Okaramines H and I, New Okaramine Congeners, from *Aspergillus aculeatus*

Hideo Hayashi,* Kouji Furutsuka, and Yoshihito Shiono

Department of Applied Biological Chemistry, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan

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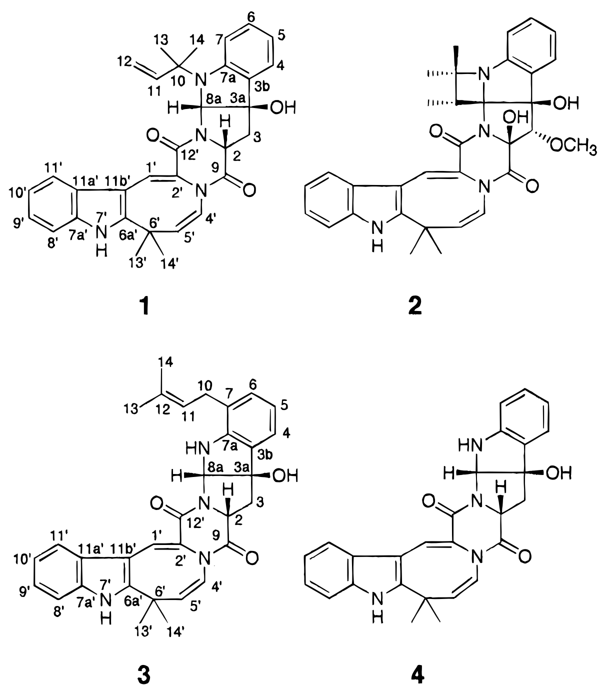
Two new congeners of okaramine, okaramines H (**3**) and I (**4**), were isolated from okara fermented with *Aspergillus aculeatus* KF-428. Their structures were elucidated by spectroscopic methods. Neither okaramine H nor I showed insecticidal activity against silkworms.

During the course of a search for microbial metabolites that exhibit activity against insects, we isolated and described insecticidal okaramines A (**1**),[1] B (**2**),[1] D,[2] E,[2] F,[2] and G,[3] and convulsive compounds, penitrem A[4] and 6-bromopenitrem E,[5] from a strain of *Penicillium simplicissimum* ATCC 90288. In further screening for insecticides, we obtained an isolate of *Aspergillus aculeatus* KF-428 from a soil sample, which showed activity against silkworms when cultured on okara (the water-insoluble residue of whole soybean). Active principles were isolated and identified as okaramines A (**1**) and B (**2**) by spectroscopic evidence. In addition, two other okaramine-related compounds were isolated and termed okaramines H (**3**) and I (**4**). This paper describes the isolation and structure elucidation of **3** and **4**.



Purification of the okaramines was guided by their insecticidal activity against silkworms (*Bombyx mori*) and characteristic coloration by TLC as described previously.[2] *Aspergillus aculeatus* KF-428 was cultured on okara (15 kg) at 25 °C for 14 days. A MeOH extract of the fermented okara was fractionated by sequential solvent partitioning, Si gel column chromatography, and finally purification by ODS column chromatography. Three compounds were isolated from the active fraction: two were identified as **1** and **2**; the third was termed okaramine H (**3**). Purification of the other fraction also gave a new okaramine-related compound, which was named okaramine I (**4**).

Table 1. ^{1}H NMR Data of **3** and **4** in Me$_2$CO-d_6

position	3	4
2	4.77 (dd, 11.2, 6.7)	4.76 (dd, 10.7, 6.7)
3	2.71 (dd, 13.4, 6.7)	2.71 (dd, 13.4, 6.7)
	2.32 (dd, 13.4, 11.2)	2.30 (dd, 13.4, 10.7)
4	7.19 (d, 7.3)	7.31 (dd, 7.3, 0.9)
5	6.74 (dd, 7.6, 7.3)	6.74 (td, 7.3, 0.9)
6	6.98 (d, 7.6)	7.11 (ddd, 7.9, 7.3, 0.9)
7		6.69 (d, 7.9)
8a	5.52 (d, 3.1)	5.53 (d, 3.1)
10	3.28 (dd, 15.9, 7.0)	
	3.20 (dd, 15.9, 7.6)	
11	5.28 (m)	
12		
13	1.75 (d, 0.9)	
14	1.73 (d, 0.6)	
1′	7.64 (s)	7.62 (s)
4′	5.82 (d, 8.2)	5.83 (d, 8.2)
5′	5.97 (d, 8.2)	5.97 (d, 8.2)
8′	7.41 (dd, 7.0, 1.5)	7.40 (dd, 6.4, 1.5)
9′	7.15 (td, 7.0, 1.2)	7.15 (td, 6.4, 1.8)
10′	7.17 (td, 7.0, 1.5)	7.17 (td, 6.4, 1.5)
11′	7.70 (dd, 7.0, 1.2)	7.70 (dd, 6.4, 1.8)
13′	1.79 (s)	1.79 (s)
14′	1.68 (s)	1.68 (s)
3a-OH	5.07 (s)	5.10 (s)
8-NH	5.54 (d, 3.1)	6.06 (d, 3.1)
7′-NH	10.81 (br s)	10.68 (br s)

The molecular formula of **3** was determined to be $C_{32}H_{32}N_4O_3$ by HREIMS. The ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) data also indicated that **3** had the same molecular formula as **1**. The UV spectrum of **3** showed absorptions at 233 and 286 nm (indole ring) and 374 nm (azocinoindole moiety). The IR spectrum showed hydroxyl absorption at 3430 cm^{-1} and amide carbonyl absorption at 1670 cm^{-1}.

