Aurovertins F–H from the Entomopathogenic Fungus *Metarhizium anisopliae*[1]

Miwa Azumi,[†] Kei-ichi Ishidoh,[‡] Hiroshi Kinoshita,[‡] Takuya Nihira,[‡] Fumio Ihara,[§] Tsuyoshi Fujita,[⊥] and Yasuhiro Igarashi*[,†]

Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan, The International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, National Institute of Fruit Tree Science, 92-24 Nabeyashiki, Shimo-kuriyagawa, Morioka, Iwate 020-0123, Japan, and Suntory Institute for Bioorganic Research, 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka 618-8503, Japan

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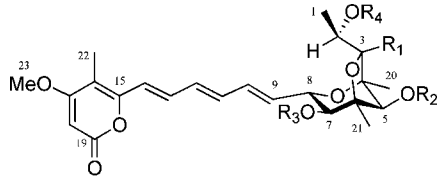
Analysis of fermentation broth of the entomopathogenic fungus *Metarhizium anisopliae* has led to isolation of aurovertin D (**1**) and three new aurovertin-type metabolites, aurovertin F (**2**), aurovertin G (**3**), and aurovertin H (**4**). Their structures were determined on the basis of spectroscopic analyses and chemical conversions.

Aurovertins are secondary metabolites initially identified as toxic substances in fermentation broth of the fungus *Calcarisporium arbuscula*.[2] Aurovertins are of polyketide origin and are characterized by a 2,6-dioxabicyclo[3.2.1]octane ring system with a conjugated α-pyrone moiety.[3] There have been five aurovertins (A−E) structurally elucidated to date. Relative and absolute configurations of aurovertin B were determined by spectroscopic analysis and total synthesis, respectively.[4,5] Structures of aurovertins A, C, and D were determined on the basis of spectroscopic comparison with aurovertin B.[3] Aurovertin E was also isolated from the fungus *Albatrellus confluens*.[6] Aurovertin B binds to F1-ATPase and thus inhibits ATP synthesis and hydrolysis in mitochondorial enzyme systems.[7] During our continuing investigation of bioactive microbial secondary metabolites, three new aurovertins, aurovertin F (**2**), aurovertin G (**3**), and aurovertin H (**4**), were isolated from the culture broth of the entomopathogenic fungus *Metarizhium anisopliae* HF260 along with previously reported aurovertin D (**1**). The structures of these compounds were determined by combined spectroscopic and chemical methods.



1: R_1 = H, R_2 = Ac, R_3 = R_4 = H
1a: R_1 = H, R_2 = R_3 = R_4 = Ac
2: R_1 = R_2 = R_3 = R_4 = H
3: R_1 = OH, R_2 = Ac, R_3 = R_4 = H
3a: R_1 = OH, R_2 = R_3 = R_4 = Ac
4: R_1 = OH, R_2 = H, R_3 = Ac, R_4 = H

The producing strain *M. anisopliae* HF260 was isolated from a soil sample collected in Okinawa, Japan, by the baiting method.[8] In the course of screening for plant growth regulating substances, the 1-butanol extract of strain HF260 inhibited seed germination of barnyard millet. Investigation of active principles led to the isolation of aurovertin D (**1**) and three new aurovertins. The major aurovertin produced by strain HF260 was aurovertin D (**1**). Compound **1** has an additional OH group at C-2 of aurovertin B, but the relative and absolute configurations are obscure. Therefore, the structural analysis of a series of aurovertins from our strain

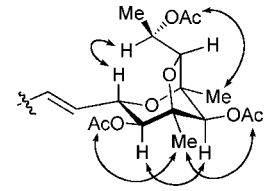


Figure 1. Key NOESY correlations for **1a**.

was started from **1**. The ^{1}H and ^{13}C NMR data of **1** were identical to those reported in the literature.[3] The relative configuration of **1** was elucidated by analyzing the NOESY spectrum of its acetate (**1a**). A NOESY correlation between H-2 and H-8 indicated the anti-relationship of H-2 and H-3, which was consistent with the large coupling constant between H-2 and H-3 (9.0 Hz). The key NOESY correlation was observed between the methyl of 2-OAc and the angular methyl at C-20, illustrating the preferable conformation where the 2-OAc group is oriented on the same side as Me-20 while Me-1 is on the opposite side. Accordingly, the relative configuration at C-2 was determined as depicted in Figure 1. Since the optical rotation of **1** shows a negative value of $[\alpha]^{23}_D$ −55.7 (*c* 0.5, EtOH) similar to aurovertin B {natural compound: $[\alpha]^{20}_D$ −50.6 (EtOH);[4] synthetic compound: $[\alpha]^{27}_D$ −57.9 (*c* 0.125, EtOH)[5]}, it is clear that **1** has the same absolute configuration as aurovertin B.

