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NEW INTERMEDIATES IN THE OXIDATIVE POLYMERISATION OF 5,6-DIHYDROXYINDOLE TO MELANIN PROMOTED BY THE PEROXIDASE/H_2O_2 SYSTEM.

Marco d'Ischia, Alessandra Napolitano, Kostantino Tsiakas and Giuseppe Prota*

Department of Organic and Biological Chemistry,
University of Naples
Via Mezzocannone 16, I-80134 Naples, Italy.

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Abstract. According to the generally held view, the biosynthesis of melanins, the major determinants of skin colour differences in man, involves ultimately the oxidative polymerisation of 5,6-dihydroxyindole (**1**) promoted by the enzyme tyrosinase. We have now found that such a process is brought about more efficiently by the peroxidase/H_2O_2 system at physiological pHs, under which conditions the oxidation reaction leads in the early stages to a distinct pattern of oligomer products. These were isolated after acetylation and identified as the tetraacetoxybiindolyls **2** and **3** and the related trimers **4** and **5**. The observed mode of polymerisation of **1** seemingly involves the attack of the nucleophilic 2-position of the indole to the electron deficient C-4 and C-7 sites of 5,6-indolequinone arising from further oxidation or disproportionation of peroxidase generated phenoxyl radicals.

Among naturally occurring pigments, melanins occupy a special position because of their involvement in human pigmentation and related pathological conditions, such as albinism, vitiligo and malignant melanoma[1-4]. Distinguishing features of these pigments, which are widely distributed in the animal kingdom, include high molecular weight, complete insolubility in all solvents, lack of well defined physical and chemical characteristics; in consequence direct structural investigation by current spectral techniques as well as by X-ray crystallography has been defeated.

Most of what is presently known on the chemistry of melanins has been gained from model studies by Raper[5] on the *in vitro* oxidation of tyrosine catalysed by the enzyme tyrosinase. These have led to the elucidation of the melanin pathway as far as the formation of 5,6-dihydroxyindole (**1**), and it is generally agreed that the subsequent steps of the process involve the polymerisation of 5,6-indolequinone arising by tyrosinase catalysed oxidation of **1** .

However, in spite of a large amount of work carried out over the years, no stage between **1** and the melanin polymer has been adequately characterised, the main difficulties arising from the fast kinetics of the process and the elusive nature of the intermediates[6].

Recently, we found that, under appropriate conditions, enzymic or aerial oxidation of 5,6-dihydroxy-1-methylindole leads in the early stages to a discrete pattern of oligomer products, the major of which could be isolated and characterised[7]. From the structures of the compounds identified, evidence was obtained for an unexpected tendency of the 5,6-dihydroxyindole system to undergo oxidative coupling at the 2- and 4-positions rather than at the 3- and 7-positions, as previously suggested[6,8].

In the light of these results, an examination of the oxidative polymerisation of the parent indole **1**, the major postulated melanin precursor, appeared desirable.

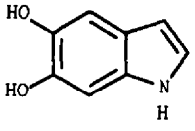
Initially, the oxidation of **1** was performed under the usual conditions of melanogenesis *in vitro*[9,10], i.e. with the enzyme tyrosinase, in aqueous buffer at neutral pH. Unexpectedly, melanin formation was found to proceed slowly even in the presence of a large amount of the enzyme. Moreover, if the reaction was stopped in the early stages by reduction with dithionite, HPLC analysis of the oxidation mixture revealed the presence of a chromatographically ill defined pattern of products, supposedly oligomers of **1**, along with much unchanged starting material. These observations indicated that **1** is a relatively poor substrate of tyrosinase, as confirmed in separate kinetic experiments[11].

In seeking for other enzymic systems capable of promoting the oxidative polymerisation of **1**, we found that the peroxidase/H_2O_2 couple is particularly effective in inducing a rapid and complete conversion of the indole to melanin in aqueous buffer at pH 6.8. Spectrophotometric monitoring of the reaction course revealed that melanin formation is preceded by a purplish chromophoric phase (λ_{max} 560 nm) reminiscent of melanochrome, the last UV detectable intermediate in the tyrosinase catalysed oxidation of tyrosine[9,12].

