prevent such contamination, and aflastatin has been reported to be an inhibitor of aflatoxin biosynthesis.[3]

Aflatoxins are biosynthesized from acetyl-CoA through steps catalyzed by more than 18 enzymes,[4] and 5′-hydroxyaverantin (HAVN) dehydrogenase catalyzed the conversion of HAVN to averufin in aflatoxin biosynthesis.[5,6] HAVN dehydrogenase was chosen as the target enzyme for inhibition of aflatoxin biosynthesis because it occupies an early step in aflatoxin biosynthesis, and both the enzyme and substrate were readily available in our laboratory. We therefore screened for aflatoxin biosynthesis enzyme inhibitors among the metabolites produced by soil fungi. One of them, identified as *Trichoderma hamatum* (Bon.) Bain., produced a metabolite inhibitory to the HAVN dehydrogenase.

Metabolite **1**, named TAEMC161, was isolated as a pale yellow powder. The yield was 0.4 mg/L from the culture



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filtrate of the fungus. On the basis of HREIMS and ^{13}C NMR data, **1** had the molecular formula $C_{20}H_{18}O_6$. DEPT data showed that two of the protons in the molecule bonded to oxygen. One ketone and one ester function were indicated, respectively, by carbon resonances at δ 206.7 and 173.4 and IR absorptions at 1671 and 1707 cm^{-1}. The 10 carbon resonances from δ 122.1 to 158.6 indicated five double bonds and suggested five rings in the molecule.

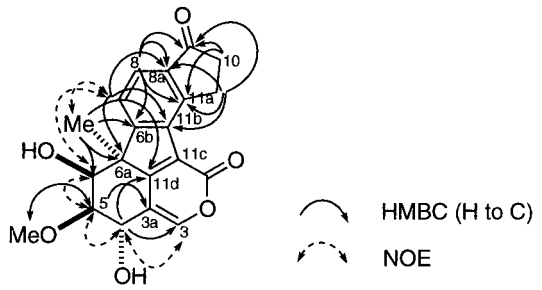

Figure 1. Selected HMBC and NOE correlations for **1**.

The structural fragments in **1**, −C(4)HOH−C(5)H(O)−C(6)HOH−, −C(7)H=C(8)H−, −C(10)H_2C(11)H_2−, and 6a-CH_3, were deduced through analysis of its $^1H-^1H$ COSY and HMQC spectra and from chemical shift considerations. These fragments were connected to other carbon atoms by analysis of the HMBC spectrum (Figure 1) as follows. The methoxy methyl protons correlated with C-5, and the methine proton of 5-H correlated with the methoxy methyl carbon, indicating that the methoxy group was attached to C-5. The methyl protons of 6a-Me correlated with C-6, C-6a, C-6b, and C-11d and indicated that C-6a was attached to the four carbons C-6, C-6b, C-11d, and 6a-Me. The connection of C-3a with the three carbons C-4, C-3, and C-11d was suggested by the correlation between 5-H and C-3a, 4-H and C-3, and 4-H and C-11d, respectively. The methylene protons of H-10 correlated with C-9 and C-11a, and those of H-11 correlated with C-8a, C-9, C-11a, and C-11b. This indicated the bonding of C-9 to C-10, of C-11 to C-11a, and of C-11a to C-8a and C-11b. The connection of C-6b to C-7 was shown by the correlation between H-7 and C-6a. The aromatic proton, H-7, correlated with C-8a and C-11b, and the aromatic proton, H-8, with the four carbons C-6b, C-8a, C-9, and C-11a. This is evidence of three bonds at C-8a, to C-8, C-9, and C-11a. Thus all atoms in **1** except for an ester function and an sp^2 carbon atom (C-11c) were bonded to each other to give a partial structure in which three bonds at C-3, C-11b, and C-11d were vacant. Connection of these vacant bonds to the ester function and C-11c afforded six possible structures, four of which were ruled out because of the extreme instability observed in the constructed molecular models. This left two possible structures, and the NMR chemical shifts of the resonances at δ 145.6 and 7.81 for C-3 and 3-H, respectively and at δ 122.1 for C-3a favored the structure **1**.

