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Detection of Melanochromes by MALDI-TOF Mass Spectrometry

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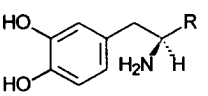
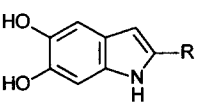
Abstract: Melanin formation from dopamine, DOPA, DHI, or DHICA, was analyzed by means of matrix-assisted laser desorption mass spectrometry. Oligomers of dihydroxyindoles, i.e. melanochromes, up to DP 11 were detected. Increments of 16 mass units provide evidence for the presence of trihydroxyindole units. The results indicate that polymerization of dihydroxyindoles towards melanins occurs by sequential coupling of monomers with concomitant oxygenation.

Melanins are naturally occurring polyphenolic pigments of largely unknown structure. They are biosynthesized by oxidative coupling of catecholamines which are ultimately derived from L-tyrosine. In humans, most important types of melanins are found in the brain where the black pigment of the *substantia nigra* is possibly a waste product from the neurotransmitter dopamine (**1**), while the dark pigment of the skin has its biosynthetic origin from L-DOPA[2] (**2**) (for a comprehensive textbook, see[3]). Due to the clinical relevance of melanins in relation to the neurodegenerative disorder Parkinson's disease as well as the increasing occurrence of malignant melanomas of the skin, research on the structure, biosynthesis, and function of the pigments has remarkably intensified in recent years.

The early steps of melanin biosynthesis are reasonably well understood: enzymatic oxidation of dopamine (**1**) or DOPA (**2**) with tyrosinase/O_2 or peroxidase/H_2O_2 to quinone intermediates and their cyclization and further oxidation leads to DHI (**3**) and DHICA (**4**), respectively (Scheme 1). The classical work of Raper and Mason and, later, also Swan (reviewed by Prota[3]), has led to the assumption the melanins are composed mostly of oligomers of **3** or **4**, respectively, and the incorporation of **4** into DOPA melanin has been confirmed.[4] However, very little is known on the chemical nature of melanochromes, i.e. intermediates between the monomeric indole quinones and the polyphenolic polymers. Prota et al.[5,6] obtained from reaction mixtures of the autoxidation or enzymatic oxidation of DHI and DHICA a series of dimers and trimers that were coupled via the indolic 2-2', 2-4', 2-7', 4-4', and 4-7' carbon atoms. Ito and Nicol[7] isolated from the tapetum lucidum of the sea catfish *Arius felis* a mixture of DHICA derivatives and suggested that this contained mainly tetramers of DHICA.



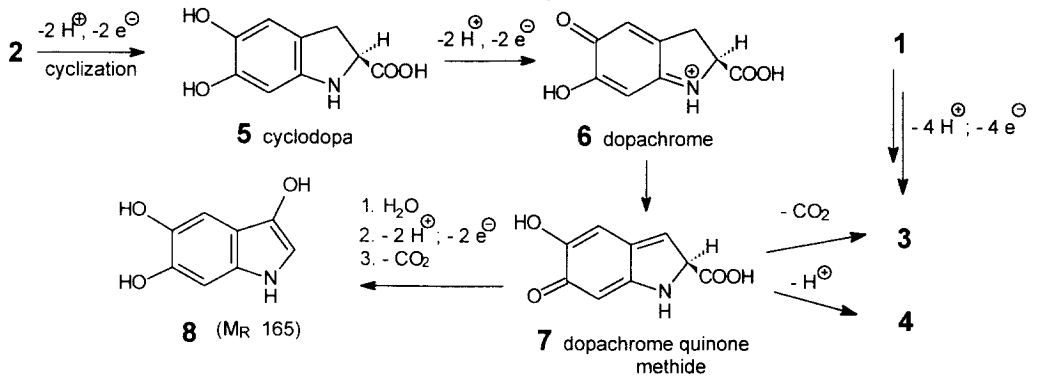
1: R = H: dopamine (M = 153)
2: R = COOH: DOPA (M = 197)



