Chemical and Biological Characterization of Corticrocin, a Yellow Pigment Formed by the Ectomycorrhizal Fungus *Piloderma croceum*

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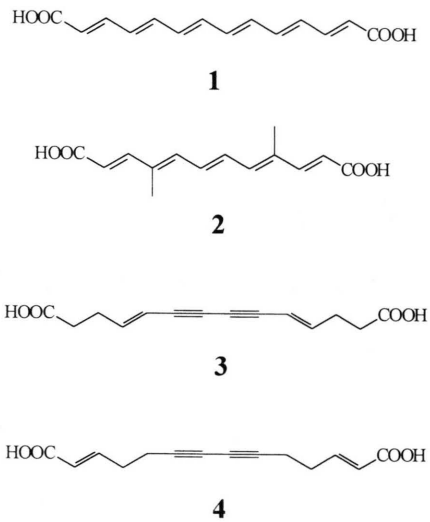
Corticrocin is synthesized by mycelia of *Piloderma croceum* in contrast to mycorradicin, which is deposited into the vacuoles of maize root cells upon colonization with arbuscular mycorrhizal fungi. Corticrocin is formed only when *P. croceum* is grown in darkness. This indicates that the compound is unlikely of a carotenoid origin which also follows from the chemical structure. Corticrocin is deposited at the surface of the fungal hyphae. The all-*E*-geometry of the polyunsaturated chain is confirmed by comparing the spectral and chromatographic properties of the natural product with a synthetic reference.

Introduction

The mycelia of *Piloderma* (= *Corticium*) *croceum* form bright yellow hyphae when colonizing roots of Norway spruce, Scots pine, worthleberry (*Vaccinium vitis-idaea*) and others (Erdtman, 1948). The pigment was purified, identified as all-*E*-tetradeca-2,4,6,8,10,12-hexaene-1,14-dioic acid (**1**) and termed corticrocin (Erdtman, 1948). The recently characterized mycorradicin (**2**) (Klingner *et al.*, 1995a), the major component of the yellow pigment formed in maize and other grasses upon colonization by arbuscular mycorrhizal fungi, is also a dicarboxylic acid with 14 C-atoms in total. Mycorradicin is deposited into the vacuoles of the root cells in large quantities during the course of this symbiosis (Klingner *et al.*, 1995b). In contrast to corticrocin (**1**), the polyene chain of mycorradicin (**2**) is substituted by two methyl groups and the latter is probably of carotenoid origin. Corticrocin unlikely is an isoprenoid as these methyl groups are missing. The structure elucidation of corticrocin by *Erdtman* (Erdtman, 1948) was based on its acidic nature, its UV-spectrum and its behaviour towards oxidation and hydrogenation. In 1954, the compound was synthesized independently by two research groups (Shaw and Whiting, 1954; Weedon, 1954), who used the rearrangement of tetradeca-2,12-diene-6,8-diyne-1,14-dioic acid (**4**), resp. its 4,10-diene-6,8-diyne isomer (**3**), in alkaline solution to obtain the all-*E*-polyene chain. The melting point of the dimethyl esters of the natural and the synthetic products compared well and it was undepressed in a mixture of the two compounds (Shaw and Whiting, 1954; Weedon,



Scheme 1. Chemical structure of corticrocin **1**, mycorradicin **2** and intermediates (**3**, **4**) of the first syntheses of corticrocin (Shaw and Whiting, 1954; Weedon, 1954).

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1954). Differences were, however, observed in the UV- and infrared-spectra of the two products (Shaw and Whiting, 1954), which disappeared after further recrystallization from acetic acid. Since the techniques in structure elucidation and isomer separation were not so advanced as nowadays, *Erdtman* (Erdtman, 1948) and the other groups (Shaw and Whiting, 1954; Weedon, 1954) may have missed some structural features. Therefore, the structure of corticrocin, in particular its all-*E*-geometry, was reexamined, and the findings were corroborated by chromatographic and spectroscopic data. Additionally, experiments on the light dependence of its biosynthesis and on its deposition in the hyphae will be presented in the present communication.

