Trichoflectin, a Bioactive Azaphilone from the Ascomycete *Trichopezizella nidulus*

Eckhard Thines,[†] Heidrun Anke,*[,†] and Olov Sterner*[,‡]

Department of Biotechnology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany, and Department of Organic Chemistry 2, Lund University, P.O. Box 124, S-22100 Lund, Sweden

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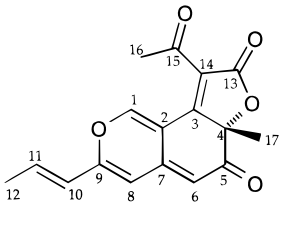
Trichoflectin (**1**), a new azaphilone belonging to the deflectin series, 6-deoxy-7-*O*-demethyl-3,4-anhydrofusarubin (**2**), and 6-deoxy-3,4-anhydrofusarubin (**3**) were isolated from submerged cultures of the ascomycete *Trichopezizella nidulus*. All compounds showed antimicrobial activity and inhibited dihydroxynaphthalene melanin biosynthesis in fungi. The structure of trichoflectin (**1**) was elucidated by spectroscopic methods.

The formation of melanized appressoria is a prerequisite for successful invasion of host plants by *Magnaporthe grisea* and *Colletotrichum* species. Inhibitors of the dihydroxynaphthalene melanin (DHN-melanin) biosynthesis, like tricyclazole or pyroquilone, can prevent disease caused by these fungi.[1] In the course of our screening for inhibitors of the dihydroxynaphthalene melanin (DHN-melanin) biosynthesis in fungi, three compounds exhibiting activity in vitro[2] were isolated from fermentations of the ascomycete *Trichopezizella nidulus*, strain A73-95. In the following paper, we report the growth of the strain and the isolation, structure elucidation, and biological activities of the compounds, which are the first metabolites described from this fungal species.

The three active metabolites (**1**−**3**) were isolated from the crude extract of the culture fluids of submerged cultures of the ascomycete *Trichopezizella nidulus* (Hyaloscyphaceae) by bioactivity-guided fractionation according to the details given in the Experimental Section.Spectral analysis of the pure compounds revealed



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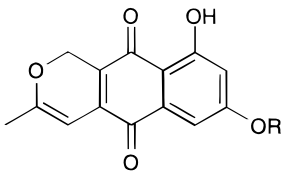

2, R=H
3, R=CH_3

that two of them, 6-deoxy-7-*O*-demethyl-3,4-anhydrofusarubin (**2**)[3] and 6-deoxy-3,4-anhydrofusarubin (**3**),[4] were known metabolites of fusarubin biosynthesis in blocked mutants of *Nectria haematococca* (the sexual form of the phytopatogenous fungus *Fusarium solani*). The third compound was a new azaphilone, with the same basic structure as the deflectins,[5] for which we propose the name trichoflectin (**1**). The EI and CI mass spectra of **1** indicated its molecular weight to be 298, and high resolution measurements indicated that the elemental composition was $C_{17}H_{14}O_5$. This was confirmed by the ^1H and ^{13}C NMR spectra in which the signals for all hydrogens and carbons were visible. The number of unsaturations in **1** is consequently 11. The signals for all 14 protons in the ^1H NMR all correlated to ^{13}C NMR resonances in the HMQC spectrum, indicating that **1** does not contain any exchangeable protons. The structure could then be elucidated by analysis of the data obtained in 2D NMR experiments, of which the correlations observed in the HMBC spectrum are summarized in Figure 1. The presence of a 1(*E*)-propenyl group was established by the $^1H-^1H$ couplings between CH_3-12 and H-11 and H-11 and H-10. The positioning of this on C-9 of the C-8/C-9 double bond was shown by HMBC correlations from H-11 to C-9, which according to its ^{13}C chemical shift should be oxygenated, and from H-10 to both C-9 and C-8. H-8 gave HMBC correlations to C-2 and C-6 in addition to C-10. A strong NOESY correlation as well as a weak COSY correlation was also observed between H-6 and H-8. C-1 is an oxygenated olefinic carbon as suggested by its ^{13}C chemical shift as well as the $^1J_{CH}$, which had a typical value (208 Hz), and its position at the other side of the C-9 oxygen was shown by the HMBC correlation between H-1 and C-9. H-1 also gave HMBC correlations to C-2, C-3, and C-7, which was in agreement with the suggested structure. The C-4 methyl group (C-17), which gave HMBC correlations to C-3 (an olefinic carbon), C-4 (an oxygenated tertiary carbon), and C-5 (a keto carbon), must be positioned on C-4, and the second six-membered ring was indicated by the HMBC correlations observed between H-6 and C-2 and C-4. The acetyl group C-15/C-16 was shown to be positioned at C-14 by a strong HMBC correlation between CH_3-16 and C-14, which according to a weak HMBC correlation between CH_3-16 and C-3 should be part of a C-3/C-14 double bond. There were no HMBC correlations to C-13, but there is no other alternative than to put it as a lactone carbonyl group between O-4 and C-14. Trichoflectin (**1**) has only one asymmetric carbon, and the absolute configuration of C-4 has, in similar azaphilones, been shown to be