The UV and IR spectra also indicated that **3** was an okaramine-related compound. The ^{1}H and ^{13}C NMR spectra of **3** were very similar to those of **1**. In the ^{1}H NMR spectrum of **3**, signals at δ 7.41, 7.15, 7.17, and 7.70 were assigned to H-8′, H-9′, H-10′, and H-11′, respectively. These signals, together with signals at δ 5.82, 5.97, and 7.64, supported the presence of an azocinoindole moiety. In addition, signals of an α,α-dimethylallyl group observed in **1** were absent, and signals assigned to a prenyl group were observed at δ 1.73, 1.75, 3.20, 3.28, and 5.28. In the ^{1}H NMR spectrum of **3**, the doublet signal at δ 5.54 that was coupled to the methine proton at C-8a was assigned to the

* To whom correspondence should be addressed. Tel./Fax: + 81 (722) 54-9471. E-mail: hayashi@biochem.osakafu-u.ac.jp.

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Table 2. ^{13}C NMR Data for **1**, **3**, and **4** in Me_2CO-d_6

position	1	3	4
2	57.7 (d)	58.8 (d)	58.7 (d)
3	34.8 (t)	42.1 (t)	42.2 (t)
3a	84.9 (s)	87.5 (s)	87.4 (s)
3b	134.4 (s)[a]	131.8 (s)	131.9 (s)
4	124.2 (d)	121.4 (d)	123.7 (d)
5	119.9 (d)	120.1 (d)	119.2 (d)
6	129.5 (d)	129.6 (d)	130.2 (d)
7	115.2 (d)	124.1 (s)	110.8 (d)
7a	149.2 (s)	147.5 (s)	149.5 (s)
8a	85.6 (d)	85.3 (d)	85.4 (d)
9	167.5 (s)	167.4 (s)	167.4 (s)
10	59.6 (s)	29.9 (t)	
11	149.2 (d)[a]	122.5 (d)	
12	111.4 (t)	133.8 (s)	
13	25.0 (q)	25.8 (q)	
14	28.2 (q)	17.8 (q)	
1′	115.9 (d)	114.8 (d)	114.5 (d)
2′	127.9 (s)	127.4 (s)	127.6 (s)
4′	123.5 (d)[a]	123.7 (d)	123.6 (d)
5′	139.9 (d)[a]	140.2 (d)	140.1 (d)
6′	36.7 (s)	36.9 (s)	36.8 (s)
6a′	148.8 (s)	148.9 (s)	148.7 (s)
7a′	135.0 (s)	135.1 (s)	135.2 (s)
8′	112.5 (d)	112.5 (d)	112.5 (d)
9′	122.8 (d)	122.8 (d)	122.8 (d)
10′	121.6 (d)	121.7 (d)	121.6 (d)
11′	117.8 (d)	117.8 (d)	117.7 (d)
11a′	131.2 (s)	131.1 (s)	131.2 (s)
11b′	105.8 (s)	106.0 (s)	105.9 (s)
12′	165.0 (s)	163.8 (s)	163.4 (s)
13′	27.1 (q)	26.9 (q)	26.9 (q)
14′	28.2 (q)	28.1 (q)	28.1 (q)

[a] These signals were reassigned in this paper.

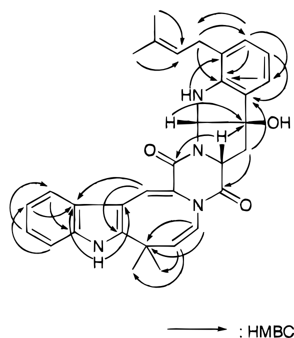

Figure 1. Significant HMBC correlations for **3**.

amino proton at N-8 because it was exchangeable on addition of D_2O; seven protons were observed, which were assigned to two indole moieties, instead of eight protons in the case of **1**. To establish the connection of the prenyl group, HMBC experiments were carried out on **3** (Figure 1). Observation of ^1H and ^{13}C long-range correlations among 10-CH_2 and C-7a and C-6 and between 6-H and C-10 revealed that the prenyl moiety was at C-7 in the pyrroloindole ring. Other correlations shown in Figure 1 indicate the relative stereochemistry of **3**. The configurations of hydrogens at C-2, C-8a and of a hydroxyl at C-3a were considered to be the same as those of **1** because protons at C-2 and C-3 in **3** had chemical shifts and coupling constants quite similar to those of **1**. In addition, the relative stereochemistry of **1** was previously determined by an X-ray study on an acetyl derivative of **1**.[1] Therefore, structure **3** was assigned to okaramine H.

