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Haematocin, a New Antifungal Diketopiperazine Produced by *Nectria haematococca* Berk. et Br. (880701a-1) Causing Nectria Blight Disease on Ornamental Plants

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A new antifungal diketopiperazine named haematocin was isolated from the culture broth of *Nectria haematococca* Berk. et Br. (880701a-1) causing blight disease on ornamental plants, *Phalaenopsis* spp. and *Doritanopsis* spp. Its structure was established by spectroscopic methods. Haematocin inhibited the germ-tube elongation and spore-germination of *Pyricularia oryzae* at the ED_{50} values of 30 μg/ml and 160 μg/ml, respectively.

Many classes of bioactive metabolites have been found from fungal pathogens of plants: *i.e.* antibiotics, phytotoxins, plant-growth regulators, enzyme inhibitors, and so on. These active metabolites are often accompanied by undesirable biological-activities toxic to plants and/or animals, which make it difficult to apply for practical use. To isolate new antifungal agents without any toxicity to plants, we have screened the cultures of the phytopathogenic fungi by using both bioassays of fungal-spore germination and seedlings of plants. In this report, the isolation, structure determination, and biological activity of a new antifungal agent named haematocin, produced by *Nectria haematococca* Berk. et Br. (880701a-1)[1] causing blight disease on ornamental plants, are described.

The producing organism, *Nectria haematococca* Berk. et Br. (880701a-1), was inoculated into 300-ml Erlenmeyer flasks containing 100 ml of the seed medium of the modified Czapek-Dox medium containing yeast extract 0.05% (pH 6.8). After incubation at 25°C for 3 days on a rotary shaker (160 rpm), one ml of this suspension was transferred to 300-ml Erlenmeyer flasks each containing 100 ml of the modified Czapek medium. The fermentation was carried out at 25°C for 5 days (160 rpm).

The broth filtrate (5 liters) was adjusted to pH 2.5 and extracted with ethyl acetate (5 liters). The ethyl acetate layer was re-extracted with saturated $NaHCO_3$ and the neutral fraction was obtained. The $NaHCO_3$ layer was adjusted to pH 2.5 and extracted with ethyl acetate to give the acidic fraction. The acidic fraction inhibited toward both the spore-germination of *Pyricularia oryzae* and the seedlings of rice and Chinese cabbage, while the neutral fraction did toward only the spore-germination (data not shown). The isolation and identification of the antifungal agents for the neutral fraction was undertaken. The neutral fraction (167 mg) was applied to a silica gel column (6 g) and eluted stepwise with MeOH-$CHCl_3$ (2:98, 5:95, and 10:90 v/v). The active portion (53 mg) was subjected to the preparative silica gel TLC with ethyl acetate-dichloromethane (20:80 v/v), followed by with ethyl acetate-benzene (30:70 v/v) to give haematocin (12.5 mg) as white powders.

Haematocin is soluble in methanol, ethyl acetate, acetone, $CHCl_3$, and dichloromethane, and insoluble in *n*-hexane, EtOH, and water. The physico-chemical properties of haematocin are shown in Table 1. The FD-MS spectrum gave the maximum ion peak at *m/z* 502 (base peak, M^+) and the fragment peak at *m/z* 455 (16%, M^+-SCH_3), while the EI and FAB-MS spectrum gave the intense ion peaks at *m/z* 455, accompanying by each the low intensity peak at *m/z* 502 (4%) and *m/z* 503 (trace). The molecular formula was determined to be $C_{24}H_{26}O_6N_2S_2$ from the HREI-MS.

The ^1H NMR spectrum (400 MHz, $CDCl_3$, Fig. 1 and

Table 2) and ^{13}C NMR spectrum of haematocin (100 MHz, $CDCl_3$, Table 2) revealed the well defined 13 protons and 12 carbons, respectively, which correspond to the half of the determined molecular formula. This indicated that haematocin is a symmetrical molecule formed from two identical units. The ^1H and ^{13}C NMR data containing the ^1H-^1H COSY and FG-HMQC experiment are summarized in Table 2. The 12 carbons consisted of $CH_3\times2$, $CH_2\times1$, $CH\times5$ and non-protonated carbon$\times4$. The IR (film) and ^{13}C NMR spectrum suggested the presence of an ester (ν 1735 cm^{-1}, δ_C 170.43) and amide group (ν 1665 cm^{-1}, δ_C 164.90). In addition, ^1H and ^{13}C NMR spectra showed, the presence of a tri-substituted and a di-substituted double-bonds (δ_C 133.98, 119.98, 125.11, 128.04), three carbons bearing O, N, or S atom (δ_C 75.39, 64.25, 74.14), an acetyl group (δ_C 21.34/δ_H 2.11) and a methylthio group (δ_C14.34/δ_H 2.25), and a methylene group (δ_C 40.13/δ_H 2.83 and 3.01) were suggested.

