6-*O*-DEMETHYL-5-DEOXYFUSARUBIN AND ITS ANHYDRO DERIVATIVE PRODUCED BY A MUTANT OF THE FUNGUS *NECTRIA HAEMATOCOCCA* BLOCKED IN FUSARUBIN BIOSYNTHESIS

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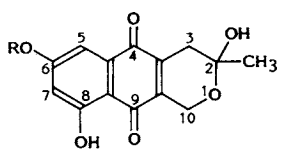
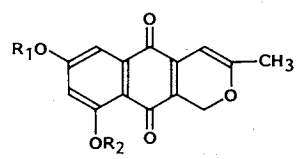
6-*O*-Demethyl-5-deoxyfusarubin and 6-*O*-demethyl-5-deoxyanhydrofusarubin have been isolated from the mutant redD169.yelY9 of the fungus *Nectria haematococca* blocked in fusarubin biosynthesis. These products were identified on the basis of physico-chemical data by comparison with known substances.

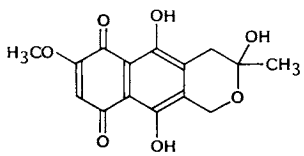
Cultures of the redD169 strain of the fungus *Nectria haematococca* (Berk. and Br.) Wr., the sexual form of *Fusarium solani*[1], contain a mixture of red substances composed primarily of the pyranonaphthoquinone fusarubin (**3**) and of related minor compounds[2~7]. Fusarubin and several accompanying products have been reported to possess moderate antibacterial, antifungal, insecticidal and phytotoxic activities[8~12]. The heptaketide origin of fusarubin was indicated by incorporation studies with ^{13}C-acetate[13]. We recently isolated fusarubinoic acid which appears to be the first cyclization product in the fusarubin series[4]. In the course of studying the biosynthetic pathway leading to fusarubin, we obtained several blocked mutants which were yellow-pigmented[14]. *N. haematococca* annA*58.yelJl, one of these mutant strains, has been reported to accumulate 4-deoxyfusarubin (**4**), 4-deoxyanhydrofusarubin (**5**) and 4-deoxyjavanicin (according to the present numbering system), together with minor, as yet unidentified compounds[15].



1a R=H
1b R=CH_3



2a $R_1=R_2=H$
2b $R_1=CH_3$ $R_2=H$
2c $R_1=R_2=CH_3$



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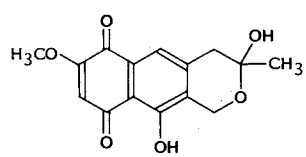

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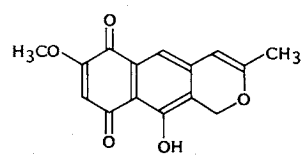

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In this paper, we describe the isolation and identification of 6-*O*-demethyl-5-deoxyfusarubin (**1a**) and of 6-*O*-demethyl-5-deoxyanhydrofusarubin (**2a**), representative of a novel series of compounds produced by another blocked mutant: redD169.yelY9.

Materials and Methods

Organism

The yellow yelY9 mutant was derived from the homothallic S1 wild-type strain of *N. haematoccocca*[14] after UV mutagenesis (350 J/m^2). A cross with the pigment overproducing red169 mutant[3] yielded the deep-yellow redD169.yelY9 double-mutant used for this study.

Culture Medium

The production medium (GAMS) was composed of (g/liter): Glucose 22.5, L-asparagine·H_2O 0.95, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.75, $CaCl_2 \cdot 2H_2O$ 0.15, $FeSO_4 \cdot 7H_2O$ 0.003, $ZnSO_4 \cdot 7H_2O$ 0.003, $CuSO_4 \cdot 5H_2O$ 0.00125, $MnSO_4 \cdot H_2O$ 0.00035, $Na_2MoO_4 \cdot 2H_2O$ 0.00025 (pH 5.5). For large scale cultivation, 2 liters of GAMS were distributed into 80 Petri dishes (25 ml each).