Aurovertin F (**2**) was obtained as a yellow powder that analyzed for $C_{23}H_{30}O_8$ by HRFABMS and NMR data. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** except for the loss of the acetyl group. The upfield shift of H-5 clearly indicated that **2** was a deacetylated analogue of **1**, which was supported by the observed HMBC correlations. Compound **2** was alternatively obtained by methanolysis of **1** in MeOH with K_2CO_3. Additionally, acetylation of **2**gave the triacetate of which ^{1}H and ^{13}C NMR spectra and the optical rotation were identical to those of acetate **1a** prepared from **1**.

Aurovertin G (**3**) had a UV spectrum nearly identical to that of **1**, but the molecular formula indicated that compound **3** contained an additional oxygen atom. Since the downfield chemical shift of C-3 (δ 105.4) was characteristic of an acetal or hemiacetal carbon, it was clear that **3** had an OH rather than a hydrogen atom at C-3. This was also consistent with the quartet signal of H-2 and HMBC correlations of H-1 to C-3 and 3-OH (δ 3.60) to C-2 and C-3. The key NOESY correlations between H-2 and H-8, and Me-20 and 2-OAc, were observed in the NOESY spectrum of the acetate **3a**, which was prepared from **3**. The optical rotation of **3** was $[\alpha]_D$ −47.4, a value similar to that of **1**, suggesting that **3** has the same absolute configuration as **1**, but the CD spectrum of **3** ($\Delta\epsilon_{222}$ −0.614, $\Delta\epsilon_{252}$ +0.391, $\Delta\epsilon_{353}$ −1.089) was somewhat different from that of **1** ($\Delta\epsilon_{213}$ +2.317, $\Delta\epsilon_{269}$ −3.128, $\Delta\epsilon_{361}$ −0.525). Then, we examined the CD spectra of the acetates **1a** and **3a**. The CD spectrum of **3a** showed a positive Cotton effect at 269 nm ($\Delta\epsilon$

* To whom correspondence should be addressed. Tel: +81-766-56-7500. Fax: +81-766-56-2498. E-mail: yas@pu-toyama.ac.jp.
[†] Toyama Prefectural University.
[‡] Osaka University.
[§] National Institute of Fruit Tree Science.
[⊥] Suntory Institute for Bioorganic Research.

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Table 1. NMR Data for Aurovertins F−H (**2**−**4**)

	aurovertin F (**2**)		aurovertin G (**3**)			aurovertin H (**4**)	
position	δ_H, mult (*J* Hz)[a]	δ_C[b]	δ_H, mult (*J* Hz)[a]	δ_C[b]	HMBC	δ_H, mult (*J* Hz)[a]	δ_C[b]
1	1.39 d (6.4)	22.2	1.36 d (6.4)	17.6	2, 3	1.37 d (6.4)	16.2
2	4.25 dq (9.0, 6.4)	65.7	4.15 q (6.4)	66.7	1, 3, 4	4.23 q (6.4)	64.9
3	3.81 d (9.0)	86.2		105.4	2, 3		104.4
4		84.0		84.5			83.8
5	3.44 s	81.0	4.83 s	80.8	3, 4, 7, 24	3.55 d (5.2)	79.8
6		84.1		84.5			83.5
7	3.20 d (7.9)	76.4	3.35 d (8.0)	76.4	6, 8, 9	4.84 d (8.6)	74.4
8	4.10 dd (7.9, 6.2)	78.1	4.02 dd (8.0, 5.6)	78.0	7, 9, 10	4.11 dd (8.6, 7.0)	73.5
9	5.91 dd (14.3,6.2)	134.5	5.92 dd (13.9, 5.6)	133.5	8, 10, 11	5.71 dd (15.4, 7.0)	131.5
10	6.44 dd (14.3, 10.8)	131.6	6.46[c] m	131.8	8, 9, 12	6.35[c] m	131.2
11	6.48 dd (14.3, 10.8)	137.0	6.47[c] m	136.8	9, 13	6.43[c] m	135.5
12	6.37 dd (14.3, 11.0)	132.1	6.37[c] m	132.3	13, 14	6.42[c] m	131.3
13	7.18 dd (14.9, 11.0)	135.6	7.17 dd (14.6, 10.8)	135.6	12, 14, 15	7.14 dd (15.1, 10.8)	134.6
14	6.36 d (14.9)	119.6	6.37 d (14.6)	119.6	13, 15, 16	6.37 d (15.1)	120.2
15		154.3		154.2			154.2
16		108.1		108.2			108.4
17		170.6		170.7			170.7
18	5.50 s	88.8	5.50 s	88.8	16, 17, 19	5.51 s	87.7
19		163.5		163.8			163.8
20	1.49 s	17.9	1.48 s	16.7	3, 4, 5	1.57 s	15.4
21	1.37 s	14.6	1.33 s	15.6	5, 6, 7	1.32 s	14.2
22	1.97 s	8.9	1.97 s	8.8	15, 16, 17	1.97 s	7.7
23	3.83 s	56.2	3.84 s	56.2	17	3.84 s	55.0
24				168.6[d]			169.9[f]
25			2.21[e] s	20.7[e]		2.10[g] s	19.6[g]
3-OH			3.60 br.s		2, 3		
5-OH						4.05 d (5.2)	

