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# Oxidative Degradation of Melanins to Pyrrole Acids: a Model Study.

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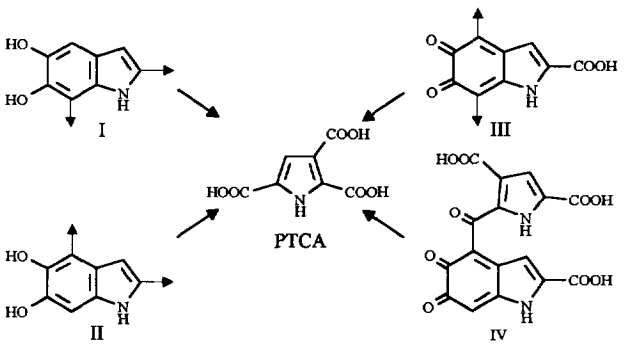
***Abstract***: The origin of pyrrole-2,3,5-tricarboxylic acid (PTCA), the most characteristic degradation product of melanins, was investigated by use of synthetic pigments prepared from the major biosynthetic precursors, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Under the reported conditions, i.e. acidic permanganate, oxidative degradation of DHI- and DHICA-melanins afforded PTCA in 0.15 and 3.0% w/w yield, respectively. A significant improvement of PTCA yields up to 7% was obtained using alkaline hydrogen peroxide as the oxidising agent. Under these conditions pyrrole-2,3-dicarboxylic acid (PDCA) was also obtained in significant yields. Investigation of the oxidative degradation of some model indole oligomers (**1-4**) provided unambiguous evidence that PTCA may originate from 2-linked DHI-units in the pigment polymer as well as from DHICA-derived units, whereas PDCA arises from DHI-units not substituted at 2-position. Monitoring of the oxidation of dimer **1** to PTCA showed the intermediate formation of DHICA. Accordingly, a mechanistic route is proposed for the degradation of 2-substituted DHI-units in melanin polymer to PTCA, which also accounts for the observed yields of formation of the pyrrole acid from the model oligomers.

Among the natural pigments, melanins have attracted considerable interest because of their involvement in human pigmentation and related phenomena of intrinsic importance at the biological and physiological level e.g. skin photoprotection, suntanning, hair greying and aging. [1,2] Additional interest in these pigments derives from their association with a number of pathological conditions, including malignant melanoma. [3]

Despite extensive investigations carried out over the years, the structure of melanins is still largely speculative. Model biosynthetic studies suggest that these pigments are polymers or mixtures of polymers arising by oxidative coupling of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). [1,4,5] Considerable insight into the mechanism of formation of melanin polymers has also been gained by investigation of the oxidation chemistry of the putative indole precursors under biomimetic conditions. [6,7] However, attempts to characterise the natural pigments by direct analysis have been so far little rewarding owing to the high degree of insolubility and the lack of well defined physico-chemical properties of the material. [5]

One approach which was developed by Nicolaus and associates in the sixties involves oxidative degradation of melanins with peracetic acid or alkaline permanganate. [4] Under these conditions, sepiomelanin was found to give pyrrole-2,3,5-tricarboxylic acid (PTCA) in 1-2% w/w yield along with trace amounts of pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-tetracarboxylic acid. [8-10] Similar results were obtained by degradation of synthetic melanins prepared from different precursors such as tyrosine-, dopa-, dopamine-, and DHI-melanins. [10-12]




Scheme 1.

Formation of PTCA was originally interpreted in terms of the oxidative breakdown of indole units in the pigment polymer, at different oxidation levels, either linked through the 2-position (I, II) or bearing a carboxyl group at the same position (III) (Scheme 1). [12-14] Likewise, PDCA could arise from degradation of DHI-units with a free 2-position. β-Diketone-like degradation of terminal units of the type IV was also envisaged as a possible source of PTCA. [15] However, in some more recent studies on the structure of natural and synthetic melanins, [16,17] Ito *et al.* suggested that PTCA could arise from oxidative degradation of DHICA-, but not of DHI-derived monomer units. By comparison of the yields of PTCA with other data including C/N ratio and carboxyl content it was then concluded that DHICA-derived units were about 10% in synthetic dopa-melanins, and up to 50% in intact natural melanins. [16]