The ^1H-^1H COSY experiment revealed the sequence of coupled signals from H-5 to H-9 (Table 2). Therefore, the remaining, olefinic-carbon having no proton at δ_C 133.98 can be assigned to the C-4. The FG-HMBC experiment (Fig. 3) unambiguously indicated the positions of the acetyl and methylthio group; the correlation of the C-8 methine (δ_H 6.13) and acetyl methyl (δ_H 2.11) to the carbonyl carbon (δ_C 170.43) of acetyl group, and of the methylthio (δ_H 2.25) to the quaternary carbon C-2 (δ_C 74.14). In addition to these results, the correlation of the methylene protons (δ_H 2.83 and 3.01) to the carbons C-4 (δ_C 133.98), C-5 (δ_C 119.98), C-9 (δ_C 64.25), and C-2 (δ_C 74.14)

Table 1. Physico-chemical propeties of haematocin.

Appearance	White powder
MP	97.5-101.2°C
$[\alpha]_D^{25}$ (c 0.076, $CHCl_3$)	- 216°
Molecular formula	$C_{24}H_{26}O_6N_2S_2$
HREI-MS (*m/z*)	
Found:	502.1234 (M^+)
Calcd:	502.1232
EI-MS (m/z, rel. int.)	502 (4), 455 (65), 395 (59),368 (20), 347 (66), 335 (37),305 (77), 288 (81), 287 (95), 286 (100)
FD-MS (m/z, rel. int.)	502 (100), 455 (16)
FAB-MS (m/z)	503 (trace), 455
UV (MeOH) λmax nm (ε)	269.4 (8000), 209.2 (14000)
CD (MeOH) nm (Δε)	284 (+1.15), 258 (s, +0.5), 222 (-38.5)
IR (film) ν cm^{-1}	3100, 2900, 1735, 1665, 1380, 1230, 1040, 960, 750, 700
Rf values	0.50*, 0.45**

*EOAc-CH_2Cl_2 =20:80 **EOAc-benzene =30:70

Fig. 1. 400 MHz ^1H NMR spectrum of haematocin in $CDCl_3$.

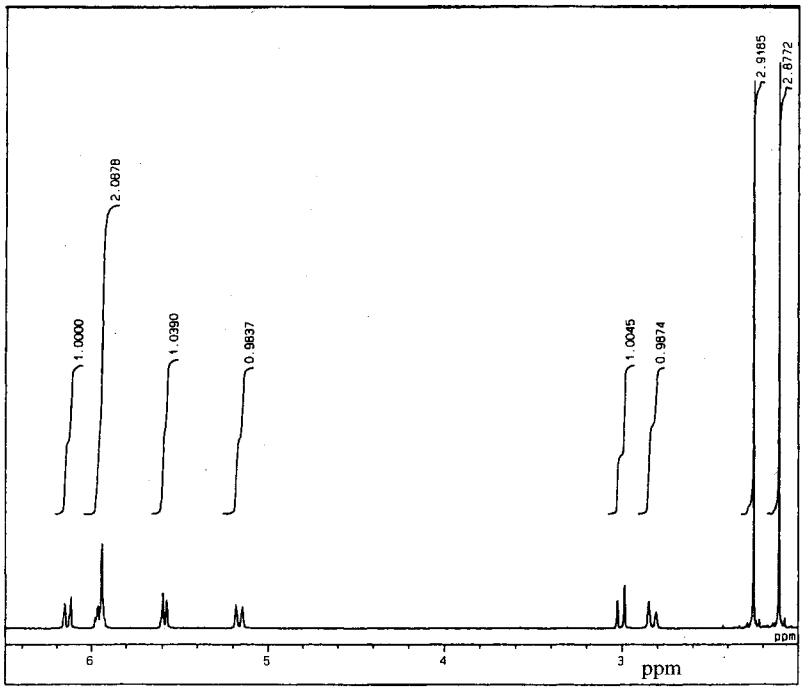


Table 2. ^{1}H and ^{13}C NMR spectral data of haematocin.