Cultivation

Stock cultures were maintained on V8-vegetable juice-agar plates at 26°C and transferred monthly. Precultures were made by spreading microconidia removed from stock cultures onto plates of V8-vegetable juice-agar and incubated for 2 days at 26°C. The conidia-bearing surface of a preculture was scraped into 10 ml of sterile distilled water and 0.1 ml of the conidial suspension was added to each 25 ml of GAMS in Petri dishes. The cultures were incubated for 5 days at 26°C.

Isolation of Metabolites

The mycelium was removed by filtration through filter paper on a Büchner funnel and washed with distilled water. The filtrate and washing were pooled and concentrated under reduced pressure to a volume of 500 ml. The concentrate (pH 5.5) was extracted four times with 500 ml of ethyl acetate. The pH of the aqueous phase was then adjusted to 3 with acetic acid and the acidified solution was extracted twice with 500 ml of EtOAc.

Purification by Chromatography

The extracts were pooled, concentrated under reduced pressure and fractionated by flash chromatography on silica gel. The eluting solvents consisted of pentane (200 ml) followed by 9:1, pentane-EtOAc (100 ml); 3:1, pentane-EtOAc (2 liters); 1:1, pentane-EtOAc (1 liter); 1:3, pentane-EtOAc (500 ml); EtOAc (1 liter); 49:1, EtOAc-acetic acid. The eluates were monitored by SiO_2 TLC and appropriate fractions were then combined to give 7 fractions. Further purification of these fractions was accomplished by preparative TLC (elution with pentane-EtOAc (1:1)) or SiO_2 column chromatography (elution with pentane-EtOAc mixtures).

Analytical Instruments

MP's were determined with a Kofler block under the microscope and are corrected. Electronic absorption spectra were recorded with a Varian Lambda-5 UV-visible spectrophotometer. IR spectra were obtained in pressed KBr discs using a Nicolet 205 FT-IR apparatus. EI mass spectra were measured on an AEI MS-50 spectrometer. ^1H and ^{13}C NMR spectra were recorded on Bruker 250 and 400 MHz FT spectrometers (DMSO-d_6, δ ppm from TMS as internal standard).

Methylation of Compounds **1a** and **2a**

The compound **1a** was dissolved into EtOAc (5 mg/1 ml) and an excess of ethereal diazomethane (4 ml) was added. The mixture was left at room temperature for about 1 hour. The reaction mixture was then evaporated to dryness under a nitrogen stream. SiO_2 TLC in CH_2Cl_2-MeOH (97:3) showed a major yellow substance and at least four minor spots. These were separated by preparative SiO_2 TLC in

CH_2Cl_2-MeOH (19:1) to afford the methylated derivative **1b**. When substance **2a** was treated with diazomethane as reported above, a dry residue was obtained which consisted (according to TLC) of at least 6 products. A purple **2b** and a red **2c** major compounds were isolated by preparative SiO_2 TLC using the mixture pentane-CH_2Cl_2-MeOH (4:15:1).

Dehydration of Compound **1a**

The compound **1a** was dissolved in acetic acid (11 mg/1 ml) and heated for about 40 minutes at 55°C. The color changed from yellow to purple. The reaction mixture was then diluted with 50 ml EtOAc and evaporated to dryness under reduced pressure. SiO_2 TLC in CH_2Cl_2-MeOH (19:1) showed a yellow minor spot corresponding to the starting product and a major purple compound with a Rf identical to that of substance **2a**. Preparative TLC in the same solvent mixture afforded 8.5 mg of pure product.