The suggested structural significance of PTCA coupled with its general use as a melanin marker in microanalysis of tissues and clinical samples [1,18] prompted us to re-examine the origin of this acid by oxidative degradation of melanins. Under the reported degradation conditions, involving addition of 3% $KMnO_4$ to a suspension of the pigment in 1 *M* $H_2SO_4$,[18] DHICA-melanin afforded PTCA in about 3% yield by weight, whereas DHI-melanin gave less than 0.15 % yield. A remarkable increase in the yields of PTCA was obtained when hydrogen peroxide in alkaline media, particularly in $K_2CO_3$ solutions, was used as the oxidising system. Swelling of the pigment sample in the alkaline medium prior to oxidation and brief heating of the mixture after degradation resulted in additional improvements of the PTCA yields.

Table 1 reports the yields of PTCA from synthetic DHI- and DHICA- melanins prepared by oxidation of the appropriate indoles by the two major enzymic systems involved in melanogenesis, i.e. tyrosinase and peroxidase.[1] HPLC analysis of the degradation mixture of DHI-melanins under these conditions revealed the presence of another major component which proved to be PDCA (Table 1). Identification of this product was secured by spectral analysis. [19] Notably formation of PDCA by melanin degradation was observed by early investigators, [10-12] but did not receive further attention as a structural marker, probably because it is not obtained by the more recently adopted degradation procedure, using acidic $KMnO_4$. In fact, control experiments showed that under these conditions, PDCA is degraded to a significant extent.

PTCA yields from DHI-melanins were about 10% those of the corresponding DHICA-melanins. Alkaline hydrolysis of DHI- and DHICA-melanins, under nitrogen atmosphere, afforded PTCA in yields which were less than 1% of those observed after the complete degradation procedure. This would indicate that, in contrast with previous suggestions, [15] the hydrolytic release of PTCA does not represent a significant mode of formation of the acid, at least for the synthetic melanins examined. On the other hand, the yields of

Table 1. Yields of PTCA and PDCA by Degradation of DHI- and DHICA-melanins

| Sample | Preparation conditions | Yield % w/w[a] | |
|---|---|---|---|
| | | PTCA | PDCA |
| DHI-melanin | peroxidase/$H_2O_2$ | 0.76 | 0.54 |
| DHI-melanin | tyrosinase/$O_2$ | 0.39 | 0.46 |
| DHICA-melanin | peroxidase/$H_2O_2$ | 7.10 | -- |
| DHICA-melanin | tyrosinase/$O_2$ | 6.12 | -- |

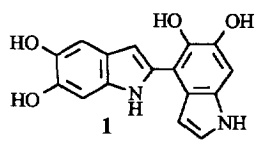
[a] Average of three separate experiments. S.D. $\leq$ 5%.

PDCA obtained from these melanins were comparable to those of PTCA. Given the mode of polymerisation of DHI involving 2,4' or 2,7' coupling of the indole units, [6] one could argue that the yields of PDCA may roughly be proportional to the terminal DHI-units, whereas PTCA yields reflect either the terminal 2-linked or the di-substituted DHI moieties within the pigment backbone. Thus, from the relatively high PDCA/PTCA ratio it may be concluded that, for the pigments under investigation, the degree of polymerisation is rather low.

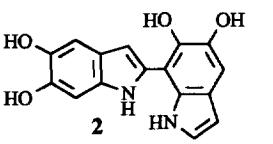
In the light of these results, we next investigated the degradation with $H_2O_2/K_2CO_3$ of some DHI- and DHICA-oligomers which could serve as a model of the melanin polymer. These included the DHI-dimers **1-3** and the DHICA-dimer **4**, obtained by enzymic [6,7] or metal catalysed [7,20] oxidation of the corresponding indoles. Use of the alkaline reaction medium allowed *in situ* deprotection of these oligomers, stored in the *O*-acetyl form, prior to $H_2O_2$ oxidation, without requiring isolation of the highly unstable tetrahydroxybiindolyls. Air was rigorously excluded during the hydrolytic treatment and the degradation reaction to prevent concurrent alkali catalysed oxidative polymerisation of the products to melanin pigments.