Materials and methods

General

HPLC: Kontron model 200, column: LiChrocart RP 18 (125 mm, Merck); solvent: MeOH/H_2O 2:8 (5 min), linear gradient to 100% MeOH (15 min), 100% MeOH (10 min), flow: 1 ml/min; Hewlett-Packard 1040A diode-array detector. – CC: silicagel 60, 63–200 (Merck). – UV: Lambda 15 UV/VIS spectrophotometer (Perkin Elmer) and DU 7000 (Beckman). – Fluorescence-spectra: Jobin-Yvon JV3D fluorometer, solvent: MeOH. – MS: Finnigan-MAT 4510 GC/MS, solid probe (EI: 70 eV). – NMR: Bruker AM-300 (^{1}H: 300 MHz, ^{13}C: 75 Mhz). All solvents were dried and distilled prior to use. All glassware was dried carefully and reactions were carried out in inert (Ar) atmosphere.

Culture conditions

The axenic isolate of *Piloderma croceum* var. EVA was kindly supplied by Prof. T. Unestam, S-Uppsala and grown on 1.5% agar in a medium containing per l in g: malt extract 1.5, D-glucose 5, $(NH_4)_2HPO_4$ 0.25, $MgSO_4$ x 7 H_2O 0.15, $CaCl_2$ 0.05, NaCl 0.025 and 1.2 ml $FeCl_3$ (1% solution). 100 µg thiamine-HCl.

Measuring the formation of corticrocin

Freeze dried hyphae were ground with a mortar and extracted with MeOH by stirring at room temperature. After centrifugation (10 min, 12 000 x *g*) and filtration, excess of CH_2N_2 in Et_2O was added followed by evaporating the solution to dryness. Polar impurities were removed by flash-chromatography on RP18 with MeOH. Spectra were recorded from this material.

Isolation of crude corticrocin

Freeze dried hyphae (108.3 mg) were extracted with hot acetone as described by *Erdtman* (Erdtman, 1948). Corticrocin (0.8 mg, 60% purity as determined by HPLC-analysis at 210 nm) crystallized from the filtrate in orange needles. The crude product was esterified with CH_2N_2 and the resulting dimethyl ester was used for comparison with the synthetic reference. Both compounds showed identical chromatographic (HPLC) behaviour and spectroscopic (UV and MS) properties.

all-E-*Deca-2,4,6,8,-tetraene-1,10-dioic acid dimethyl ester* (**6**)

E,E-Mucondialdehyde (**5**) (100 mg, 0,91 mmol) (Bohn and Koßmehl, 1974) and carbomethoxymethylenetriphenylphosphorane (1.5 g, 4.5 mmol) (Isler *et al.*, 1957) were dissolved in 30 ml toluene. The reaction mixture was heated to reflux for 8 h. The solvent was removed *in vacuo* and the residue chromatographed on silicagel (100% Et_2O) to give 171.1 mg (85%) **6**. UV λ_{max} (MeOH) nm (ε): 327 (sh, 28700), 339 (42850), 356 (43050); EIMS *m/z* (rel. int.): 222 $[M]^+$ (20), 207 $[M\text{-}CH_3]^+$ (1), 191 $[M\text{-}OCH_3]^+$ (11), 158 (23), 131 (86), 103 (100), 77 (42), 51 (48); ^{1}H-NMR ($CDCl_3$): δ 7.31 (2H, dd , *J* =15.3 and 10.9 Hz, H-3 and H-8), 6.59 (2H, m, H-5 and H-6), 6.43 (2H, m, H-4 and H-7), 5.95 (2H, d, *J* =15.3 Hz, H-2 and H-9), 3.74 (6H, s, OCH_3); ^{13}C-NMR ($CDCl_3$): 167.2 (s, C=O), 143.6 (d, C-3 and C-8), 139.0 (d, C-5 and C-6), 133.6 (d, C-4 and C-7), 122.5 (d, C-2 and C-9), 51.7 (q, CH_3).

all-E-*Deca-2,4,6,8-tetraene-1,10-diol* (**7**)

