Fusarin C, (7*Z*)-Fusarin C and (5*Z*)-Fusarin C; Inhibitors of Dihydroxynaphthalene-melanin Biosynthesis from *Nectria coccinea* (*Cylindrocarpon* sp.)

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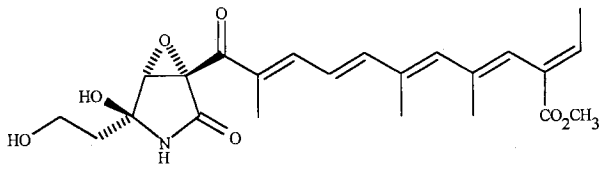
In Ascomycotina and related Deuteromycotina, the dark-brown to black pigments in cell walls are generally synthesized *via* the pentaketide pathway with 1,8-dihydroxynaphthalene (DHN) as a precursor[1]. DHN melanin has been implicated as a pathogenicity factor in fungal plant diseases and animal mycoses[2]. Inhibitiors of melanin biosynthesis like tricyclazole, pyroquilone or fthalide are used to prevent rice blast disease caused by *Pyricularia oryzae*[3]. During the search for natural inhibitors of DHN melanin biosynthesis, extracts from the culture broth of *Nectria coccinea* A56-95 exhibited high activity. Bioactivity-guided isolation of the compounds revealed that fusarin C (**1**), (7*Z*)-fusarin C (**2**) and (5*Z*)-fusarin C (**3**) were responsible for the inhibitory activity. This note describes the fermentation, isolation and biological evaluation of fusarin C, (7*Z*)-fusarin C and (5*Z*)-fusarin C.

Mycelial cultures of *Nectria coccinea* A56-95 were derived from ascospores. The fruiting bodies growing on a twig were collected near Oberjoch, Germany. The bright red perithecia, the asci and ascospores fit the description for *Nectria coccinea* (Person ex Fries) Fries[4]. A voucher specimen of the fungus is deposited in the herbarium of the department Biotechnology, University of Kaiserslautern. On agar cultures the strain produces conidia of the *Cyclindrocarpon* type (37~45 μm long, 3~5 septate) which is in accord with the literature[4]. For maintenance on agar slants as well as for submerged cultivation, the fungus was grown in a YMG medium composed of (g/liter): yeast extract 4, malt extract 10, glucose 4, pH 4.8. For the production of fusarin C, (5*Z*)-fusarin C and (7*Z*)-fusarin C a well grown seed culture was used to inoculate 20 liters of YMG in a Biolafitte C6 fermentation apparatus. The fermenter was incubated at 22°C with stirring (120 rpm) and an aeration of 3 liters/minute for 66 hours. Inhibition of melanin biosynthesis was followed using a test system based on the production of DHN melanin by *Lachnellula* sp., A32-89, in agar cultures[5].

The active components located in the culture filtrate (19 liters) were extracted by adsorption onto Mitsubishi DIAION HP-21 resin. The column (5.5 × 35 cm) was washed with 2 liters of water. The bioactive components were eluted with 1.5 liters of acetone-water (1 : 1). The acetone was evaporated *in vacuo* to an aqueous residue, which was extracted four times with EtOAc. After evaporation of the solvent from the combined EtOAc extracts, 890 mg of an oily crude extract were obtained. Chromatography on silica gel (Merck 60, 0.063~0.2 mm, column size 150 × 25 mm) in cyclohexane-EtOAc (25 : 75) yielded 195 mg of an intermediate product. From this product, two fractions were obtained by preparative HPLC on LiChrogel PS 1 (7 μm; 250 × 25 mm; flow rate 5 ml/minute) in 2-propanol. Fraction I containing (7*Z*)-fusarin C (**2**) and (5*Z*)-fusarin C (**3**) eluted at 23 minutes (yield: 19 mg). Fraction II containing fusarin C (**1**) was obtained at 32 minutes (yield: 81 mg). Final purification was achieved by preparative HPLC on



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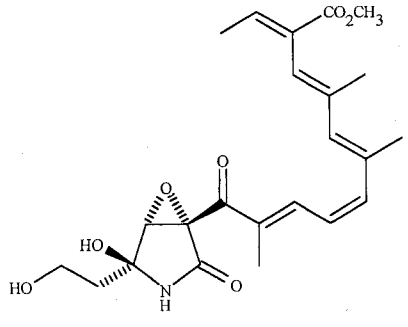

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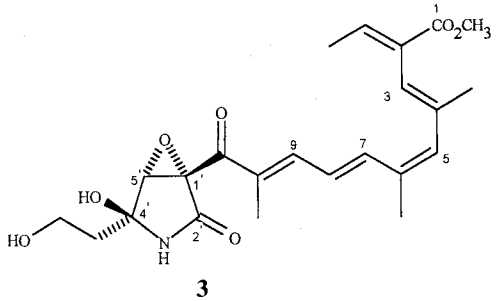

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LiChrosorb-Diol (7 μm; 250 × 25 mm; flow rate 5 ml/minute; detection at 360 nm) with a linear gradient (40% EtOAc in cyclohexane - 100% EtOAc; 0 ~ 160 minutes). The Rt for (7*Z*)-fusarin C (**2**) was 98 minutes, yield 5.6 mg, 2.2 mg of (5*Z*)-fusarin C (**3**) eluted after 114 minutes and 16 mg of fusarin C after 127 minutes. As the formation of compounds **2** and **3** could result from an isomerization of the all-*E* isomer, precautions were taken during the isolation and the analysis to prevent photoisomerization[6]. Therefore we conclude, that all three compounds are natural products.

