# Melanocrocin, a Polyene Pigment from *Melanogaster broomeianus* (Basidiomycetes)

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A new polyene pigment, melanocrocin, has been isolated either from fruit bodies or mycelial cultures of the subterranean fungus *Melanogaster broomeianus*. The structure of the pigment was determined by spectroscopic methods and chemical transformations. Melanocrocin is the *N*-acyl derivative of L-phenylalanine methyl ester with a polyolefinic carboxylic acid.

## Introduction

The tuberous, reddish-brown fruit bodies of the Gasteromycete *Melanogaster broomeianus* Berk. are completely or partly embedded in the soil. From this fungus the cyclopentenone derivatives (–)-chamonixin (**1**) and (–)-involutin (**2**) (Fig. 1) have been isolated, which supports the inclusion of *Melanogaster* in the order Boletales (Besl *et al.*, 1996; Binder *et al.*, 1997).

In this communication we report the structure of melanocrocin (**4**), a polyene pigment that is responsible for the brilliant orange colour of extracts from this fungus.

## Results and Discussion

Extraction of the fruit bodies or the mycelial cultures of *Melanogaster broomeianus* with ethyl acetate and chromatography of the extract on a polyamide column yielded the crude pigment that was further purified by HPLC on reversed phase (RP-18). The isolation procedure has to be carried out with care to avoid the formation of isomerisation products.

The UV/Vis spectrum of the polyene exhibits strong absorption maxima at 306, 319 and 404 nm indicating a polyene chromophore. The HR EI-MS of the pigment shows a molecular ion at *m/z* 459 which corresponds to the molecular formula $C_{29}H_{33}NO_4$. The HPLC-APCI-MS of the pigment gives a $[M + H]^+$ ion at *m/z* 460. Characteristic fragment ions are visible in the HPLC-APCI-MS/MS at *m/z* 400 $[M + H - CO - CH_3OH]^+$ and 281 [M + H - phenylalanine methyl ester (H-Phe-OMe)]$^+$, respectively 180 $[H\text{-Phe-OMe} + H]^+$, indicating a H-Phe-OMe unit in the pigment.

The $^1H$ and $^{13}C$ NMR spectra of the pigment (Table I) exhibit characteristic signals for a $-CH_2(CH{=}CH)_6-$ unit and three methyl singlets, one belonging to a methyl ester group and two to a $-CH{=}C(CH_3)COCH_3$ moiety. Since NOESY correlations are detectable between 15-H and 13-H, and between 14-H and 12-H, the all-*trans* configuration is assigned to the polyene chain of **4**. In addition, all discernible olefinic couplings in the $^1H$ NMR spectrum of **4** are in the range of $J = 15$ Hz. NOESY correlations between 14-H and 19-$CH_3$ as well as between 15-H and 18-$CH_3$ revealed the *E*-stereochemistry at the double bond between C-15 and C-16.

Extensive high-field NMR measurements including $^1H,^1H$-COSY, NOESY, HMQC and HMBC led to structure **4** for melanocrocin.

Catalytic hydrogenation of melanocrocin with $PtO_2/H_2$ yielded the colourless perhydromelanocrocin (**3**) (Fig. 1). The APCI-MS of this compound shows a $[M+H]^+$-ion at *m/z* 474 in accordance with the presence of seven double bonds in

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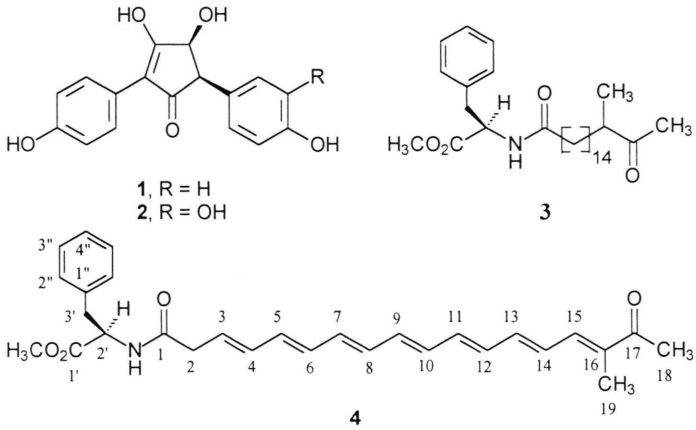



Fig. 1. (−)-Chamonixin (**1**), (−)-involutin (**2**), perhydromelanocrocin (**3**) and melanocrocin (**4**).