3: R = H: DHI (M = 149)
4: R = COOH: DHICA (M = 197)

We have shown earlier by solid-state NMR spectroscopy of melanin samples, prepared by peroxidase/H_2O_2 catalyzed oxidation of side chain ^{13}C labelled dopamine, that the pigment contains C-3 oxygenated indole units (c.f. **8**) which originate from the addition of hydroxy groups to quinone methide **7**.[8] In order to study intermediates of polymerization of melanin precursors, we have now analyzed reaction mixtures at various times after start of the oxidation of dopamine (**1**), DOPA (**2**), DHI (**3**), or DHICA (**4**), using MALDI-TOF mass spectrometry[9] as a tool for the detection of the corresponding melanochromes. Among the matrices tested, α-cyano-4-hydroxycinnamic acid in a 35-fold molar excess over the monomeric analyte proved to be superior to 2,5-dihydroxybenzoic acid or sinapic acid in terms of ion abundance and resolution. However, in presence of α-cyano-4-hydroxycinnamic acid as a matrix, a considerable number of ions appear in the low mass range (*m/z* < 400).

Scheme 1



Enzymatic oxidation of dopamine (**1**) was performed by means of peroxidase/H_2O_2 in sodium phosphate buffer. Immediately after addition of the enzyme, the solution turned wine-red. Samples were withdrawn after various time periods and subjected to mass spectrometry (Table 1). The spectrum recorded after 1 min (Fig. 1A) shows an intensive peak of DHI (**3**) at *m/z* 151 (calcd. $[M+H]^+$: *m/z* 150) which also could be assigned to dopamine quinone (calcd. $[M+H]^+$: *m/z* 152). The shoulder of the matrix peak at *m/z* 295 indicates the presence of a dimer (calcd. $[M+H]^+$: *m/z* 297). A trimer of DHI (calcd. $[M+H]^+$: *m/z* 444) cannot be assigned unambiguously because the presence of a matrix ion at *m/z* 443 ± 2. Ions of masses *m/z* > 450 are detectable after 3 min. A tetramer of DHI appears after 8 min at *m/z* 589 (calcd. $[M+H]^+$: *m/z* 591), and a pentamer is indicated by a broad low intensity peak at *m/z* 740 (calcd. $[M+H]^+$: *m/z* 738) (Fig. 1B). Those are clearly resolved in the spectrum recorded 20 min after start of the reaction while higher ions up to *m/z* 1179 are present (Fig. 1C). The peak centers correspond to a DHI hexamer at *m/z* 885 ± 2 (calcd. $[M+H]^+$: *m/z* 885), heptamer at *m/z* 1032 (calcd. $[M+H]^+$: *m/z* 1032), and octamer at *m/z* 1179 (calcd. $[M+H]^+$: *m/z* 1179). The ion peaks of higher oligomers are situated within rather broad groups of signals, as is expected not only from isotope composition but also from an increasing chemical heterogeneity of the samples. A nonamer appears after 50 min at *m/z* 1326 (calcd. $[M+H]^+$: *m/z* 1326), a decamer at *m/z* 1472 (calcd. $[M+H]^+$: *m/z* 1473) and an undecamer at *m/z* 1619 (calcd. $[M+H]^+$: *m/z* 1620) (Fig. 1D and 1E). At this time, continuing turnover of the monomer is indicated by the relatively low intensitiy of ion *m/z* 151 as compared with the intensity of matrix ions. Oligomers are barely detectable after 90 min due to the ongoing polymerization of the melanochromes.