[a] 400 MHz, $CDCl_3$. [b] 100 MHz, $CDCl_3$. [c] Overlapping signals. [d] 5-O*C*OCH$_3$. [e] 5-OCO*CH$_3$*. [f] 7-O*C*OCH$_3$. [g] 7-OCO*CH$_3$*.

+0.825) and negative Cotton effects at 231 ($\Delta\epsilon$ −1.480) and 351 nm ($\Delta\epsilon$ −0.812). This Cotton effect pattern was in good agreement with that of **1a** ($\Delta\epsilon_{233}$ −0.451, $\Delta\epsilon_{265}$ +0.887, $\Delta\epsilon_{350}$ −0.467), finally establishing that **1** and **3** have the same absolute configuration.

Aurovertin H (**4**) had the same molecular formula as **3**. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** with those of **3** revealed the upfield shift of H-5 (δ 3.55) and the downfield shift of H-7 (δ 4.84), indicating that the C-7 OH group was acetylated. This was supported by the HMBC correlation from H-7 to the acetyl carbonyl (δ 169.9). Aurovertin H (**4**) was thus determined to be the regioisomer of **3**. The NMR spectra and the optical rotation of the acetate derived from **4** were identical to those for the acetate **3a**, thereby confirming that **3** and **4** have the same relative and absolute configurations.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-3000 polarimeter. CD spectra were recorded in EtOH on a JASCO J-720 spectropolarimeter. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were recorded on a Shimadzu FTIR-300 spectrophotometer. NMR spectra were obtained on a JEOL JNM-LA400, a Bruker AV400 M, or a Bruker AVANCE 500 spectrometer. HRFABMS was measured on a JEOL JMS-HX110 spectrometer. HRESIMS were recorded on a Bruker microTOF focus spectrometer. Silica gel 60-C18 (Nakalai Tesque 250–350 mesh) was used for ODS column chromatography. HPLC separation was performed using a Cosmosil 5C18-AR-II (Nacalai Tesque Inc., 20 × 250 mm) with a photodiode array detector.

Microorganism. The entomopathogenic fungus *Metarhizium anisopliae* HF260 was isolated from a soil sample collected in Okinawa, Japan, by using the baiting method.[8] The isolated strain was identified as *M. anisopliae* according to its morphology. After the conidia had developed on PDA slants in test tubes at 25 °C, the strain was kept at −30 °C as a stock culture.

Fermentation. The strain *M. anisopliae* HF260 cultured on a potato dextrose agar medium (Difco Laboratories) was inoculated into 500 mL flasks each containing 100 mL of production medium consisting of soluble starch 2%, glucose 0.5%, glycerol 2%, Pharmamedia (Traders Protein) 1.5%, yeast extract (Difco Laboratories) 0.4%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were cultured on a rotary shaker (200 rpm) at 30 °C for 5 days. For the isolation of aurovertins F and H, fermentation was continued for an additional 10 days.