* To whom correspondence should be addressed. H.A. Tel.: +49-631 205 2615. Fax: +49-631 205 2999. E-mail: anke@rhrk.uni-kl.de. O.S. Tel.: +46-46 222 8213. Fax: +46-46 222 8209. E-mail: Olov.Sterner@orgk2.lth.se.
† University of Kaiserslautern.
‡ Lund University.

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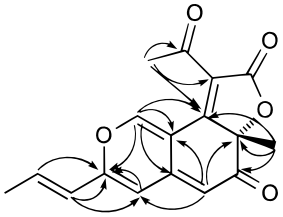

Figure 1. HMBC correlations observed with trichoflectin (**1**).

controlled by the sign of the specific rotation.[6] According to this, **1** should have the *S* configuration at C-4.

All three compounds are inhibitors of DHN-melanin biosynthesis. In our test system, using *Lachnellula* sp. A32-89 as a test organism,[2] 50 μg per paper disk (6 mm) of **1** resulted in a pigment inhibition zone 19 mm in diameter, while the same amounts of compounds **2** and **3** both gave inhibition zones of 14 mm. The mycelial growth of the test organism was not affected. Trichoflectin (**1**) exhibits moderate antimicrobial activities; 50 μg/per paper disk (6 mm) of compound **1** in the standard plate diffusion assay[7] resulted in inhibition zones of 23 mm against *Mucor miehei* and *Bacillus subtilis*. Compounds **2** and **3** also show moderate antimicrobial activities.[8]

Experimental Section

General Experimental Procedures. Preparative HPLC was carried out on a JASCO HPLC (PU 980, MD 910), analytical HPLC was carried out on a Hewlett-Packard 1090 Type II HPLC with LiChrosphere RP18 (10 μm; 125 × 4 mm) and a H_2O:MeOH gradient. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed, and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a JEOL SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting points (uncorrected) were determined with a Reichert microscope, and the optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Producing Strain. Fruiting bodies of *T. nidulus* were collected from rotten wood in Oberjoch, Germany. The fungus showed characteristics of the genus and species.[9] A mycelial culture was obtained from ascospores and maintained on YMG medium with 2% agar. A voucher specimen (no. A73-95) of the fungus is deposited in the herbarium of the department of Biotechnology, University of Kaiserslautern.