Table 2 summarises the yields of PTCA and PDCA obtained from dimers **1-4** in comparison with those of the monomer indoles. Notably, oxidation of DHI-dimers by the acidic permanganate method gave extremely low yields of PTCA (< 0.03 % w/w). This may be probably due to the known instability of the indoles in acidic media, [21] as well as to the extreme harshness of the oxidising agent.

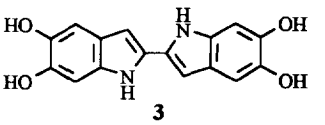
DHI-dimers **1** and **2** gave significant yields of PTCA, whereas only trace amounts of the acid were obtained from DHI indicating that the oxidative polymerisation process is negligible under the degradation conditions adopted. Dimers **1** and **2** afforded comparable yields of PTCA, much higher than those obtained



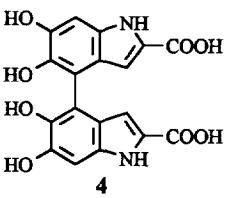

**1**




**2**




**3**




**4**

Table 2. Yields of PTCA and PDCA by Degradation of DHI- and DHICA-Dimers

| Compound | Yield (%) [a,b] | |
|---|---|---|
| | PTCA | PDCA |
| DHI | 0.01 | 4.82 |
| DHICA | 14.1 | - |
| **1** | 1.00 | 3.39 |
| **2** | 1.08 | 3.87 |
| **3** | 0.08 | - |
| **4** | 8.71 | - |

[a] Average of three separate experiments. S.D. $\leq$ 5%.
[b] Expressed as % by weight to allow direct comparison with the yields of synthetic melanins

from **3**. This would indicate a marked difference in the ease to oxidative breakdown of the catechol moiety with respect to the pyrrole ring in the 5,6-dihydroxyindole system. As expected, degradation of dimer **3** did not afford PDCA, whereas considerably high yields of the acid were obtained from dimer **1** and **2** and the precursor DHI. DHICA and dimer **4**, having a carboxyl group at 2-position gave much higher yields of PTCA than those obtained from DHI-dimers **1-2**. All together, these data provide unambiguous evidence that, in line with the original hypothesis, [12-14] PTCA can arise from oxidative degradation of 2-substituted DHI-derived units present in the melanin polymer as well as of DHICA-derived units.

To get an insight into the mechanism of formation of PTCA from 2-substituted DHI-units in melanins, the course of the oxidative degradation of DHI-dimer **1** was examined in more detail. Periodical HPLC analysis of the reaction mixture at the addition of the oxidant, after complete hydrolysis of the acetyl groups, revealed the rapid depletion of biindolyl **1** with concomitant formation of a species which accumulated in the early stages of the degradation process and then decayed while the concentration of the final product PTCA increased with time (Fig. 1). The intermediate product was identified as DHICA by coinjection with an authentic sample.