Extraction and Isolation. A 5-day-old fermentation broth of strain HF260 (100 mL × 20 flasks) was extracted with 1-butanol (100 mL per flask) on a rotary shaker (200 rpm) for an additional 1 h. The mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent provided approximately 1 g of extract per 1 L of culture. The crude extract was subjected to reversed-phase ODS column chromatography with a step gradient of MeCN−0.15% KH_2PO_4 buffer solution (pH 3.5) (2:8, 3:7, 4:6, 5:5, 6:4, and 8:2 v/v). Evaporation of fractions 4 and 5, followed by extraction with EtOAc, provided 0.6 and 0.7 g of yellow powders, respectively. Final purification was conducted by reversed-phase HPLC separation with 35% MeCN in 0.15% KH_2PO_4 buffer solution (pH 3.5) to yield aurovertin D (**1**, 106 mg) from fraction 5 and aurovertins F (**2**, 7 mg), G (**3**, 30 mg), and H (**4**, 7 mg) from fraction 4.

Aurovertin F (2): yellow powder; $[\alpha]^{25}_D$ −63.7 (*c* 0.07, EtOH); IR (KBr) 3430, 1680; UV (MeOH) λ_{max} (log ϵ) 229 (4.07), 268 (4.31), 274 (4.32), 367 (4.29); ^{1}H and ^{13}C NMR (Table 1); HRFABMS *m*/*z* $[M + H]^+$ 435.2019 (calcd for $C_{23}H_{31}O_8$435.2023).

Aurovertin G (3): yellow powder; $[\alpha]^{24}_D$ −47.4 (*c* 0.5, EtOH); IR (KBr) 3280, 1700; UV (MeOH) λ_{max} (log ϵ) 230 (3.91), 268 (4.19), 274 (4.20), 366 (4.19); ^{1}H and ^{13}C NMR (Table 1); HRESIMS *m*/*z* $[M + Na]^+$ 515.1889 (calcd for $C_{25}H_{32}O_{10}Na$ 515.1891).

Aurovertin H (4): yellow powder; $[\alpha]^{25}_D$ −36.8 (*c* 0.5, EtOH); IR (KBr) 3430, 1690; UV (MeOH) λ_{max} (log ϵ) 228 (3.97), 268 (4.11), 274 (4.12), 356 (3.98); ^{1}H and ^{13}C NMR (Table 1); HRESIMS *m*/*z* $[M + H]^+$ 493.2079 (calcd for $C_{25}H_{33}O_{10}$ 493.2068).

Conversion of Aurovertin D (1) to Aurovertin F (2). A mixture of **1** (10 mg) and K_2CO_3 (25 mg) in MeOH (1.5 mL) was stirred for 8 h at room temperature. The reaction mixture was diluted with water and loaded onto a column of Diaion HP-20 resin (Mitsubishi Chemical Co.). After washing with water, the column was eluted with methanol and the eluate was concentrated to yield **2** (7 mg). Its NMR data and HPLC retention time were in good agreement with those of natural **2**. Optical rotation was $[\alpha]^{25}_D$ −62.5 (*c* 0.2, EtOH).

Acetylation of Aurovertin D (1). A mixture of **1** (10 mg) and acetic anhydride (1 mL) in dry pyridine (1 mL) was stirred for 8 h at room temperature. The reaction mixture was quenched with ice–water

and extracted with EtOAc. The organic layer was successively washed with diluted aqueous HCl, saturated aqueous $CuSO_4$, saturated aqueous $NaHCO_3$, and brine and dried over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo* and purified by HPLC separation to give 2,7-diacetylaurovertin D (**1a**, 9 mg): $[\alpha]^{25}_D$ −79.6 (*c* 0.5, EtOH); CD (EtOH) $\Delta\epsilon_{233}$ −0.451, $\Delta\epsilon_{265}$ +0.887, $\Delta\epsilon_{350}$ −0.467; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.13 (3H, s, H-21), 1.26 (3H, s, H-20), 1.44 (3H, d, J = 6.2 Hz, H-1), 1.97 (3H, s, H-22), 2.04 (3H, s, 2-OCOCH_3), 2.08 (3H, s, 7-OCOCH_3), 2.18 (3H, s, 5-OCOCH_3), 3.83 (3H, s, H-23), 3.97 (1H, d, J = 9.6 Hz, H-3), 4.28 (1H, dd, J = 8.7, 7.2 Hz, H-8), 4.83 (1H, d, J = 8.7 Hz, H-7), 4.89 (1H, s, H-5), 5.33 (1H, dq, J = 9.6, 6.2 Hz, H-2), 5.51 (1H, s, H-18), 5.70 (1H, dd, J = 14.3, 7.2 Hz, H-9), 6.33 (1H, m, H-12), 6.37 (1H, d, J = 15.1 Hz, H-14), 6.38 (1H, m, H-11), 6.40 (1H, m, H-10), 7.12 (1H, dd, J = 15.0, 10.5 Hz, H-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.9 (C-22), 15.1 (C-21), 17.3 (C-20), 18.4 (C-1), 20.7 (2-OCO*C*H$_3$), 20.7 (7-OCO*C*H$_3$), 21.3 (5-OCO*C*H$_3$), 56.2 (C-23), 67.3 (C-2), 74.8 (C-8), 75.1 (C-7), 81.0 (C-5), 82.7 (C-3), 82.9 (C-6), 84.1 (C-4), 89.0 (C-18), 108.2 (C-16), 120.0 (C-14), 131.9 (C-9), 132.8 (C-11), 133.4 (C-12), 135.4 (C-10), 136.5 (C-13), 154.2 (C-15), 163.6 (C-19), 169.5 (2-O*C*OCH$_3$), 169.7 (5-O*C*OCH$_3$), 169.7 (7-O*C*OCH$_3$), 170.5 (C-17); HRESIMS *m*/*z* [M + Na]$^+$ 583.2149 (calcd for $C_{29}H_{36}O_{11}Na$ 583.2155).