The molecular formula of **4** was determined to be $C_{27}H_{24}N_4O_3$ by HREIMS. Compound **4** showed IR and UV spectra similar to those of **1**, indicating that **4** was also an okaramine congener. In the ^1H NMR spectrum (Table 1), signals of an α,α-dimethylallyl group that was present in **1** were not observed, but a signal assigned to an amino proton that coupled to a methine proton at C-8a was found. The proposed structure was also supported by the ^{13}C NMR data (Table 2). Furthermore, the ^1H and ^{13}C NMR spectra of **4** corresponded to those of despentenyl okaramine A obtained by hydrogenolysis of **1** with Pd/C as reported previously.[1] Based on these results, the structure **4** was assigned to okaramine I.

Table 3. Toxicity of Okaramines against Silkworm

compound	LD_{50} (μg/g diet)
okaramine A (**1**)	8
okaramine B (**2**)	0.2
okaramine H (**3**)	>100
okaramine I (**4**)	>100

The insecticidal activities of **1**−**4** against the third instar larvae of silkworms are shown in Table 3. The LD_{50} values of **1** and **2** were 8 and 0.2 μg/g diet, respectively, while **3** and **4** showed no activity. These results indicate that the α,α-dimethylallyl side chain at N-8, in **1**, is important for insecticidal activity. A similar conclusion was reported previously.[1]

Experimental Section

General Experimental Procedures. Melting points were uncorrected. Optical rotation was measured with a Horiba model SEPA-300 polarimeter. The IR spectra were recorded with a Perkin−Elmer 1760X FT-IR spectrophotometer, and the UV spectra were recorded with a Hitachi model U-3210. Mass spectra were recorded with a JEOL JMS-DX300 instrument, and ^1H and ^{13}C NMR spectra were obtained with a JEOL JNM A-500 spectrometer. Chemical shifts were given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was performed with Wakogel C-200 (Wako Pure Chemical Industries), Kieselgel-60 (Merck), and ODS (Chromatorex ODS). The okara used as a medium in this experiment was kindly supplied by Kitagawa Tofu (bean-curd) Shop, Sakai. The insect bioassay was carried out according to a procedure described previously.[1]

Organism and Fermentation. *Aspergillus aculeatus* KF-428 was isolated from a soil sample collected in Kansai area, Japan, and identified by the Centraalbureau Voor Schimmelcultures, the Netherlands. A loopful of spores from a slant culture of the *A. aculeatus* KF-428 was inoculated into 30 g of okara in a petri dish 9 cm in diameter, and cultivation was carried out at 25 °C for 14 days.

Extraction and Isolation. Okara (15 kg), fermented with strain KF-428, was soaked in MeOH. Evaporation of the MeOH gave an aqueous concentrate that was extracted with CH_2Cl_2. The CH_2Cl_2 extract was partitioned between *n*-hexane and MeOH containing 10% H_2O, the lower layer was concentrated and extracted with EtOAc. The EtOAc layer was subsequently dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue (6.9 g) was chromatographed on Wakogel C-200 by eluting with *n*-hexane and increasing the ratio of EtOAc to afford 60−80% EtOAc eluates. These fractions were further flash-chromatographed on Kieselgel 60 by eluting with $CHCl_3$ and increasing volumes of EtOAc. The fraction (250 mg) eluted with 10−15% EtOAc was crystallized from MeOH to give **2** (30 mg). The filtrate (200 mg) was subjected to ODS flash chromatography using a H_2O−MeOH (4:6) solvent system to yield **1** (60 mg) and **3** (55 mg), which were finally crystallized from toluene. The fraction (186 mg) eluted with 20% EtOAc was further purified by ODS flash chromatography using a H_2O−MeOH (5:5) solvent system to give crude okaramine I. Recrystallization from a hexane−EtOAc mixture afforded **4** (3 mg).

Okaramine H (1): yellow needles: mp 204−206 °C; $[\alpha]^{20}_D$ +559° (*c* 0.22,MeOH); UV (MeOH) λ_{max} (ε) 233 (30 300), 286 (18 600), 374 (19 400) nm; IR (KBr) ν_{max} 3430, 3340, 1670, 1610, 1460, 1425, 735 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table

2; EIMS *m/z* 520 [M]$^+$ (32), 502 (93), 445 (14), 319 (70), 304 (38), 250 (44), 222 (65), 207 (64), 195 (50), 167 (62); HREIMS *m/z* 520.2484 (calcd for $C_{32}H_{32}N_4O_3$, 520.2457).

Okaramine I (2): pale yellow powder; mp 266−269 °C; $[\alpha]^{20}_D$ +645° (*c* 0.09,MeOH); UV (MeOH) λ_{max} (ϵ) 233 (31 000), 285 (16 600), 371 (16 600) nm; IR (KBr) ν_{max} 3360, 1700, 1658, 1613, 1425, 1380, 742 cm^{-1}; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 452 [M]$^+$ (1), 319 (11), 206 (5), 196 (7), 220 (4), 180 (4), 167 (7); HREIMS *m/z* 452.1854 (calcd for $C_{27}H_{24}N_4O_3$, 452.1848).

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