Table I. $^{13}C$ and $^{1}H$ NMR data of melanocrocin (**4**) (600 MHz, $CDCl_3$).

| C-atom | $\delta_C$ [ppm] | $\delta_H$ [ppm] | m | $J_{HH}$ [Hz] |
|---|---|---|---|---|
| 1 | 170.7 | – | – | – |
| 2 | 41.3 | 3.04 | d | 7.5 |
| 3 | 126.8 | 5.73 | dt | 14.7, 7.5 |
| 4–12 | 133.4, 133.6, 133.7, 133.8, 134.1, 135.9, 136.1, 137.5, 140.7 | 6.18–6.51 (4-H, 6.19; 12-H, 6.41) | m | – |
| 13 | 135.0 | 6.63 | m | – |
| 14 | 130.1 | 6.59 | m | – |
| 15 | 139.9 | 7.09 | d | 10.5 |
| 16 | 136.9 | – | – | – |
| 17 | 200.0 | – | – | – |
| 18 | 26.3 | 2.35 | s | – |
| 19 | 12.3 | 1.91 | s | – |
| 1′ | 172.6 | – | – | – |
| 2′ | 53.7 | 4.87 | m | – |
| 3′ | 38.5 | 3.07 | dd | 14.5, 6.1 |
| | | 3.15 | dd | 14.5, 7.6 |
| 1″ | 136.3 | – | – | – |
| 2″, 6″ | 130.0 | 7.06 | m | – |
| 3″, 5″ | 129.3 | 7.25 | m | – |
| 4″ | 127.9 | 7.23 | m | – |
| $OCH_3$ | 53.0 | 3.72 | s | – |
| NH | | 3.95 | d | 7.4 |

Assigned by $^{1}H$, $^{1}H$ COSY, HMQC, NOESY and HMBC measurements.

the original pigment. In the $^{1}H$ NMR spectrum of **3** the characteristic signals of the phenylalanine residue can be readily discerned.

The amino acid was unambiguously identified by hydrolysis of melanocrocin (**4**) with 6 N HCl and subsequent GC analysis of the trimethylsilylated amino acids. The L-configuration of the amino acid was established by conversion into the (*S*)-MTPA-Phe-OMe derivative and comparison with the corresponding derivatives of D- and L-phenylalanine by GC-MS (Dale *et al.*, 1969).

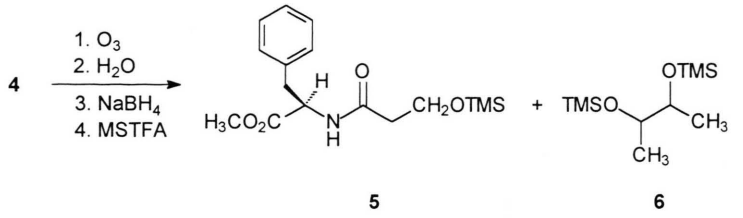
The structure of melanocrocin was confirmed by ozonolysis followed by reduction of the ozonides with sodium borohydride and GC-MS analysis of the trimethylsilylated reduction products. The detection of *N*-(3-trimethylsilyloxypropanoyl)-phenylalanine methyl ester (**5**) and 2,3-(bis-trimethylsilyloxy)butane (**6**) supports the position of the methylene group and the structure of the terminus at the polyene chain, respectively.

The structure of melanocrocin (**4**) resembles that of piptoporic acid and related pigments from *Piptoporus australiensis*, which, however, lack the amino acid unit (Gill, 1982). Conjugates of unbranched polyolefinic dicarboxylic acids with amino acids have been found in *Boletus laetissimus* and *B. rufo-aureus (Kahner et al.*, 1998).