The dimethyl ester **6** (100 mg, 0.45 mmol), dissolved in 30 ml Et_2O, was added dropwise at −40 °C to a solution of 240 mg $LiAlH_4$ (6.31 mmol) in 40 ml THF. After stirring for 4h at this temp. and hydrolysis with ice cold 2 N H_2SO_4 the mixture was extracted with CH_2Cl_2, the organic phase was dried with $MgSO_4$ and the solvent

evaporated *in vacuo* to yield 24 mg (32%) of crude diol (**7**). Since the compound tends to polymerize, when isolated, the product was used without further purification. EIMS *m/z* (rel. int.): 166 $[M]^+$ (26), 148 $[M\text{-}H_2O]^+$ (8), 135 (35), 114 (44), 91 (100), 79 (87), 67 (74).

all-E-*Deca-2,4,6,8-tetraene-1,10-dial* (**8**)

The crude diol (**7**) (50 mg, 0.3 mmol) was dissolved in 5 ml acteone. MnO_2 (0.9 g, 10.3 mmol) was added and the mixture was stirred at room temp. for 2 h. After filtration the solvent was evaporated *in vacuo*. The remaining oil was chromatographed on silicagel, deactivated with 10% H_2O, using a gradient of cyclohexane/Et_2O (8:2, v/v) to Et_2O (100%) as eluent, to give 6 mg (12%) of the dialdehyde (**8**). UV λ_{max} (MeOH) nm (ε): 334 (sh, 19100), 346 (26300), 361 (28200); EIMS *m/z* (rel. int.): 162 $[M]^+$ (40), 133 $[M\text{-}CHO]^+$ (28), 115 (34), 94 (97), 77 (100), 51 (54); ^{1}H-NMR (acetone-d_6): δ 9.62 (2H, d, *J*=7.9 Hz, H-1 and H-10), 7.4 (2H, dd, *J*=15.2 and 10.4 Hz, H-3 and H-8), 6.97 (2H, m, H-5 and H-6), 6.84 (2H, m, H-4 and H-7), 6.25 (2H, dd, *J*=15.2 and 10.7 Hz, H-2 and H-9); ^{13}C-NMR ($CDCl_3$): 193.8 (d, 2 C=O), 151.3 (d, C-3 and C-8), 141.3 (d, C-5 and C-6), 135.7 (d, C-4 and C-7), 134.0 (d, C-2 and C-9).

all-E-*Tetradeca-2,4,6,8,10,12-hexaene-1,14-dioic acid dimethyl ester* (**9**)

Carbomethoxy-methylenetriphenylphosphorane (45 mg, 0.13 mmol) is added to a solution of the dialdehyde (**8**) (6 mg, 0.037 mmol) in 5 ml toluene. The mixture is refluxed for 9 h. Upon cooling of the solution the diester (**9**) precipitates as yellow solid. It is recrystallized from methanol to yield 6.3 mg (63%) of corticrocin dimethyl ester. UV λ_{max} ($CHCl_3$) nm (ε): 374 (60300), 393 (93500), 416 (93700); λ_{max} (MeOH) nm (ε): 371 (68400), 388 (107200), 409 (107200): EIMS *m/z* (rel. int.): 274 $[M]^+$ (26), 243 $[M\text{-}OCH_3]^+$ (6), 215 $[M\text{-}COOCH_3]^+$ (9), 183 (34), 155 (100), 153 (45), 59 (61); ^{1}H-NMR ($CDCl_3$): δ 7.32 (2H, dd, *J*=15.2 and 11.4 Hz, H-3 and H-12), 6.6 (2H, dd, *J*=14.8 and 10.5 Hz, H-5 and H-10), 6.45 (2H, m, H7 and H8), 6.41 (2H, m, H6 and H9), 6.37 (2H, dd, *J*=14.8 and 11.4 Hz, H4 and H11), 5.89 (2H, d, *J*=15.2 Hz, H2 and H13), 3.73 (6H, s, OCH_3); ^{13}C-NMR ($CDCl_3$): 167.6 (s, 2 C=O), 144.4 (d, C3 and C12), 140.4 (d, C5 and C10), 135.6 (d, C7 and C8), 134.1 (d, C6 and C9), 131.3 (d, C4 and C11), 120.8 (d, C2 and C13), 51.7 (q, 2 OCH_3).

Results and Discussion

After a lag phase of 3–5 d, *P. croceum* linearly grew in the dark on agar plates for at least 7 weeks (Fig. 1). Fungi on illuminated plates (lower light intensity of 15 μE m^{-2} s^{-1}) showed sluggish growth, which ceased after 20–25 d. Almost no growth was observed at 45 μE m^{-2} s^{-1} (Fig. 1).