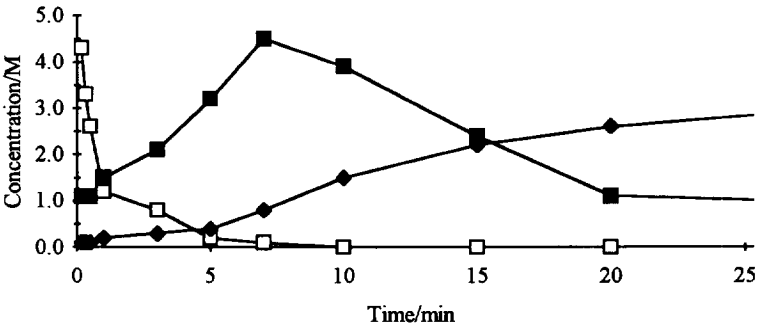
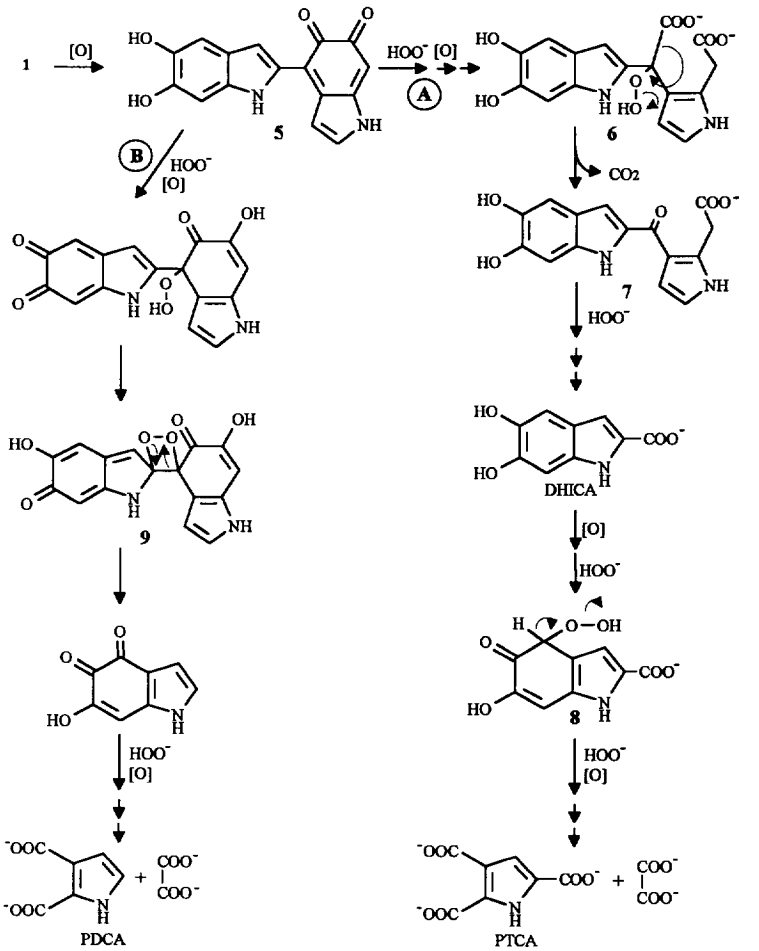



Fig. 1 Time course of the oxidative degradation of **1** to PTCA.
(---□---, Dimer **1**, $10^{-4}$ *M*; —■—, DHICA, $10^{-6}$ *M*; ---◆---, PTCA, $10^{-6}$ *M*)

A possible mechanism accounting for the intermediacy of DHICA in the degradation of dimer **1** to PTCA is depicted in Scheme 2, path A. Repeated attack of the highly nucleophilic hydroperoxide anion [22] to the *o*-quinone **5** may lead *via* a muconic-type C-C cleavage [23] to the dicarboxylic acid **6**. The intermediacy of a 1,2-dioxetane species or an acyclic Baeyer-Villinger type mechanism as demonstrated in the case of 3,5-di-*tert*-butyl-*o*-quinone [24] may be considered for this ring fission which is typical of alkaline $O_2$ or $H_2O_2$ oxidation of catechols. [24,25] Decarboxylation of **6** by a carbonyl-forming elimination [26] followed by Baeyer-Villinger oxidation [27] of the resulting ketone **7** may eventually give rise to DHICA. Other mechanistic options are possibly offered to **7** for the conversion to DHICA, including a 1,2-dioxetane mediated C-C cleavage. Attempts to substantiate either of the proposed routes by identification of intermediary products met with failure because of the complexity of the reaction mixtures under analysis. A likely pathway of conversion of DHICA to PTCA involves sequential oxygenation of the *o*-quinone of DHICA at 4- and 7-positions. This