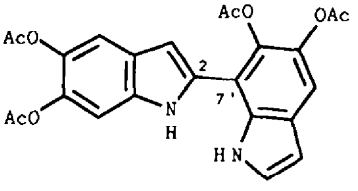
Direct analysis of the reaction mixture at the purple chromophoric stage proved to be difficult, owing to the high instability and the unfavourable solubility properties of the material. However, after reduction and acetylation, according to a recently developed procedure,[7,13] a well defined pattern of products

was observed, four of which, accounting for about 30% of the starting indole, could be isolated after careful fractionation by flash chromatography and preparative TLC.

On analysis, two of these, obtained in higher yield, proved to be isomeric dimers, $C_{24}H_{20}N_2O_8$, and were formulated as 5,5',6,6'-tetraacetoxy-2,7'-biindolyl (**2**) and 5,5',6,6'-tetraacetoxy--2,4'-biindolyl (**3**).



1



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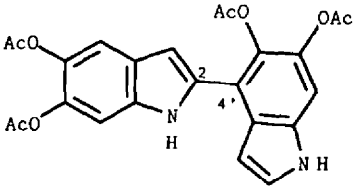

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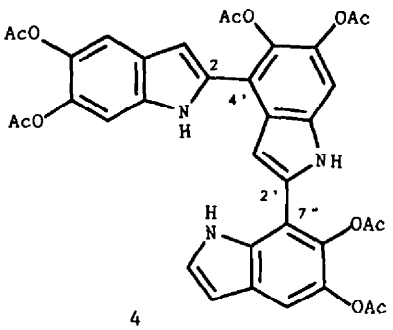
Identification of the dimer **2** followed from analysis of the aromatic region of the ^1H NMR spectrum, in which the resonances of one H-2 and one H-7 protons were clearly missing. Moreover, of the two H-3 proton resonances at δ 6.58 and 6.73, only the latter displayed the characteristic long range coupling (J= 0.7 Hz) with the H-7 indole proton[14]. Additional support to the proposed structure was provided by the significant NOE effects observed between the H-3 signal and the H-4 singlet at δ 7.41, and between the H-3' resonance and that of the H-2' and H-4' protons. The mode of linking of the indole units in the isomer **3** was likewise apparent from the ^1H NMR spectrum (absence of the H-2 and H-4' protons) and was further confirmed by the ^{13}C NMR spectrum, exhibiting two singlets at δ 118.69 and 134.25, typical of substituted C-2 and C-4 indole carbons, respectively[7]. Notably, dimer **3** was found to correspond to one of the major products formed in the early stages of the tyrosinase catalysed oxidation of **1**, as evidenced by HPLC analysis of the reaction mixture after similar work up.

The other two oligomers, which were isolated in smaller amounts, exhibited virtually identical mass spectra with molecular ion peaks at m/z 695, consistent with hexaacetoxyterindolyl structures. The ^1H

NMR spectrum of the more polar isomer comprised, as expected, eight aromatic resonances, ascribable to three H-3, two H-4, two H-7 and only one H-2 protons. Of the three H-3 signals, two (δ 6.80 and 6.89) were splitted by long range coupling with NH and H-7 protons (J=2.1, 0.7 Hz, respectively), whereas the remaining one (δ 6.90) displayed coupling with the vicinal H-2 (J= 3.1 Hz) and the NH protons. These data, coupled with weak but distinct NOE effects between one of the H-4 protons (δ 7.44) and the H-3 proton at δ 6.90, and between the H-3 resonance at δ 6.80 and the remaining H-4 signal (δ 7.43), pointed to a central indole unit linked through the 2- and 4- positions to the 2- and 7-positions of the outer indole rings. Comparison of the proton and carbon chemical shifts with those of the dimers **2** and **3** led eventually to formulate the compound as 5,5′,5",6,6′,6"-hexaacetoxy-2,4′:2′,7"-terindolyl (**4**).