## Experimental

### *General*

TLC: silica gel 60 $F_{254}$ (Merck), toluene-$HCO_2Et$-$HCO_2H$ (10:5:3). Column chromatography: Polyamide SC 6-Ac (Macherey-Nagel). Prep.-HPLC: Column: Nucleosil RP-18 (Macherey-Nagel, 250 × 20 mm; 7 μm), UV detection at 400 nm). UV: Perkin-Elmer Lambda spectrophotometer. IR: Bruker FTIR spectrophotometer IFS 45. NMR: Bruker AMX-600 spectrometer ($^{1}H$ at

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Scheme 1. Ozonolysis of **4**.

600.13, $^{13}C$ at 150.9 MHz), chemical shifts in δ rel. to $CDCl_3$ ($\delta_H$ 7.26, $\delta_C$ 77.7) as internal standard. HR EI-MS: Finnigan MAT 95Q instrument using EI at 70 eV. GC-MS: Varian Model 3400 GC with a split-splitless capillary injector coupled to a Finnigan MAT 90 instrument using EI at 70 eV. Capillary column DB-5 (25 m × 0.25 mm id, 0.25 μm df, J & W scientific). Temperature programmes: Determination of the configuration of (*S*)-MTPA-Phe-OMe: 200 °C for 2 min, then to 300 °C with 5K/min and isotherm at 300 °C for 10 min. Identification of the ozonolysis products: 50 °C for 2 min, then to 300 °C with 10K/min and isotherm at 300 °C for 8 min. HPLC-APCI-MS: Gynkotek-HPLC equipped with a Nucleosil RP-18 column (Macherey-Nagel, 250 × 2 mm, 5 μm operation temperature 40 °C, flow: 300 μl/min [solvent A: $H_2O$ solvent B: $CH_3CN$; gradient: 50% A/50% B → 100% B in 40 min]) coupled with a Finnigan TSQ 7000, Finnigan API ion source interface, positive APCI mode: ionisation 4.5 kV, capillary temperature 200 °C, mass range 50–800 mu, multiplier 1000 V (scan modus), MS/MS: argon collision gas 1.5 mTorr, sheath gas ($N_2$) 40 psi, multiplier 1400 V, collision energy automatically rotated at −20, −30, −40 eV.

*Fungal material*

M. broomeianus was collected at the campus of the University of Regensburg, Germany, in September 1996 (leg. et det. H. Besl). Voucher specimens were deposited in the Herbarium of the Institute of Botany, University of Regensburg.

*Extraction and isolation*

Fresh fruit bodies (20 g) or mycelial cultures of *M. broomeianus* were carefully extracted with ethyl acetate in the dark. The extracts were concentrated under reduced pressure at 40 °C to yield an orange-brown residue. The pigments were separated by column chromatography on polyamide first by elution with petroleum (bp 60–80 °C), then with ethyl acetate. The yellow ethyl acetate fraction contained mainly melanocrocin (**4**) that was purified by prep. HPLC on a RP-18 column [solvent A: $H_2O$, solvent B: $CH_3CN$; gradient: 50% A/50% B → 100% B (40 min)]. Yield of **4** in form of an amorphous solid: 14 mg. Due to the instability of the polyene pigment, NMR measurements should be performed immediately after the isolation. Nevertheless, the formation of isomers could not be completely avoided.

*Melanocrocin* (**4**)