Scheme 2

possibly takes place by OOH- addition and subsequent base-catalysed decomposition of the adduct, e.g. **8**, [23,26] followed by oxidation and C-C cleavage by a mechanism analogous to that shown above in the case of **5**. A similar series of reactions may be considered for oxidative degradation of **1** to PDCA (path B). The initial cleavage of the C-C bond between the two indole moieties of **1** may proceed *via* the 1,2-dioxetane intermediate **9** in a fashion similar to that reported for the fission of the acridine dimer lucigenin by alkaline hydrogen peroxide. [28] Sequential oxygenation and ring cleavage as in path A would then lead to PDCA along with oxalic acid. Notably, formation of oxalic acid is commonly observed during degradation of melanins. [8,10]

The proposed mechanistic scheme may be extended to the oxidative degradation to pyrrole acids of dimers **2-4** as well as of 2-substituted DHI-units in melanin polymers. It also well accounts for the much higher yields of PTCA obtained from DHICA and DHICA-oligomers in comparison with those of DHI-oligomers. Moreover, the lower PTCA yields from dimer **3** with respect to those of dimers **1** and **2** could be interpreted in terms of the relative tendency of 5,6-indolequinone ring system to undergo oxidative cleavage at C2-C3 bond by HOO- attack in comparison with other electrophilic sites. On the other hand, the facile mode of formation of PDCA from DHI-units with a free 2-position is in line with the high yields of the acid obtained from dimers **1** and **2** as well as from the parent indole DHI.

## EXPERIMENTAL

Fast atom bombardment (FAB MS) mass spectra, positive ion mode (matrix: glycerol) were run on a Kratos MS 50 spectrometer. $^1$H NMR (270 MHz) and $^{13}$C NMR (67.9 MHz) spectra were recorded on a Bruker AC 270 spectrometer. Chromatographic analyses were performed on a Gilson instrument equipped with a 305 model pump and a 316 UV detector using a Spherisorb S5 ODS2 (4.6 x 250 mm) column. Detector was set at 270 nm. The mobile phase was 0.1 *M* sodium phosphate buffer, pH 2.1-MeOH 95:5 (v/v) for PTCA and PDCA analysis or a linear gradient of acetonitrile in 0.1 *M* sodium phosphate buffer, pH 2.0 from 2 to 50% v/v in 40 min, for the kinetic experiments. The flow rate was maintained at 1 ml/min. The yields of PTCA, PDCA and DHICA in the reaction mixtures were estimated by measurement of peak areas and comparison with external calibration curves obtained with an authentic sample. Mushroom tyrosinase (EC 1.14.18.1, *o*-diphenol:$O_2$ oxidoreductase, 2780 U/mg) and horseradish peroxidase (EC, 200U/mg) were from Sigma Chemicals (St. Louis, MO). Hydrogen peroxide (30% solution in water) was purchased from Aldrich Chemical Company (Steinheim, Germany). DHI and DHICA were synthesised according to Benigni and Minnis. [29] 5,5',6,6'-Tetraacetoxy-2,4'-biindolyl, [6] 5,5',6,6'-tetraacetoxy-2,7'-biindolyl, [6] 5,5',6,6'-tetraacetoxy-2,2'-biindolyl [20] and 5,5',6,6'-tetraacetoxy-2,2'-carboxy-4,4'-biindolyl [7] were obtained as previously described. PTCA [30] was prepared by degradation of 5-hydroxyindole-2-carboxylic acid as reported. [18] PDCA [19] was obtained by degradation of DHI as described below. All other chemicals were of the highest purity available. Glass distilled and deionised water was used for preparation of all solutions.