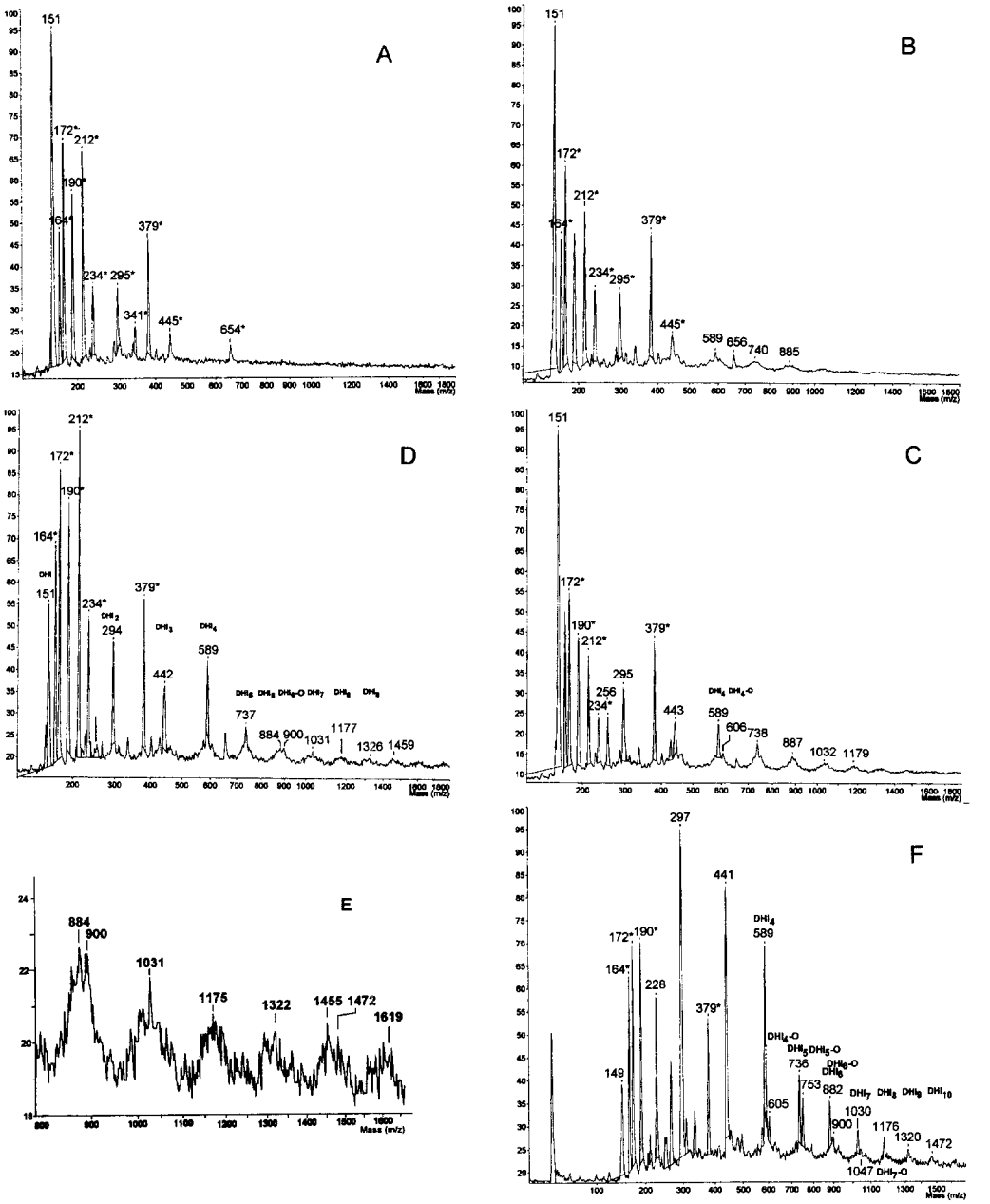


Figure 1: MALDI-TOF mass spectra of A) dopamine/peroxidase/H_2O_2 after 1 min; B) dopamine/peroxidase/H_2O_2 after 8 min; C) dopamine/peroxidase/H_2O_2 after 20 min; D) dopamine/peroxidase/H_2O_2 after 50 min; E) enlarged section of spectrum D; F) DHI autoxidation in acetone after 4 h; matrix ions are labelled with an asterisk.