Position No.	δ_C / δ_H		^{1}H-^{1}H COSY*
1	164.90 s /		/
2	74.14 s /		/
3a	40.13 t /	2.83 br.d (1H, 16.1)	H-5, H-7, H-8, H-9
3b		3.01 dd (1H, 16.1, 1.2)	H-5, H-9
4	133.98 s /		/
5	119.98 d /	5.94 m (1H)	H-6, H-7, H-9, H-3a,b
6	125.11 d /	5.96 m (1H)	H-5, H-7, H-8
7	128.04 d /	5.59 br.d (1H, 9.2)	H-5, H-6, H-8, H-3a
8	75.39 d /	6.13 br.d (1H, 14.8)	H-6, H-7, H-9
9	64.25 d /	5.16 br.d (1H, 14.8)	H-5, H-8, H-3a,b
10	14.34 q /	2.25 s (3H)	/
11	170.43 s /		/
12	21.34 q /	2.11 s (3H)	/

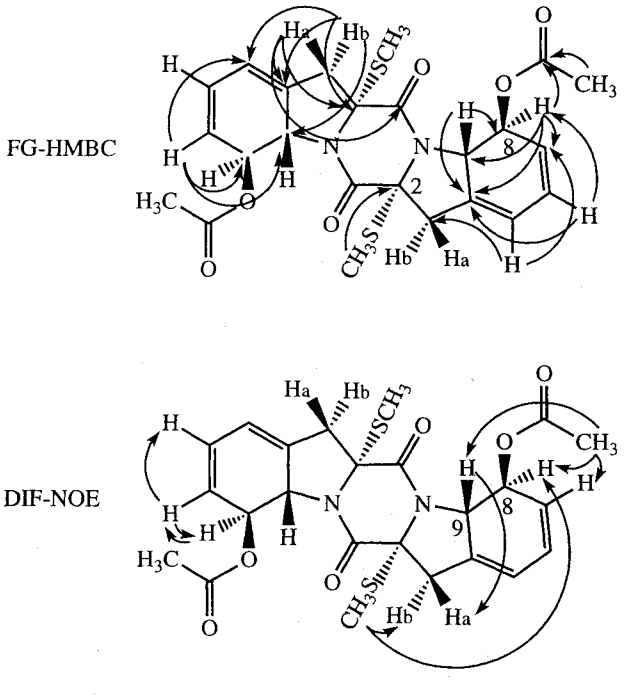
*Underlined protons are long range couplings

suggested that the nitrogen atom of the amide group bears the C-9 and C-2, and the methylene group bears the C-2 and C-4. Thus, the structure of haematocin is given as a diketopiperazine illustrated in Fig. 3. The presence of the allylic couplings between H-5 and H-3 and the correlation of the C-8 methine (δ_H 6.13) to the C-4 also supported the deduced structure. The two identical units forming haematocin are present in those of bisdethio-di(methylthio)-acetylapoaranotin[2] (BDAA). The proton chemical shifts for the common structural unit of haematocin and BDAA were roughly identical. This supported also the plain structure of haematocin.

The relative stereochemistry on the C-2, C-8, and C-9 of haematocin was determined by the values of the proton-proton coupling constants and the DIF-NOE experiment (Fig. 2). The vicinal coupling constants of H-8 and H-9, 14.8 Hz, indicated that H-8 and H-9 are in a trans-diaxial relationship. The DIF-NOE experiment showed that the methylthio group is the same side as H-8 and Hb, and H-9 is the same side as Ha. Thus, the relative stereostructure of haematocin was illustrated as Fig. 3-(1).

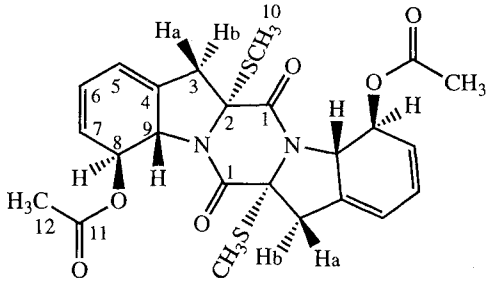
The absolute stereostructure was determined by a comparison of the CD spectrum of haematocin with that of acetylaranotin[3] (Fig. 3-(2)). The relative stereochemistry on the common three asymmetric carbon atoms of haematocin and acetylaranotin is identical, although acetylaranotin differs from haematocin in having the dihydrooxepin and disulfide moieties in place of the cyclohexadiene and methylthio moieties of haematocin. The absolute

Fig. 2. Correlations observed by FG-HMBC and DIF-NOE experiments.