*Preparation of melanins*

Melanins by tyrosinase catalysed oxidation of DHI and DHICA were prepared by a modification of the reported procedure. [16] In brief, the appropriate indole (1 mmol) in 80 ml of 0.1 *M* phosphate buffer, pH 6.8 was incubated for 4 h at 25°C in the presence of tyrosinase (16,000 units) under a stream of oxygen. The melanin formed was precipitated by acidification of the incubation mixture to pH 3, collected by

centrifugation and washed repeatedly (4-5 times) with water. DHI- or DHICA-melanin by peroxidase/$H_2O_2$ oxidation were prepared by sequential addition of the enzyme (1300 units) and 30% $H_2O_2$ (120 µl) to the substrate (1 mmol) dissolved in 80 ml of 0.1 M phosphate buffer pH 6.8. After 2 h under stirring at 25 °C, the pigment formed was collected and washed as above. All melanins were dried over silica gel and NaOH overnight and then equilibrated with saturated $CaCl_2$ until a costant weight was obtained.

*Oxidative degradation of melanins.*

The appropriate melanin (5 mg) was added in a rubber capped cuvette to 1 *M* $K_2CO_3$ (5 ml) which had thoroughly been purged with oxygen-free nitrogen for at least 40 min prior to the addition. The resulting suspension was taken under stirring at 80°C for 30 min and, after cooling, 30% $H_2O_2$ (35 µl/ml suspension) was added *via* a syringe. The mixture was allowed to stand under stirring overnight at room temperature and then heated for additional 30 min at 80°C. After addition of 5% $NaHSO_3$ (1.5 ml), the mixture was acidified with 6 *M* HCl to pH 1 and extracted 5 times with AcOEt. The combined organic layers were evaporated to dryness and the yields of PTCA and PDCA determined by HPLC analysis. In some experiments, a suspension of melanin in 1 *M* NaOH is taken under nitrogen atmosphere, at 80°C for 2 h. After acidification to pH 1 and work up as above the mixture was analysed by HPLC.

*Oxidative degradation of DHI, DHICA and dimers* ***1-4***

Dimers **1-4** were obtained by alkaline hydrolysis of the corresponding *O*-acetyl derivatives. A solution of the acetylated compound (2 mg) predissolved in methanol (200 µl) was added to a rubber capped cuvette containing 1 *M* $K_2CO_3$ (4 ml) which had been thoroughly fluxed with oxygen-free nitrogen for at least 40 min prior to the addition. After 30 minutes, when the hydrolysis was complete as evidenced by HPLC analysis, 30 % $H_2O_2$ was added (40 µl). In the case of DHI and DHICA the hydrolytic step was omitted. The mixture was allowed to stand at 25 °C overnight, the residual $H_2O_2$ was destroyed by addition of 5% $NaHSO_3$ (400 µl) and the solution, acidified with 6 *M* HCl, was worked up as above. In the kinetic experiments of oxidation of dimer **1**, after hydrolysis of the acetylated precursor as above, and addition of the oxidant, aliquots of the reaction mixture were periodically withdrawn, treated with 5% $NaHSO_3$ to stop the reaction, acidified with AcOH and injected for analysis of DHICA, PTCA and residual dimer **1**. A standard solution of dimer **1** was prepared by alkaline hydrolysis under nitrogen as above.

## ACKNOWLEDGEMENTS

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19 FAB MS, m/z: 156 $(M+H)^+$, $^1H$ NMR (DMSO-$d_6$) δ (ppm): 6.65 (1H, dd, J= 3.0, 2.7 Hz, H-4), 7.05 (1H, dd, J= 2.7, 2.1 Hz, H-5), 9.04 (1H, bs, NH); $^{13}C$ NMR (DMSO-$d_6$) δ (ppm): 113.30 (d, C-4), 117.96 (s, C-3), 123.02 (d, C-5) 125.24 (s, C-2), 160.87 (s, C-2-COOH), 168.91 (s, C-3-COOH)
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30. FAB MS, m/z: 200 $(M+H)^+$;$^1H$ NMR (DMSO-$d_6$) δ (ppm): 7.10 (1H, d, J= 2.4 Hz, H-4), 9.21 (1H, bs, NH); $^{13}C$ NMR (DMSO-$d_6$) δ (ppm): 117.72 (d, C-4), 118.80 (s, C-3), 126.06, 129.16 (s, s, C-2, C-5), 160.71 (s, s, C-2-COOH, C-5-COOH), 167.35 (s, C-3-COOH).

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