The spectral data of (5*Z*)-fusarin C (**3**) are very similar to those of fusarin C (**1**) and (7*Z*)-fusarin C (**2**) (see Table 1 for NMR data and the MS data below) although distinct differences can be noted. All three compounds gave the same ^{1}H-^{13}C long-range correlations through the unsaturated chain and in the bicyclic system, as well as from the two C_2 units to C-2 and C-4′ (see structure of compound **3** for the numbering of the atoms, which is different from the one used in some publications), indicating that they are stereoisomers. The differences between the three compounds were revealed by the NOESY spectra, which showed that compound **1** is all-trans (*i.e.* fusarin C[7]) and **2** is (7*Z*)-fusarin C[8]. For compound **3**, strong NOESY correlations were observed between 7-H and 3-H as well as 4-CH_3, and between 5-H and 6-CH_3, clearly demonstrating that it is the (5*Z*)-isomer of fusarin C.

(5*Z*)-Fusarin C (**3**) or 2-ethylidene-11-[4-hydroxy-4-(2-hydroxyethyl)-2-oxo-6-oxa-3-azabicyclo[3.1.0]hex-1-yl]-4,6,0-trimethyl-11-oxo-3,5,7,9-undecatretraenoic acid methyl ester ($C_{23}H_{29}NO_7$) was obtained as a slightly yellow oil. EI-MS (70 eV, *m/z*: 399 (5%), 387 (4%), 358 (11%), 326 (19%), 309 (48%), 281 (73%), 213 (92%), 211 (96%), 185 (100%). CI-MS (NH_3): 449 (14%), 432 (22%), 431 (26%), 414 (25%), 360 (29%), 290 (35%), 273 (90%), 222 (56%), 141 (100%). The NMR data are listed in Table 1.

All three compounds inhibited the formation of DHN melanin by *Lachnellula* sp. A32-89 to the same extent (Table 2). The concentrations needed for inhibition of melanin synthesis started at 1 ~ 2 μg/disk. At 5 μg/disk the growth of aerial mycelia was also inhibited. The enzymatic step inhibited in the biosynthetic pathway of melanin remains to be elucidated. Cytotoxic and phytotoxic activities were determined as reported by ZAPF *et al.*[9] and ANKE *et al.*[10]. All three compounds showed cytostatic activity against L1210 and HL60 cells at 10 and 20 μg/ml. At 25 μg/ml, BHK cells were lysed (80%). Fusarin C and (7*Z*)-fusarin C were phytotoxic against *Setaria italica* and *Lepidium sativum*. The germination of the seeds and growth of seedlings of *Setaria italica* was inhibited for 50% at a concentration of 300 μg/ml. The antimicrobial activity of compounds **1** ~ **3** was tested in the serial dilution assay[10] against six Gram-positive and three Gram-negative bacteria, five yeasts and four fungi. The fusarins showed only bacteriostatic activity against *Salmonella typhimurium* at 100 μg/ml. Upon incubation with L-cysteine, the fusarins lost their activity in the melanin bioassay, suggesting that the C_{13}-C_{14}

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for (5*Z*)-fusarin C (**3**).

	^{1}H (δ, mult., *J*)	^{13}C (δ, mult.)
1	—	168.1; s
2	—	130.3; s
3	5.90; s	125.1; d
4	—	137.1; s
5	6.16; s	138.3; d
6	—	133.9; s
7	7.44; d; 15.1	141.9; d
8	6.67; dd; 11.2, 15.1	124.9; d
9	7.49; d; 11.2	146.2; d
10	—	133.8; s
11	—	190.0; s
1′	—	61.5; s
2′	—	167.7; s
4′	—	85.1; s
5′	3.99; s	63.9; d
1″	6.97; q; 7.2	140.4; d
2″	1.78; d; 7.2	15.9; q
1‴	2.04; m	36.2; t
2‴a	4.05; m	58.1; t
2‴b	3.90; ddd; 5, 5, 11.0	—
4-CH_3	1.63; s	18.6; q
6-CH_3	1.99; s	20.6; q
10-CH_3	1.99; s	11.4; q
1-OCH_3	3.78; s	52.1; q

The spectra were recorded in $CDCl_3$ with the solvent signals (at 7.26 and 77.0 ppm, respectively) as reference, the chemical shifts (δ) are given in ppm and the coupling constants in Hz.

Table 2. Inhibition of melanin biosynthesis in agar cultures of *Lachnellula* sp. A32-89 by fusarin C, (7*Z*)-fusarin C and (5*Z*)-fusarin C.

Compound	Concentration (μg/ disk)	Inhibition zone (mm)	
		Melanin synthesis	Aerial mycelium
Fusarin C	2	11	n. i.
	5	15	13
(7*Z*)-Fusarin C	2	11	n. i.
	5	15	13
(5*Z*)-Fusarin C	2	11	n. i.
	5	15	13

n. i. = No inhibition.

epoxide group of the fusarins is responsible for inhibition of melanin biosynthesis. Similar results were obtained, when the mutagenic activity was tested[11].

Compound **3**, so far, has not been described as a natural product and all *Fusarium* strains which have been described to produce fusarin C (**1**) and (7*Z*)-fusarin C (**2**) are anamorphs of *Gibberella* species[12,13]. This is the first time that a *Nectria* or *Cylindrocarpon* species has been found to produce these mycotoxins.

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