In the other trimer the linking between the indole units was apparently restricted to the 2- and 4-positions, as indicated by the presence of two sets of three H-3 and H-7 signals in the ^{1}H NMR spectrum, and of four singlets, due to two C-4 (δ 118.03, 118.21) and two C-2 (δ 133.40, 134.10) carbons, in the ^{13}C NMR spectrum. These data were compatible with two possible isomeric structures, one having a 2,4′:2′-4"-terindolyl and the other a 2,2′:4′,4"--terindolyl skeleton. Attempts to discriminate between the two isomers by spectral techniques, including NOED and 2D-hetero-correlation long range experiments, failed to provide conclusive evidence. However, the predominant formation in the reaction mixture of the 2,4′-dimer **3**, and the lack of any detectable 2,2′- and 4,4′-dimers, would make the 5,5′,5",6,6′,6"-hexaacetoxy--2,4′:2′,4"-terindolyl structure **5** a more likely candidate on a chemical basis. Indirect support for this assignment can be found in the chemical shift values of the H-3 protons, none of which falls in the relatively upfield region at δ 6.0-6.1, distinctive of H-3 protons on a 4,4′-biindolyl system[15,16].

As far as the mechanism of formation of compounds **2-5** is concerned, all attempts to gain direct information by conventional techniques were defeated, owing to the intrinsic complexity of the chemistry involved. However, on the basis of the current knowledge of peroxidase promoted oxidations[17], a realistic reaction pathway could involve initial one electron transfer from **1** to the peroxidase-hydrogen peroxide complex to yield the 5,6-dihydroxy-



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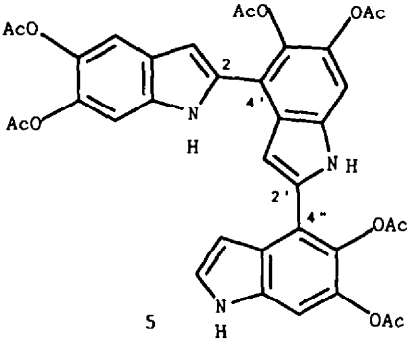

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indole semiquinone (or an electronically equivalent species) to which a number of mechanistic options are offered, i.e. homolytic self-coupling, further one-electron oxidation and disproportionation. A choice between these possible routes can be dictated by the regiochemical features of the chain elongation process which, as apparent from the structures **2-5**, occurs invariably *via* the 2,4′ or 2,7′ coupling of the indole monomer units. This mode of polymerisation is strongly suggestive of an ionic type coupling mechanism, in which the 2-position of **1**, experiencing a pronounced electron donating effect from the C-6 hydroxyl, brings about nucleophilic attack to the electron deficient C-4 and C-7 sites of an oxidised counterpart, presumably the elusive 5,6-indolequinone. Whether this latter species arises from further oxidation of the postulated semiquinone intermediates or from their spontaneous disproportionation can hardly be inferred from available data.

Interestingly enough, a mechanistic pathway for one electron oxidation of **1**, derived from pulse radiolysis kinetic experiments, has recently been proposed[18,19], which involves disproportionation of the initially formed semiquinone radicals to yield 5,6-indolequinone, largely in the form of its hypothetical quinone methide and quinone imine tautomers. Once generated, these latter would undergo nucleophilic addition of water to give the highly reactive trihydroxyindole intermediates, which would fastly interact with the remaining 5,6-indolequinone tautomers thereby initiating the polymerisation process. While it is possible that this pathway is operative under the particular conditions of pulse radiolysis experiments, it does not seem to match with the chemistry emerging

from our biomimetic study, expecially for what concerns the postulated significant involvement of trihydroxyindole intermediates in the polymerisation process.