Yield 14 mg (0.07% of fresh weight), amorphous orange solid, TLC: $R_f$ 0.60; HPLC: $R_t$ = 24 min; UV: $\lambda_{max}^{CH_3OH}$ nm (log ε): 243 (3.884), 283 (3.891), 306 (3.914), 319 (3.902), 404 (4.307); IR (KBr): $\tilde{\nu}$ ($cm^{-1}$): 3428 (s), 2926 (s), 2669 (m), 1742 (m), 1648 (s), 1542 (m), 1498 (m), 1456 (m), 1366 (w), 1215 (m), 1008 (m), 897 (w), 744 (m), 696 (m), 608 (w); $^1H$ and $^{13}C$ NMR: see Table I; EI-MS: *m/z* (rel. int.): 459 (65) $[M]^+$, 180 (30), 162 (100), 161 (23), 146 (21), 131 (29), 121 (21), 120 (89), 108 (26), 107 (54), 106 (39), 105 (47), 104 (59). HR EI-MS: *m/z*: found 459.2395 $[M]^+$ (calc. for $C_{29}H_{33}NO_4$ 459.2410); HPLC-APCI-MS: $R_t$ = 21.2 min; *m/z* 460 $[M + H]^+$; HPLC-APCI-MS/MS: (parent ion *m/z*: 460, 20eV) *m/z* (rel. int.): 400 (25), 281 (56), 186 (81), 180 (100), 120 (60).

*Perhydromelanocrocin* (**3**)

**4** (2 mg) in MeOH (10 ml) was hydrogenated with $PtO_2/H_2$ at 25 °C under atmospheric pressure for 1 h. After filtration of the mixture, the solvent was evaporated to yield 2 mg of perhydromelanocrocin (**3**) as a colourless solid. IR (KBr): $\tilde{\nu}$ ($cm^{-1}$): 3435 (s), 2922 (s), 2851 (s), 2475 (w), 1744

(m), 1709 (m), 1641 (s), 1536 (m), 1498 (w), 1445 (m), 1375 (w), 1252 (m), 1172 (m), 721 (w), 700 (m), 607 (m), 497 (m); $^1$H NMR: δ (ppm): 1.06 (d, $J$ = 6.9 Hz, 3H, 18-H), 1.15–1.35 (m, 22H), 1.49 (tt, $J$ = 7.5, 6.9 Hz, 2H, 3-H), 1.62 (m, 2H), 2.13 (s, 3H, 18-H), 2.14 (t, $J$ = 7.3 Hz, 2H, 2-H), 2.55 (tq, $J$ = 6.9, 6.8 Hz, 1H, 16-H), 2.94 (dd, $J$ = 13.9, 9.4 Hz, 1H, 3′-H), 3.16 (dd, $J$ = 13.9, 5.5 Hz, 1H, 3′-H), 3.69 (s, 3H, $OCH_3$), 4.67 (dd, $J$ = 9.4, 5.5 Hz, 1H, 2′-H), 7.18–7.21 (m, 3H, 3″-H, 4″-H, 5″-H), 7.26 (dd, $J$ = 7.6, 7.3 Hz, 2H, 2″-H, 6″-H). APCI-MS: $m/z$: 474 $[M + H]^+$.

*Acid hydrolysis of* **4** *and determination of the absolute configuration*

**4** (2 mg) was hydrolysed with 6 N HCl (10 ml) in a pressure tube at 120 °C for 24 h. After cooling to 25 °C, the solution was concentrated *in vacuo*.

1 mg of the residue was treated at 40 °C for 2 h with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (20 µl), and the amino acid was identified as phenylalanine by GC-MS.

To another sample (1 mg) was added MeOH (100 µl) and trimethylsilyl chloride (10 µl), and the mixture was kept for 1 h at 50 °C. After concentration with a $N_2$ stream, the residue was dissolved in pyridine (10 µl) and treated with (*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl (MTPA) chloride (2 µl) and a catalytic amount of 4-(dimethylamino)-pyridine (DMAP). After 4 h, the solvent was removed with $N_2$ and the residue dissolved in MeOH. GC-MS comparison with authentic samples prepared from L- and D-phenylalanine by the same procedure proved the L-configuration of the amino acid.

*Ozonolysis of* **4**

A solution of **4** (1 mg) in dry $CH_2Cl_2$ (10 ml) was cooled to −78 °C and treated for 5 min with ozone and then for 15 min with oxygen. The reaction mixture was reduced with sodium borohydride, and after removal of the solvent and treatment with MSTFA subjected to GC-MS analysis. Two main products were detected: **5** ($R_t$ = 8.16 min) and **6** ($R_t$ = 21.20 min).

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