Results and Discussion

Characterization of the yelY9 and redD169.yelY9 Mutant Strains

Pure cultures of the yelY9 and redD169.yelY9 strains produced no pigments of the fusarubin series in any nutritional condition which was tested, but did produce yellow compounds. Red pigmented cultures containing fusarubin and related substances were obtained in co-synthesis experiments between redD169.yelY9 and the previously studied redD169.yelJl yellow double-mutant[14]. These results indicated that the yelY9 mutation was not an allele of yelJl although both prevented synthesis of fusarubin. As further confirmation of the non-identity of the yelY9 and yelJl mutant strains of *N. haematococca*, TLC analysis of EtOAc extracts from their culture filtrates showed two series of substances differing principally in their polarity. The two components **1a** and **2a** were isolated from redD169.yelY9 culture filtrates and identified, respectively, as 6-*O*-demethyl-5-deoxyfusarubin and 6-*O*-demethyl-5-deoxyanhydrofusarubin on the basis of spectral and chemical analyses.

Isolation and Structure Elucidation of Substance **2a**

Fraction I from flash chromatography contained a major purple compound **2a**. After purification by SiO_2 TLC, substance **2a** was crystallized from heptane-CH_2Cl_2 as dark-purple needles with mp 181~184°C. The yield of crystalline product was of the order of 2 mg/liter of culture filtrate. The spectral data are summarized in Table 1. The UV-visible spectrum of **2a** exhibited maxima at 279, 355 and 509 nm; the IR spectrum showed hydroxyl groups (3440 cm^{-1}), a non-chelated (1640 cm^{-1}) and a chelated (1613 cm^{-1}) quinone carbonyl groups. The above data, together with a *meta*-coupling (J=2.4 Hz) of the aromatic protons at 6.50 and 6.93 (5-H and 7-H) and two phenolic protons at 11.72 and 12.88 (6-OH and 8-OH) in the NMR spectrum (Table 2) suggested the presence of a dihydroxynaphthoquinone moiety in **2a**. The ^1H NMR spectrum of **2a** (Table 2) contained also three methyl protons at 2.03, two methylene protons at 5.07 and a vinylic proton at 5.85. The EI-MS showed the molecular ion at m/z 258 and fragments at 243,

Table 1. Physico-chemical characteristics of compounds **1a** and **2a**.

	1a	**2a**
MP (°C)	194~196	181~184
MS (m/z) (%)	276 M^+ (8), 258 $(M-18)^+$ (100), 216 (45), 188 (31), 160 (8), 43 (62)	258 M^+ (100), 243 $(M-15)^+$ (16), 228 (15), 215 (24)
UV λ_{max}^{MeOH} nm (ε)	215 (4.20), 268 (3.89), 288 (sh 3.73), 442 (3.34)	279, 355, 509
IR (KBr) cm^{-1}	3429 (H-bonded OH), 1649 (C=O), 1620 (H-bonded C=O)	3437 (H-bonded OH), 1644 (C=O), 1613 (H-bonded C=O)

Table 2. ^{1}H NMR data for substances **1a**, **1b**, **2a**, **2b** and **2c**.

Protons	**1a**	**1b**	**2a**	**2b**	**2c**
2-CH_3	1.43 s	1.44 s	2.03 s	2.03 s	2.02 s
2-OH	6.10 s	6.11 s			
3-H	2.33, 2.43, 2.50, 2.60 dd	2.47, 2.55, 2.65, 2.71 dd	5.85 s	5.90 s	5.87 s
5-H[a]	6.93 d	7.04 d	6.93 d	7.0 d	7.17 d
6-OH	11.20 s		11.72 s		
6-OCH_3		3.91 s		3.93 s	3.92 s
7-H[a]	6.50 d	6.80 d	6.50 d	6.80 d	7.0 d
8-OH	12.0 s	12.05 s	12.88 s	12.40 s	
8-OCH_3					3.98 s
10-H	4.53 s	4.51 s	5.07 s	5.10 s	5.06 s

DMSO-d_6, 250 MHz, δ ppm.
[a] J=2.4 Hz.

215, 186, thirty mass units lower than found for anhydrofusarubin[13]. This fragmentation pattern suggested that substance **2a** differed from anhydrofusarubin (**4a**) by the lack of an oxygen atom (16 mass units less) and a methyl group (14 mass units). Presumably, substance **2a** was biogenetically related to anhydrofusarubin.