Table 1: Mass Spectra of Reaction Mixture of the Enzymatic Oxidation of Dopamine (**1**).

found *m/z*	calcd.[a)]	assignment	observed after time period (min)	comments
151	149	DHI or	0.2; 1; 3; 6; 8; 20; 42; 50	most intensive up to 50 min; not detected after 67 min
	151	dopamine quinone		
295	296	$(DHI)_2$	0.2; 1; 3; 6; 8; 20; 42; 50	matrix superimposed, recognized as a shoulder during first 6 min
445	443	$(DHI)_3$	1; 3; 6; 8; 20; 42; 50	matrix superimposed; the matrix ion peak is of low relative intensity
589	590	$(DHI)_4$	6; 8; 20; 42; 50	max. 26-67 min; strong
606	606	$(DHI)_4$+O	20; 42; 50	max. 26-42 min
737 ± 3	737	$(DHI)_5$	20; 42	max. 42-50 min
753	753	$(DHI)_5$+O		weak
885 ± 2	884	$(DHI)_6$	20; 42; 50	max. 42-90 min
900	900	$(DHI)_6$+O	42; 50	max. 42-50 min
1030 ± 2	1031	$(DHI)_7$	20; 42; 50	
1047	1047	$(DHI)_7$+O	42	14-42 min
1179	1178	$(DHI)_8$	20	
1324 ± 2	1325	$(DHI)_9$	20; 50	
1472	1472	$(DHI)_{10}$	50	
1619	1619	$(DHI)_{11}$	50	

a) Calculated values are for the non protonated molecular species. Possible quinonoid and semiquinonoid oxidation states are not considered.

Oxygenated indole units are indicated by increments of 16 mass units: *m/z* 590 → 606; 884 → 900. It is reasonable to assume that the oxygen is introduced *via* quinone methide **5** leading to **8** and incorporation of the latter into the growing polymer.

The ions at *m/z* 606 and 900 could be assigned also to oligomers of dopamine or to mixed dopamine-DHI oligomers [calcd. for $(dopamine)_4$: $[M+H]^+$: *m/z* 607; $(dopamine)_4$-$(DHI)_2$: $[M+H]^+$: *m/z* 901)]. However, when DHI is autoxidized in acetone, the spectrum recorded after 4 h shows a similar pattern of DHI- and hydoxylated DHI-oligomers at *m/z* 149, 297, 441, 589, 605, 736, 753, 882, 900, 1030, 1047, 1176, 1320, and 1472 (Fig. 1F). In particular, the ions of the higher oligomers are remarkably well resolved in this case. Thus, oligomerisation of DHI proceeds rapidly without participation of free dopamine in the early steps.

The reaction of a tyrosinase/O_2 catalyzed oxidation of dopamine has been studied recently with MALDI-MS by Bertazzo et al..[10] The results are consistent with our findings though the reaction proceeds slower than the peroxidase/H_2O_2 catalyzed oxidation and ions corrsponding to defined oligomers are difficult to identify. In our hands, spectra recorded from dopamine/tyrosinase reaction mixtures show more complex compositions of melanochromes.

Analysis of an tyrosinase/O_2 oxidation of DOPA (**2**) by MALDI-TOF yields very heterogeneous mixtures as compared with that of dopamine or DHI. However, detection of oligomers of DHICA in addition to mixed oligomers of DHI and DHICA leads to the conclusion that decarboxylation of DHICA to DHI occurs to some extent in the course of the reaction.

An autoxidation reaction mixture of DHICA (**4**) carried out at pH 9 shows a transient peak of a tetramer in the first few min. After 20 min, higher masses are detected. Assigned ion peaks correspond to $(DHICA)_3$ at *m/z* 574 (calc. 575), $(DHICA)_2(DHI)_2$ at 677 (calcd. 678), $(DHI)(DHICA)_3$ at 722 (calcd. 722), $(DHI)_5$ at 736 (calcd. 737), hydroxylated $(DHI)_5$ at 753 (calcd. 753), and $(DHICA)_3(DHI)_2$ at 867 (calc. 869). Mixed oligomers arise from oxidative coupling of DHI that is generated by partial decarboxylation of DHICA. Furthermore, oxygenation of dihydroxyindole units takes place as observed in the oxidation of dopamine and DHI.