Experimental

M.ps. were determined with a Kofler hot-stage apparatus and are uncorrected. UV spectra were performed with a Perkin-Elmer Lambda 7 spectrophotometer. ^{1}H NMR (270 MHz) and ^{13}C NMR (67.9 MHz) spectra were recorded on a Bruker AC 270 spectrometer, using acetone-d_6 as the solvent and TMS as the internal standard. Electron impact mass spectra were determined with a Kratos MS-50 mass spectrometer. Beside molecular ions, the most abundant ions in the mass spectra (above m/z 100) are given with their relative intensities. Analytical and preparative TLC were carried out on precoated silica gel F-254 plates from Merck (0.25 and 0.50 mm layer thickness). Flash chromatography was performed on a silica column packed with Merck Kieselgel (230-400 mesh). HPLC analyses were carried out on a Waters model 6000 A instrument using a 4 mm x 25 cm RP-18 Lichrosorb column (Merck) and the flow rate was maintained at 0.8 ml/min. Detection was carried out with a UV spectrophotometer Waters model 480 (λ=300 nm). Horseradish peroxidase (donor:H_2O_2 oxidoreductase EC 1.11.1.7) type II (220 units/mg, RZ E_{430}/E_{275}=2.0) and mushroom tyrosinase (*o*-diphenol:O_2 oxidoreductase,EC 1.14.18.1, 4800 units/mg) were purchased as lyophilised powders from Sigma Chemical Co. 5,6-Dihydroxyindole (**1**) was prepared by a standard synthetic procedure[20].

Oxidation of **1**

A. by horseradish peroxidase.

To a solution of **1** (300 mg, 2.0 mmol) in 0.1 M phosphate buffer at pH 6.8 (120 ml) containing horseradish peroxidase (2640 units), hydrogen peroxide (177 µl of a 35% solution diluted with 8 ml of water) was added under vigorous stirring. The reaction mixture immediatedly turned purple and a precipitate began to separate. After 3 minutes, conversion of the starting material was complete, as evidenced by HPLC analysis (0.1 M acetate buffer,pH 4-MeOH 75:25, mobile phase), and the reaction was stopped by addition of sodium dithionite. The resulting mixture was repeatedly extracted with ethyl acetate and the combined organic layers, dried over sodium sulphate, were taken to dryness. The residue thus obtained was acetylated with acetic anhydride-pyridine at room temperature for 12 h. Flash chromatography of the acetylated mixture on a 4x40 cm column, using $CHCl_3$ containing from 0 to 20% ethyl acetate as the eluent, gave three main fractions. Crystallisation of the first pooled fractions with AcOEt afforded pure **2** (54 mg, Rf 0.65 in $CHCl_3$/MeOH 98:2). The second and the third pooled fractions were further chromatographed on silica gel plates ($CHCl_3$/MeOH 98:2) to give dimer **3** (70 mg, Rf 0.50 in $CHCl_3$/MeOH 98:2) and the trimers **4** (10 mg, Rf 0.30 in $CHCl_3$/MeOH 98:2) and **5** (10 mg, Rf 0.60 in $CHCl_3$/MeOH 98:2), respectively.

B. by mushroom tyrosinase

To a solution of **1** (100 mg, 0.67 mmol) in 0.1 M phosphate buffer, pH 6.8 (90 ml), mushroom tyrosinase (48,000 units) in the same buffer (10 ml) was added, under vigorous stirring. The reaction course was followed by periodical HPLC analysis of the oxidation mixture (0.1 M acetate buffer, pH 4-MeOH 75:25, mobile phase). After

60 min, when about 50% of the starting material was consumed, the reaction was stopped by addition of sodium dithionite and the resulting mixture worked up as described in A. TLC analysis (Et_2O-MeOH 98:2) of the acetylated mixture revealed the presence, beside substantial amounts of the starting material as diacetoxyindole (Rf 0.8) and a complex mixture of polar high molecular weight species (Rf < 0.3), of a major compound (Rf 0.6) identified as dimer **3** by NMR spectroscopy and comparative chromatographic analysis on TLC and HPLC (CH_3CN: H_2O 55:45, mobile phase).