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Compound **1** has four chiral centers, and determination of their stereochemistry was attempted by measuring NOE. The key NOEs observed (Figure 1) indicated that **1** has the relative stereochemistry as shown.

The IC_{50} of **1** to HAVN dehydrogenase activity was 250 μM. Three millimoles of **1** had no effect on malate dehydrogenase, which needs the same coenzyme, NAD^+, as HAVN dehydrogenase. This is a strong evidence of the specific inhibition by **1** of HAVN dehydrogenase. To our surprise, even 1 mM of **1** produced no inhibition of aflatoxin biosynthesis in vivo. The reason is not yet clear.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Shimadzu UV-2200 UV−vis recording spectrophotometer and IR spectra on a JASCO FT/IR-5300 spectrometer. Optical rotation was measured with a Horiba SEPA-200 high sensitive polarimeter. EIMS were obtained with a JEOL AX505HA spectrometer (direct probe, 70 eV). NMR spectra were measured in $CDCl_3$ with a JEOL Lambda 400 NMR spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.20).

Fungal Material. The fungus isolated from a soil sample collected in Chizu-cho, Tottori Prefecture, Japan, which was identified as *Trichoderma hamatum* (Bon.) Bain. based on its morphological features,[7] has been maintained on potato dextrose agar.

Fermentation and Extraction. The fungus was grown without shaking at 24 °C for 16 days in the dark in 50 500-mL conical flasks containing liquid medium (200 mL/flask) composed of glucose (30 g/L), the extract from 200 g/L of potato, and H_2O. Metabolites were extracted from the culture filtrate with EtOAc (3 × 10 L) after adjusting the pH to 2 with HCl. The EtOAc extracts were dried over Na_2SO_4, concentrated, washed with 1 M $NaHCO_3$ (2 × 0.5 volume), and then dried over Na_2SO_4 and concentrated to dryness.

Purification. The residue (172 mg) was purified by silica gel CC (Daisogel IR-60, 15 × 220 mm). Elution was done with 250 mL (50 mL × 5) each of 0, 10, 20, and 40% acetone in *n*-hexane. The first and second fractions eluting with 40% acetone in *n*-hexane (20 mg) were purified by HPLC, giving **1** as a pale yellow powder (3.6 mg, t_R 26.6 min). HPLC was done in a Cosmosil $5C_{18}$-AR column (Nacalai Tesque, 10 × 250 mm) with 60% MeOH as the solvent at the flow rate of 1.0 mL/min, monitoring at 220 nm.