Obviously, the positions of linkages between oligomers cannot be deduced by this experimental approach. Uncertainties in mass determinations (about +/- 0.1-0.2%) and the increasing complexity in the higher mass ranges cause limitations in estimations of the redox status of oligomers. Further improvements in the quality of the spectra could be achieved by optimising the crystallisation process towards homogeneous layers of the matrix on the target. However, MALDI-TOF-MS appears to be a rapid and direct method for a kinetic studies on polymerization reactions. Evaluation of the relative peak intensities allows quantitative estimations within close limits.

The results of this study clearly show the transient formation of melanochromes as oligomers of indolic units of at least DP 11 in the process of melanogenesis. The addition of water to early intermediates is demonstrated for the first time in this context.

Experimental Section

Mass spectra were recorded on a Finnigan LaserMAT time-of-flight mass spectrometer (Finnigan, Hemel Hempstead, Herts, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse) in linear modus. The laser power was set for all measurements to 16 (maximum: 100). The matrix solution was prepared by dissolving 1 g of α-cyano-4-hydroxycinamic acid (Sigma) in 100 ml acetonitrile/H_2O/TFA 70:30:0.1 (v/v/v). Sample preparation was carried out by mixing by 3 µl of a reaction mixture and 10 µl of the matrix solution. An aliquot (1.2 µl) of this solution was deposited in a well of a a gold coated stainless-steel target (3.5 x 3.5 cm) and dried in a gentle stream of air. Spectra were recorded after focusing the laser always to the same quadrant of the wells (2 mm diameter). Data from 20 laser pulses were accumulated. - Horseradish peroxidase (250 U/mg) and mushroom tyrosinase (3200 U/mg) were from Sigma. Deionised and over charcoal and a Micropore filter (0.2 µm) filtered water was used. Dopamine hydrochloride was from E. Merck, Darmstadt. DHI (**3**) and DHICA (**4**) were prepared from DOPA using, with some modifcations, the procedure of Wakamatsu and Ito.[11] A vigorous stream of argon was bubbled for several min through all solvents before use in preparative operations.

5,6-Dihydroxyindole (**3**): A solution of 5.3 g (15 mmol) $K_3[Fe(CN)_6]$ and 2.5 g (30 mmol) $NaHCO_3$ in 60 ml H_2O is added under an argon atmosphere within 5 min to a stirred solution of 1.0 g (5.1 mmol) DOPA. Stirring is continued for 2.5 h after which the reaction is terminated by addition of a saturated solution of 2.8 g (15 mmol) of $Na_2S_2O_7$ in water. After extraction with EtOAc (4 × 250 ml), the combined extracts are washed with a saturated solution of NaCl which contains 2 g $\cdot L^{-1}$ $Na_2S_2O_7$ (2 × 100 ml) and once with 80 ml H_2O. Drying over Na_2SO_4 is followed by evaporation of the solvent. The greenish-brown powder is dissolved in toluene. A light-grey powder is obtained after precipitation with hexane, and stored under argon at -30°C. Yield: 342 mg (45 %) of **3**. - R_f: 0.35 ($CHCl_3$/MeOH 9:1). - IR: (KBr): 3436 cm^{-1} (ss, br), 1473 (m), 1416 (w), 1337 (m), 1185 (s, br), 1120 (m), 854 (m), 757 (m), 639 (w), 486 (w). - ^{1}H-NMR: (250 MHz, d_6-DMSO): δ = 10.41 (N*H*), 8.45, 8.28 (br., 2 O*H*,), 7.01 (m, 3-H), 6.82 (s, 4-H), 6.74 (s, 7-H), 6.11 (br., 2-H). - ^{13}C-NMR: (62.5 MHz, d_6-DMSO): δ = 142.4 (C-5), 140.3 (C-6), 130.2 (C-8), 122.5 (C-2), 120.2 (C-9), 104.4 (C-4),