Cultivation and Isolation. Submerged cultures were carried out in YMG medium (malt extract 10 g/L, glucose 10 g/L, yeast extract 4 g/L) in a 20 L fermenter (Braun Biostat U) at 24 °C with an aeration rate of 3.2 L/min and agitation (120 rpm). As inoculum, a well-grown culture (140 h, 200 mL) in the same medium was used. During fermentation, aliquots of the culture fluid (100 mL) were extracted twice with ethyl acetate. The combined extracts were dried with Na_2SO_4. After evaporation of the solvent in vacuo (40 °C), the oily residue was dissolved in 1 mL of MeOH. For HPLC analysis, 10 μL of the methanolic solution were used. The fermentation was terminated after 185 h, when the glucose was used up and the biological activities had reached their peaks. The mycelium, containing no active compounds, was separated from the culture fluid by filtration and discarded. The culture fluid was applied onto a column with Mitsubishi HP21 (styrene and divinylbenzene copolymer) resin. The column was washed with 2 L of H_2O, before the active compounds were eluted with 2 L of acetone. The eluate was evaporated in vacuo to an aqueous residue. The residue was extracted twice with ethyl acetate. Evaporation of the solvent yielded 2.6 g of crude extract. The crude extract was applied to silica gel (300 g), and the active compounds were eluted using a cyclohexanes−ethyl acetate gradient. Fractions containing **1** were eluted with cyclohexanes−ethyl acetate 6:4. Purification was achieved by preparative HPLC (250 × 25 mm, 7 μm, LiChrosphere RP18) using a water−acetonitrile gradient (flow 5 mL/min; 0−100% acetonitrile in 120 min; retention time 69 min, retention volume 345 mL). Fractions containing compound **2** were eluted with cyclohexanes−ethyl acetate 8:2, and **2** was isolated from these fractions by preparative HPLC (250 × 25 mm, 7 μm, LiChrosorb CN) with a cyclohexane−methyl *tert*-butyl ether (MTBE) gradient (flow 5 mL/min; 15 min 30% MTBE, 70% cyclohexane; 30% MTBE to 100% MTBE in 75 min; retention time 32 min, retention volume 160 mL). Compound **3** was purified from a fraction eluted from silica gel with cylohexanes−ethyl acetate 9:1, by the same preparative HPLC system (retention time 36 min, retention volume 180 mL). From 18 L of culture filtrate 10.9 mg of **1**, 1.7 mg of **2**, and 1.9 mg of **3** were obtained.

Trichoflectin (**1**) was obtained as orange crystals (heptane): mp 185−187 °C; $[\alpha]_D$ −121° (*c* 0.7, $CHCl_3$); UV (MeOH) λ_{max} (ϵ) 351 nm (16 100), 294 (24 800), 216 (14 500); IR (KBr) 3435, 1760, 1685, 1625, 1535, 1420, 1355, 1310, 1245, 1180, 1120, 870, 615 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.82 (1H, s, H-1), 6.62 (1H, dq, J = 7.0, 15.5 Hz, H-11), 6.06 (1H, s, H-8), 6.01 (1H, dd, J = 1.4, 15.5 Hz, H-10), 5.33 (1H, s, H-6), 2.59 (3H, s, CH_3-16), 1.95 (3H, dd, J = 1.4, 7.0 Hz, CH_3-12), 1.68 (3H, s, CH_3-17); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.4 (s, C-15), 190.0 (s, C-5), 168.2 (s, C-13), 165.6 (s, C-3), 155.2 (s, C-9), 153.2 (d, C-1), 144.1 (s, C-7), 136.3 (d, C-11), 123.2 (s, C-14), 122.3 (d, C-10), 110.8 (s, C-2), 107.5 (d, C-8), 105.7 (d, C-6), 87.6 (s, C-4), 30.1 (q, C-16), 26.3 (q, C-17), 18.7 (q, C-12); HREIMS *m/z* 298.0848 (calcd for $C_{17}H_{14}O_5$ 298.0841), 256.0762 (calcd for $C_{15}H_{12}O_4$ 256.0736); EIMS *m/z* 298 (6), 256 (100), 227 (20), 213 (11), 200 (18), 185 (21), 157 (7), 128 (9), 43 (8); CIMS (CH_4) *m/z* 339 (M + $C_3H_5^+$, 7), 327 (M + $C_2H_5^+$, 11), 299 (M + H^+, 100), 297 (M − C_2H_2O + $C_3H_5^+$, 3), 285 (M − C_2H_2O + $C_2H_5^+$, 9), 257 (M − C_2H_2O + H^+, 55).

Biological Assays. Inhibition of DHN-melanin biosynthesis was assayed in an agar diffusion assay as

described previously,[2] while antimicrobial activity was assayed as described in ref 7.

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