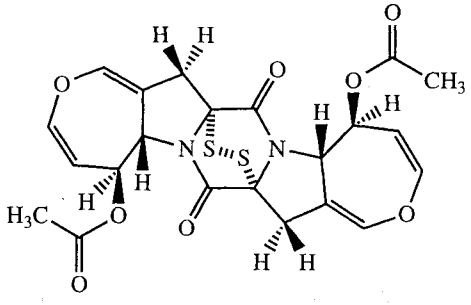


stereochemistry of acetylaranotin[4] has determined from a comparison of the CD spectra between L-prolyl-L-proline diketopiperazine and the desulfulization and deacetylation product of acetylaranotin. The CD spectrum of haematocin in MeOH consisted of a large negative Cotton effect at 222

Fig. 3. Absolute stereostructure of haematocin.



haematocin (1)



acetylaranotin (2)

nm ($\Delta\varepsilon$ +38.5), and small positive Cotton effects at 258 nm (shoulder, $\Delta\varepsilon$ +0.5) and 284 nm ($\Delta\varepsilon$ +1.15). These Cotton effects are qualitatively in good agreement with those of acetylaranotin [229 nm ($\Delta\varepsilon$ −52.2) and 268 nm ($\Delta\varepsilon$ +5.0)][4]. Since the large amplitude Cotton effect at 222 nm in haematocin and at 229 nm in acetylaranotin which is assigned as that due to the n-π^* transition of a diketopiperazine chromophore showed an identical negative sign, it was deduced that the absolute stereochemistry of haematocin is the same as that of acetylaranotin on the asymmetric carbon atoms. Thus, the complete stereostructure for haematocin was demonstrated as shown in Fig. 3-(1).

The biological activity of haematocin was examined by the spore-germination assay using *Pyricularia oryzae* and by the seedling assay using Chinese cabbage and rice. Haematocin inhibited both the spore-germination and germ-tube elongation. The inhibition rate for the spore-germination after 5 hour-incubation was 97.5, 71.2, 20.0, and 6.4% at each concentration of 500, 250, 125, and 60 μg/ml. At a concentration lower than 30 μg/ml, no spore-germination inhibition was observed, however, the inhibition for the germ-tube elongation after 18 hour-incubation was observed, and the rate at a concentration of 30 μg/ml was approximately 50%. Haematocin did not show any biological activity toward the germination and the growth of rice and Chinese cabbage at a concentration of 160 μg/ml which is comparable to the ED_{50} value for the spore-germination

Haematocin is the first metabolite belonging to the class of sulfur-containing diketopiperazines derived *via* an intermediate of two benzene-oxides only or its biochemical equivalents[2].

Experimental

General

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken by a JEOL α-400 spectrometer. EI and HREI-MS spectra were recorded on a JEOL SX-102 spectrometer, and FD and FAB-MS spectra, on a JEOL HX-110 spectrometer. IR and UV spectra were measured on a Simadzu IR-435 spectrometer and UV-265 spectrophotometer, respectively. $[\alpha]_D$ was measured on a JASCO DIP-370 digital polarimeter. CD spectra were recorded on a JASCO J-720 spectrometer. MP was measured by a Yanaco micromelting point apparatus and was not corrected.

Chemicals

Silica gel 60 (230~400 mesh) for column chromatography and Silica gel 60 F_{254} pre-coated layer (0.25 mm thickness) for TLC and preparative TLC from Merck, Germany.

Spore-germination Assay Using *Pyricularia oryzae*

The spore germination test was conducted using the rice blast fungus, *Pyricularia oryzae*, according to a previously described procedure[5]. The inhibitory activities were determined after the 5 hour-incubation for the germination and after 18 hour-incubation for the germ-tube elongation, respectively.

Seedling Assay of Chinese Cabbage and Rice

Twelve seeds of Chinese cabbage (cv. Osho) and rice (cv. Sasanishiki) were placed on filter paper moistened with 2 ml of test solution in a 6-cm Petri dish, cultured under a fluorescence lamp at 28°C for 3 days. The hypocotyl (or mesocotyl) and root lengths were measured.

Isolation of Haematocin

After acidification with 1 N HCl to pH 2.5, the filtrate of

the culture broth (5 liters) at 25°C for 5 days was extracted with EtOAc (total 5 liters). The EtOAc layer was re-extracted with saturated $NaHCO_3$ and concentrated to give the neutral fraction (167 mg). The $NaHCO_3$ layer was adjusted to pH 2.5 and extracted with EtOAc. The EtOAc extracts were concentrated to give the acidic fraction (80 mg). The neutral fraction (167 mg) was subjected to a silica gel column (6 g) with a stepwise elution of MeOH-$CHCl_3$ (2:98, 5:95, and 10:90 V/V). The active portion (53 mg) eluted with MeOH-$CHCl_3$ (2:98 v/v) was subjected to a preparative TLC on silica gel with EtOAc-CH_2Cl_2 (20:80 v/v). The band of around Rf 0.50 (20 mg), having UV absorption, was subjected again to the preparative TLC with EtOAc-benzene (30:70 v/v). The band of around Rf 0.45 gave haematocin, 12.5 mg, as white powders from EtOH.

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