100.1 (C-3), 97.1 (C-7). - EI-MS: *m/z* (%) = 149.0470 (calcd. for $C_8H_7NO_2$: 149.0476) (39) $[M]^+$, 121 (11), 110 (8), 103 (8), 91 (4), 81 (4), 77 (4), 69 (6), 65 (3), 57 (8), 55 (7), 50 (7), 41 (7).

5,6-Dihydroxyindole-2-carboxylic acid (**4**): DOPA (1 g) is oxidized by means of $K_3[Fe(CN)_6]$ as described above for the preparation of DHI. The reaction is terminated after 8 min by addition of a saturated solution of 2.8 g (15 mmol) of $Na_2S_2O_7$ in water. Stirring is continued for 8 min after which the pH is adjusted to ca. 2 with 6 N HCl. A yellowish-brown powder is obtained after workup as described above. The crude product is dissolved in a small amount of acetone. Hexane is added and the brownish oil appearing is discarded. The slightly turbid acetone solution is evaporated yielding DHICA as a yellowish amorphous solid. Yield: 491 mg (51 %) of **4**. R_f: 0.57 ($CHCl_3$). - IR: (KBr): 3432 cm^{-1} (ss), 2924 (w), 1674 (ss), 1541 (s), 1438 (vw), 1258 (ss), 1173 (s), 775 (vw), 541 (vw). - ^{1}H-NMR (200 MHz, d_6-DMSO): δ = 12.3 (br., COO*H*), 11.09 (N*H*), 9.08 (br., O*H*), 8.57 (br., O*H*), 6.87 (s, 3-H), 6.80 (m, 4-H), 6.76 (s, 7-H). - ^{13}C-NMR (62.5 MHz, d_6-DMSO): δ = 162.9 (COOH), 146.3 (C-5), 142.2 (C-6), 132.8 (C-8), 125.9 (C-2), 120.1 (C-9), 107.3 (C-4), 105.1 (C-3), 97.1 (C-7). - EI-MS: *m/z* (%) = 193.0376 (calcd. for $C_8H_7NO_2$: 193.0375) (99) $[M]^+$, 175 (100), 149 (12), 148 (5), 147 (45), 146 (5), 121 (9), 119 (8), 101 (5), 63 (8), 43 (12).

Oxidation of dopamine (**1**): Peroxidase (0.8 mg) is added at 22°C to a gently stirred solution solution of 38 mg of dopamine hydrochloride (0.2 mmol) in 50 ml of a 20 mM sodium phosphate buffer pH 7.0 and 1 ml of 2.5 % H_2O_2 (w/w) in H_2O. - *Oxidation of DOPA* (**2**): Tyrosinase (0.4 mg) is added to a vigorously stirred solution of 39.5 mg (0.2 mmol) of **2** in 50 ml of a 20 mM sodium phosphate buffer pH 7.0 at 22°C. - *Oxidation of DHI* (**3**): A solution of 30 mg (0.2 mmol) (**3**) in 50 ml of acetone is gently stirred at 22°C under air. - *Oxidation of DHICA* (**4**): -A solution of 39 mg (0.2 mmol) of (**4**) in 50 ml of a 20 mM sodium phosphate buffer pH 9.0 is vigorously stirred at 22°C under air. Samples are withdrawn by a microlitersyringe for mass spectrometry.

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References and Notes

1 This work is a part of the Doctoral Thesis of C.K., University of Bonn 1995.

2 Abbreviations: DOPA: 3,4-dihydroxyphenylalanine; DHI: 5,6-dihydroxyindole; DHICA: 5,6-dihydroxyindole-2-carboxylic acid.

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