Mycorradicin in grasses colonized by the arbuscular mycorrhizal fungus *Glomus* is deposited into the vacuole of the cortex and the endodermis cells of roots during the course of its formation (Klingner *et al.*, 1995b). In contrast, corticrocin was visible as yellow pigment in granular form at the sur-

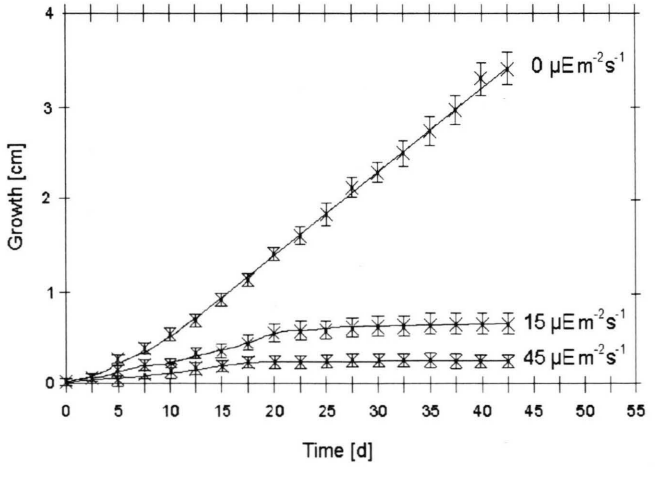


Fig. 1. Growth of *Piloderma croceum* under various light conditions. Growth represents the arithmetic means of the increase in diameter. Twelve cultures, grown on agar plates, were analyzed. Bars indicate standard deviations.

face of the hyphae (Jülich, 1984). Dark but not light grown *P. croceum* showed such corticrocin depositions on or at the cell wall of the hyphae (Fig. 2). Cultures incubated in light were greyish-white. In contrast, carotenoid synthesis in other fungi such as *Neurospora, Phycomyces* and *Fusarium* is light dependent and mediated by the B/UV photoreceptor (De Fabo *et al.*, 1976; Seger and Schmidt, 1994). This indicated that corticrocin from *P. croceum* unlikely belongs to this class of pigments.

Methanolic crude extracts from dark grown *P. croceum* showed a distinct absorption spectrum with maxima at 365, 380, and 402 nm. Compared to the spectrum of mycorradicin (Klingner *et al.*, 1995a), the maxima were shifted by about 5 nm to longer wavelengths, but the spectra showed the same fine structure. The fluorescence emission spectrum of the extracts had maxima at 498, 536, 575 and 610 nm – the maxima of mycorradicin at 498 and 533 nm show comparable values (Klingner *et al.*, 1995a). In crude preparations, corticrocin was less sensitive to exposure to white light (~15% decrease in absorbance after 24 h illumination with a conventional 40 W fluorescence bulb) than mycorradicin (Klingner *et al.*, 1995b).