TAEMC161 (1): $[\alpha]^{22}_D$ −36° (*c* 0.5, EtOH); UV (EtOH) λ_{max} (log ϵ) 250 (4.19), 319 (3.86), 399 (2.70) nm; IR (film) ν_{max} 3331 (OH), 1707 (lactone CO), 1671 (ketone CO) cm^{-1}; ^1H NMR δ 8.27 (1H, d, J = 8.1 Hz, H-7), 7.96 (1H, d, J = 8.1 Hz, H-8), 7.81 (1H, s, H-3), 5.15 (1H, br.d, J = 4.6 Hz, H-4), 4.33 (1H, dd, J = 6.3, 5.9 Hz, H-6), 3.81 (1H, m, H-11), 3.74 (3H, s, 5-OMe), 3.70 (1H, m, H-11), 3.56 (1H, dd, J = 5.9, 4.6 Hz, H-5), 3.44 (1H, br.d, J = 6.3 Hz, 6-OH), 2.94 (1H, br.s, 4-OH), 2.73 (2H, m, H-10), 1.73 (3H, s, 6a-Me); ^{13}C NMR δ 206.7 (s, C-9), 173.4 (s, C-1), 158.6 (s, C-6b), 158.0 (s, C-11a), 145.7 (s, C-11c), 145.6 (d, C-3), 142.4 (s, C-11d), 136.9 (s, C-11b), 129.8 (s, C-8a), 127.3 (d, C-8), 127.3 (d, C-7), 122.1 (s, C-3a), 81.7 (d, C-5), 71.7 (d, C-6), 61.6 (d, C-4), 60.7 (q, 5-OMe), 42.3 (s, C-6a), 36.5 (t, C-10), 30.5 (q, 6a-Me), 28.4 (t, C-11); long-range correlations in the HMBC spectrum (optimized for $J_{C,H}$ of 7 Hz) C-3 (H-4), C-3a (H-3, H-4, H-5), C-4 (H-5, H-6), C-5 (H-4, 5-OMe), C-6 (H-4, H-5, 6a-Me), C-6a (H-6, H-7, 6a-Me), C-6b (H-6, H-8, 6a-Me), C-8a (H-7, H-8, H-11), C-9 (H-8, H-10, H-11), C-10 (H-11), C-11 (H-10), C-11a (H-8, H-10, H-11), C-11b (H-7, H-11), C-11d (H-3, H-4, 6a-Me), 5-OMe (H-5), 6a-Me (H-6); EIMS *m*/*z* 354 $[M]^+$ (4), 336 (13), 308 (37), 280 (100), 265 (41), 252 (17), 237 (26), 165 (16), 152 (14); HREIMS *m*/*z* 354.1107 (calcd for $C_{20}H_{18}O_6$, 354.1103).

Enzyme Preparation. *Aspergillus parasiticus* NIAH-26[8] was grown without shaking at 28 °C for 4 days in the dark in 10 500-mL conical flasks containing liquid medium (100 mL/flask) composed of sucrose (200 g/L), yeast extract (20 g/L), and H_2O. The cytosol fraction was prepared from NIAH-26 mycelia as described previously[5,6] and brought to 30% saturation with ammonium sulfate. After centrifugation at 10000*g* for 10 min, the supernatant was brought to 60% saturation with ammonium sulfate, and the resulting pellet collected by centrifugation. The pellet was dissolved in a small amount of 20 mM Tris-HCl (pH 8.5) buffer containing 20 mM EDTA and 1 mM DTT (buffer A), and ammonium sulfate was added to give 20% saturation. This solution was loaded on a BUTYL TOYOPEAL 650 M column (2.2 × 8.0 cm) that had been equilibrated with buffer A supplemented with 0.86 M ammonium sulfate. The column was washed with 60 mL of the same buffer, and the enzyme eluted stepwise with 90 mL each of buffer A containing 0.8 M ammonium sulfate and buffer A containing 0.4 M ammonium sulfate. A 7.7 mL portion of the eluate was collected as one fraction, and fractions 25−27 with enzyme activity were combined. Glycerol was added to the combined fractions to 10% of the contents, and the solution stored at −80 °C before use in the enzyme assay.

Enzyme Assay. Inhibition of HAVN dehydrogenase by metabolites was detected as follows: 31 μL of 50 mM Tris-HCl buffer (pH 8.5) and 1 μL of a 4000 mg/L acetone solution of HAVN, 10 μL of enzyme, 5 μL of 20 mM NAD^+, and 3 μL of the sample in acetone were combined in a 1.5 mL microtube. After incubation at 33 °C for 30 min, the product, averutin, was extracted with EtOAc (3 × 100 μL), and its amount determined by HPLC in a DAISOPAK SP-50-5-ODS-AP (DAISO, 10 × 250 mm) column with MeOH−H_2O−AcOH (95:4:1 v/v/v) as the solvent at the flow rate of 0.8 mL/min, monitoring at 290 nm. Malate dehydrogenase (EC 1.1.1.37, Oriental Yeast Co., LTD, Japan, from yeast) activity was measured as reported in the literature.[9]

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