5,5',6,6'-tetraacetoxy-2,7'-biindolyl (**2**).
Colourless prisms from ethyl acetate, m.p. 224°C ; λ_{max} (EtOH) 297, 307 (shoulder) (log ϵ 4.23, 4.22); HRMS m/z 464.1251 (M^+) (calc. for $C_{24}H_{20}N_2O_8$ 464.1220); EIMS m/z 464 (M^+, 60), 422 (67), 380 (97), 338 (100), 296 (93); ^{1}H NMR δ (ppm): 2.16 (3H, s, $-COCH_3$), 2.27 (3Hx3, s, $-COCH_3$), 6.58 (1H, dd, J= 3.1, 1.9 Hz, H-3'), 6.73 (1H, dd, J= 2.2, 0.7 Hz,H-3), 7.34 (1H,d, J=0.7 Hz, H-7), 7.40 (1H,dd,J=3.1,3.1 Hz, H-2'), 7.41 (1H,s, H-4), 7.44 (1H,s, H-4'),10.61 (1Hx2, bs, NH,N'H); ^{13}C NMR δ(ppm): 20.69 (q, $-COCH_3$), 20.88 (q, $-COCH_3$), 103.45 (d,C-3), 104.07 (d,C-3'), 106.85, (d,C-7), 112.26 (s, C-7'), 114.67, 115.01 (d, d, C-4, C-4'), 126.88, 127.20 (s,s, C-9,C-9'), 128.12 (d, C-2'), 130.54 (s, C-2), 132.99, 133.27 (s,s, C-5, C-5'), 135.44, 138.05 (s,s, C-6,C-6'),138.29, 139.84 (s,s, C-8,C-8'), 169.45, 169.64, 169.81 (s,s,s, $-COCH_3$)

5,5',6,6'-tetraacetoxy-2,4'-biindolyl (**3**).
Colourless prisms from ethyl acetate-benzene, m.p. 159-161°C; λ_{max} (EtOH) 322 (log ϵ 4.35); HRMS m/z 464.1258 (M^+) (calc. for $C_{24}H_{20}N_2O_8$ 464.1220); EIMS m/z 464 (M^+, 61), 422 (59), 380 (87), 338 (100), 296 (80); ^{1}H NMR δ (ppm): 2.20 (3H,s, $-COCH_3$), 2.27 (3Hx2,s,$-COCH_3$), 2.28 (3H,s, $-COCH_3$), 6.75 (1H,ddd, J=3.1,2.9,0.7 Hz, H-3'), 6.81 (1H,dd, J=2.2, 0.7 Hz, H-3), 7.33 (1H,d, J=0.7 Hz, H-7'), 7.38 (1H,d, J=0.7 Hz, H-7), 7.40 (1H,dd, J=3.1,3.1 Hz,H-2'), 7.41 (1H,s, H-4) 10.45, 10.50 (1H each, bs, NH, N'H); ^{13}C NMR δ(ppm): 20.31 (q, $-COCH_3$), 20.58 (q, $-COCH_3$), 20.76 (q, $COCH_3$), 102.76 (d, C-3), 103.83 (d, C-3'), 106.47 (d, C-7'), 106.67 (d, C-7), 114.26 (d, C-4), 118.69 (s, C-4'), 125.32, 126.90 (s,s, C-9, C-9'), 127.76 (d, C-2'), 134.25 (s, C-2), 134.81, 134.98 (s,s, C-5, C-5'), 135.24, 137.70 (s,s, C-6, C-6'), 139.40, 139.64 (s,s, C-8, C-8'), 169.29, 169.38, 169.45, 169.54 (s,s,s,s, $-COCH_3$).