Table 3. ^{13}C NMR data of substance **1a**.

C-atom	δ (ppm)	C-atom	δ (ppm)
C-2	93.1 (Q)	C-7	107.0
C-3	32.5	C-8	163.3 (Q)
C-3a	139.9 (Q)	C-8a	107.7 (Q)
C-4	182.4 (Q)	C-9	185.8 (Q)
C-4a	141.3 (Q)	C-9a	133.4
C-5	108.2	C-10	56.6
C-6	164.6 (Q)	2-CH_3	28.1

DMSO-d_6, Q standing for quaternary C-atom.

On methylation with an excess of diazomethane, **2a** gave mainly the monomethyl derivative (m/z 272, M^+) in addition to the dimethyl derivative which appeared to be identical with anhydroherbarin[16]. The monomethyl derivative differed from the previously described 4-deoxy-anhydrofusarubin (**5**)[15] by the following properties: Purple pigment (instead of red), Rf in CH_2Cl_2 (49 : 1) 0.88 instead of 0.80, *meta*-coupling (J=2.4 Hz) between the 5-H and the 7-H in NMR (no *meta*-coupling as might be expected in the case of substance **5**).

Isolation and Structure Elucidation of **1a**

Fraction II was composed of a main yellow substance **1a** with pink and yellow products which occurred in trace amounts. The main product **1a** was isolated by preparative TLC. Further purification was achieved by crystallization from heptane-ethyl acetate. About 10 mg of yellow needles were obtained per liter of culture filtrate (mp 194~196°C). The molecular formula of **1a** was determined to be $C_{14}H_{12}O_6$ from the elemental analysis, the ^{1}H (12 protons) and ^{13}C NMR spectra.

Anal Calcd for $C_{14}H_{12}O_6$: C 60.86, H 4.35.
Found: C 60.75, H 4.46.

The absorption maxima in the UV-VIS spectrum of **1a** were found at 215, 268, 288 and 442 nm. In the ^{1}H NMR spectrum, the *meta*-coupling (J=2.4 Hz) of the aromatic proton signals was observed at 6.93 and 6.50, the two phenolic proton signals were found at 11.20 and 12.0 (Table 2) while the ^{13}C signals at 185.8, 182.4, 164.6, 163.3, 141.3, 139.9, 133.4, 108.2, 107.7 and 107.0 (Table 3) suggested the presence of a dihydronaphthoquinone moiety similar to that of **2a**. This structure was also consistent with the observation of the absorption of hydroxyl groups (3430 cm^{-1}), a chelated (1620 cm^{-1}) and a non-chelated (1650 cm^{-1})

quinone carbonyls in the IR spectrum. With regard to the signals of 2-CH_3 at 1.43, 3-CH_2 at 2.33~2.60 (dd), 10-CH_2 at 4.53 and 2-OH (tertiary) at 6.10, the ^1H NMR spectrum of **1a** also showed strong similarities to that of fusarubin (**3**). The mass spectrum was in accordance with the structure of an *O*-demethyldeoxyfusarubin. The loss of H_2O from the molecular ion at *m/z* 276 resulted in the significant $(M-18)^+$ ion (base peak) at 258. Further confirmation for the proposed structure **1a** was obtained by dehydration in acetic acid, affording a product identical to **2a**. On methylation with an ethereal solution of diazomethane, **1a** gave mainly the monomethyl derivative **1b**, MS *m/z* 290 (M^+). This derivative **1b** differed from the previously reported 4-deoxyfusarubin[15] (**4**) by a higher Rf and the observation in ^1H NMR of *meta*-coupling between the C-5 and C-7 aromatic protons, indicating that the methoxy group was bonded to the benzenoid ring as represented. Furthermore, the monomethyl derivative **1b** could be dehydrated by treatment with acetic acid affording a purple compound identical to **2b**.

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