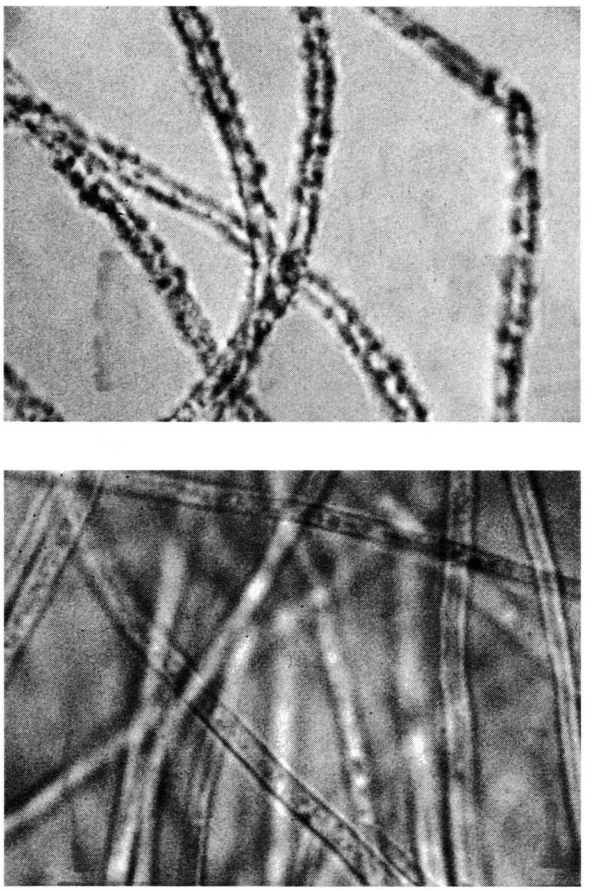
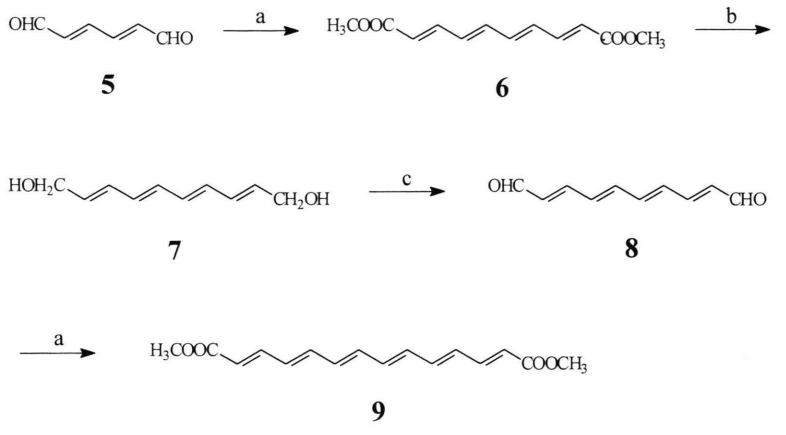


Fig. 2. Deposition of corticrocin in *P. croceum*.
a) dark grown hyphae with depositions of yellow grains at the the outer surface of the hyphae. b) hyphae grown under illumination with 45 $\mu E\ m^{-2}\ s^{-1}$ exhibiting no visible grain depositions.

A small sample of corticrocin was isolated from freeze dried hyphae of *P. croceum* following the procedure of *Erdtman* (Erdtman, 1948). Enough material was obtained by this way for HPLC analysis, UV- and mass spectroscopy, however, not for recording NMR spectra. Therefore, a synthetic reference was prepared to confirm the proposed chemical structure of the natural product.

The multistep syntheses of corticrocin, published earlier (Shaw and Whiting, 1954; Weedon, 1954), gave rise to rather small overall yields. Thus, a different reaction sequence for the preparation of the natural product was tried, as outlined in Scheme 2. All-*E*-mucondialdehyde (**5**) (Bohn and Koßmehl, 1974) was reacted with carbomethoxy-methylenetriphenylphosphorane to give the dimethyl ester **6** of all-*E*-deca-2,4,6,8-tetraene-1,10-dioic acid. Reduction of the diester with $LiAlH_4$ and oxidation of the resulting diol **7** with MnO_2 yielded the dialdehyde **8**. Finally, corticrocin dimethyl ester **9** was obtained by a second Wittig reaction in 2% overall yield. The structure of the product was ascertained to be the all-*E*-tetradeca-2,4,6,8,10,12-hexaene-1,14-dioic acid dimethyl ester by UV-, NMR- and mass-spectra. The assignment of the NMR resonances is based upon CH-correlation and long range experiments. The symmetry of the compound, documented by the presence of only eight signals in the ^{13}C- and seven in the ^{1}H-spectrum, as well as the H-H-coupling constants prove the all-*E*-geometry of the six double bonds.

The crude natural product, obtained as described above, was esterified with diazomethane. HPLC-analysis proved that a single product resulted from this reaction. The dimethyl ester of the natural product was identical with the synthetic reference, which was unequivocally shown by comparing their chromatographic (HPLC) and



Scheme 2. Synthesis of corticrocin dimethyl ester (a = Ph_3P=CH-$COOCH_3$/toluene, b = $LiAlH_4$, c = MnO_2).

spectroscopic (UV, MS) properties. Thus, *Erdtman's* structure of corticrocin was confirmed. The compound is not related to mycorradicin and is most likely a product of the polyketide metabolism (Gill and Steglich, 1987).

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