5,5',5",6,6',6"-hexaacetoxy-2,4':2',7"-terindolyl (**4**).
Colourless oil; λ_{max} 353,290 nm; HRMS m/z 695.1795 (M^+) (calc. for $C_{36}H_{30}N_3O_{12}$ 695.1751); EIMS m/z 695 (M^+, 27), 653 (51), 611 (80), 569 (100), 527 (78), 485 (54), 443 (44); ^{1}H NMR δ(ppm): 2.21 (3H,s,$-COCH_3$), 2.24 (3H,s, $-COCH_3$), 2.28 ((3Hx2,s,$-COCH_3$), 2.29 (3H,s, $-COCH_3$), 2.30 (3H,s, $-COCH_3$), 6.80 (1H,dd, J=2.1, 0.7 Hz, H-3), 6.89 (1H,dd, J=2.1, 0.7 Hz, H-3'), 6.90 (1H,dd, J=3.1, 2.2 Hz, H-3"), 7.38 (1H,d, J=0.7 Hz, H-7), 7.42 (1H,d, J=0.7 Hz, H-7'), 7.43 (1H,s, H-4), 7.44 (1H,s, H-4"), 7.52 (1H,dd, J=3.1,3.12 Hz, H-2"), 10.60, 10.70. 10.75 (1H each, bs, NH, N'H, N"H); ^{13}C NMR δ(ppm): 20.46 (q,$-COCH_3$), 103.30, 104.08, 104.18 (d,d,d, C-3,C-3', C-3"), 106.46, 106.58 (d,d, C-7, C-7'), 113.07 (s,C-7"), 114.31, 114.42 (d,d, C-4, C-4"), 120.35 (s, C-4'), 124.39, 125.46, 126.85 (s,s,s, C-9, C-9', C-9"), 128.20 (d, C-2"), 132.35, 132.93 (s,s, C-2, C-2'), 134.87, 135.07, 135.24 (s,s,s, C-5, C-5', C-5"), 135.41, 137.17, 137.80 (s,s,s, C-6, C-6', C-6"), 139.56, 139.67, 139.80 (s,s,s, C-8, C-8', C-8"), 169.15, 169.20, 169.45 (s, s,s, $-COCH_3$).

5,5',5",6,6',6"-hexaacetoxy-2,4':2',4"-terindolyl (5).

Colourless oil; λ_{max}344,307 nm; HRMS m/z 695. 1767 (M^+) (calc. for $C_{36}H_{30}N_3O_{12}$ 695.1751); EIMS m/z 695 (M^+, 29), 653 (42), 611 (64), 569 (87), 527 (100), 485 (81),443 (64); ^{1}H NMR δ (ppm): 2.21 (3H,s, $-COCH_3$), 2.24 (3H,s, $-COCH_3$), 2.28 (3Hx2,s,$-COCH_3$), 2.30 (3H,s, $-COCH_3$), 2.32 (3H,s, $-COCH_3$), 6.79 (1H,ddd, J=3.1, 2.2, 0.7 Hz, H-3"), 6.87 (1H,dd, J=2.2, 0.7 Hz, H-3), 7.11 (1H,dd, J=2.2, 0.7 Hz, H-3'), 7.36 (1H,d, J=0.7 Hz, H-7"), 7.40 (1H,d, J=0.7 Hz, H-7), 7.43 (1H,dd, J=3.1, 3.1 Hz, H-2"), 7.44 (1H,s, H-4), 7.45 (1H,d, J= 0.7 Hz, H-7'), 10.61, 10.69, 10.73 (1H each, bs, NH, N'H, N"H); ^{13}C NMR δ(ppm): 20.46 (q, $-COCH_3$), 20.52 (q, $-COCH_3$), 103.31 (d, C-3"), 103.81, 103.92 (d,d, C-3,C-3'), 106.40, 106.56, 106.75 (d,d,d, C-7,C-7',C-7"), 114.22 (d, C-4), 118.03, 118.21 (s,s, C-4', C-4"), 125.09, 125.56, 126.84 (s,s,s, C-9, C-9', C-9"), 127.79 (d, C-2"), 133.40, 134.10 (s,s, C-2, C-2'), 134.17, 134.19, 134.20(s,s,s, C-5,C-5', C-5"), 134.95, 135.59, 137.63 (s,s,s, C-6, C-6', C-6"), 139.35, 139.54, 139.96 (s,s,s, C-8, C-8', C-8"), 169.14, 169.27, 169.39 (s,s,s, $-COCH_3$).

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