Acetylation of Aurovertin F (2). Aurovertin F (**2**) was acetylated in the same manner as described above. The NMR data and HPLC retention time of the acetate derived from **2** were identical to those data of **1a** derived from aurovertin D (**1**). Optical rotation was $[\alpha]^{25}_D$ −82.8 (*c* 0.08, EtOH).

Acetylation of Aurovertin G (3). 2,7-Diacetylaurovertin G (**3a**) was prepared in the same manner as described above: $[\alpha]^{26}_D$ −80.1 (*c* 0.5, EtOH); CD (EtOH) $\Delta\epsilon_{231}$ −1.480, $\Delta\epsilon_{269}$ +0.825, $\Delta\epsilon_{351}$ −0.812; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.19 (3H, s, H-21), 1.41 (3H, s, H-20), 1.43 (3H, d, J = 6.3 Hz, H-1), 1.97 (3H, s, H-22), 2.08 (3H, s, 2-OCOCH_3), 2.10 (3H, s, 7-OCOCH_3), 2.20 (3H, s, 5-OCOCH_3), 3.83 (3H, s, H-23), 4.29 (1H, dd, J = 8.6, 7.1 Hz, H-8), 4.85 (1H, d, J = 8.6 Hz, H-7), 4.94 (1H, s, H-5), 5.39 (1H, q, J = 6.3 Hz, H-2), 5.50 (1H, s, H-18), 5.70 (1H, dd, J = 14.5, 7.1 Hz, H-9), 6.36 (1H, m, H-12), 6.37 (1H, d, J = 15.1 Hz, H-14), 6.39 (1H, m, H-11), 6.40 (1H, m, H-10), 7.16 (1H, dd, J = 14.9, 10.2 Hz, H-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.4 (C-22), 13.4 (C-1), 15.3 (C-21), 16.3 (C-20), 20.1 (2-OCO*C*H$_3$), 20.1 (7-OCO*C*H$_3$), 20.7 (5-OCO*C*H$_3$), 55.6 (C-23), 69.0 (C-2), 74.8 (C-7), 75.0 (C-8), 80.2 (C-5), 83.1 (C-4), 84.3 (C-6), 88.4 (C-18), 104.0 (C-3), 107.8 (C-16), 119.6 (C-14), 131.0 (C-9), 132.6 (C-11), 133.3 (C-12), 134.7 (C-13), 135.8 (C-10), 153.6 (C-15), 163.0 (C-19), 167.6 (5-O*C*OCH$_3$), 169.0 (2-O*C*OCH$_3$), 169.2 (7-O*C*OCH$_3$), 170.0 (C-17); HRESIMS *m*/*z* [M + H]$^+$ 577.2287 (calcd for $C_{29}H_{37}O_{12}$ 577.2279).

Acetylation of Aurovertin H (4). Aurovertin H (**4**) was acetylated in the same manner as described above. The NMR data and HPLC retention time of the acetate derived from **4** were identical to those data of **3a** derived from aurovertin G (**3**). Optical rotation was $[\alpha]^{25}_D$ −80.5 (*c* 0.02, EtOH).

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