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Tyrosinase-promoted oxidation of 5,6-dihydroxyindole-2-carboxylic acid to melanin. Isolation and characterization of oligomer intermediates.

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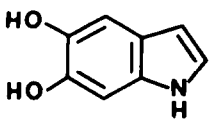
Abstract: Enzymic oxidation of 5,6-dihydroxyindole-2-carboxylic acid (2) in the presence of catalytic amounts of L-dopa led to a complex mixture of oligomeric products, three of which were isolated as the acetyl methyl ester derivatives and identified as 5,6,5',6'-tetraacetoxy-2,2'-dicarbomethoxy-4,4'-biindolyl (4), 5,6,5',6'-tetraacetoxy-2,2'-dicarbomethoxy-4,7'-biindolyl (5), 5,6,5',6',5",6"-esaacetoxy-2,2',2"-tricarbomethoxy-4,4':7',4"-terindolyl (6). A similar pattern of products was obtained when the oxidation of 2 was carried out in the presence of metal ions, e.g. Cu^{2+}. The observed tendency of 2 to undergo oxidative coupling at the 4- and, to a lesser extent, 7- position is discussed in relation to the structure and biosynthesis of eumelanins.

Eumelanins are black to dark brown pigments responsible for the diversity of colouration of skin, hair and eyes in man and other mammals[1]. Because of the lack of well defined chemical and physical characteristics, direct investigation of natural eumelanins has been little rewarding, and most of what is known about them has been gained by model experiments simulating the process of melanogenesis in vitro[2,3]. These have led to the view that eumelanins are formed by oxidative polymerization of 5,6-dihydroxyindole (1) arising from tyrosine by the action of the copper enzyme tyrosinase.

However, there are several lines of evidence suggesting that, in addition to 1, 5,6-dihydroxyindole-2-carboxylic acid (2), which is also formed in the enzymic oxidation of tyrosine, is an important ultimate precursor of eumelanins[4,5]. This view has been recently substantiated by degradative studies indicating that as much as a half of the monomeric units of intact natural eumelanins arises from the incorporation of 2 into the molecular backbone of the pigment polymer[6].



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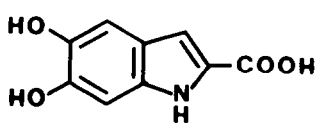
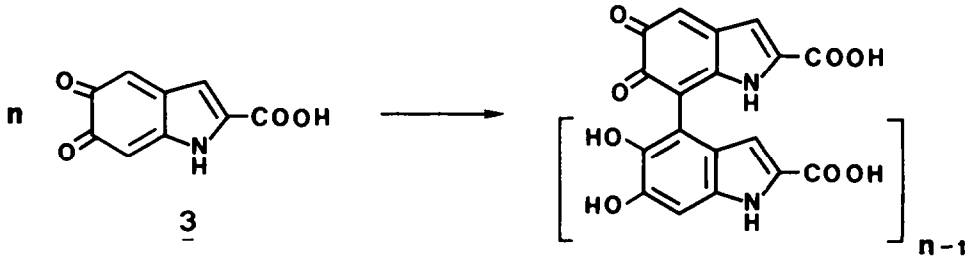


2

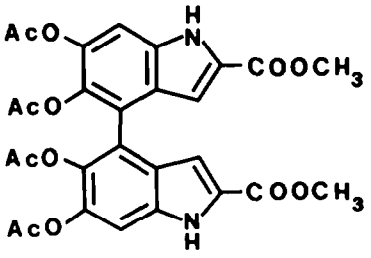
While the mode of polymerization of 1 to melanin has been extensively investigated[7,8,9,10], little is known about the oxidative behaviour of 2. Based upon the chemical and analytical properties of mixtures of polymeric products obtained by oxidation of 2 with Ag_2O, and partly on theoretical grounds, it has been suggested that the polymerization of this indole proceeds by repeated condensation of 5,6-indolequinone-2-carboxylic acid (3) through the 4- and 7-positions[11]. A similar mode of polymerization has been proposed by Ito in relation to the origin of certain oligomers of 2 found in the tapetum lucidum of the catfish[12]. However, attempts to substantiate the proposed mechanism have been hampered by difficulties encountered with the isolation of analitically pure oligomers of 2.



In this paper we report the isolation and characterization of some oligomer intermediates formed in the early stages of the oxidative conversion of 2 to melanin promoted by tyrosinase. Preliminary kinetic experiments showed that the direct interaction of 2 with tyrosinase in phosphate buffer at pH 6.8 results in an exceedingly slow and incomplete oxidation of the indole, as also observed by Mason and Peterson[13]. However, in the presence of catalytic amounts of L-dopa a fast oxidation of 2 takes place, presumably by a redox reaction with dopaquinone formed by enzymic oxidation of dopa. If the reaction is stopped after a few minutes by addition of aqueous sodium dithionite and the resulting solution extracted with ethyl acetate, a yellowish-brown residue is obtained, which becomes darker on being kept further. After esterification with MeOH-HCl and acetylation with Ac_2O-pyridine, TLC analysis on silica revealed the presence, besides considerable amounts of ill defined polymeric material, of three distinct oligomeric products accounting for about 20% of the residue. Noteworthy, a similar pattern of fluorescent compounds was obtained in better yields when 2 was oxidized with cupric ions in Tris[14] buffer at pH 7.5 in a tyrosinase - mimicking reaction. Therefore, both the enzymic and copper promoted oxidation procedures were used to obtain the three oligomers in sufficient amounts for chemical characterization.

The major of these products ($C_{28}H_{24}N_2O_{12}$, m.p. 200°C, λ_{max} 305 nm) proved to be identical with the dimer 5,6,5',6'-tetraacetoxy-2,2'-dicarbomethoxy-4,4'-biindolyl (4) recently obtained by cobalt catalysed oxidation of 2[15]. The other two oligomers were identified as 5,6,5',6'- tetraacetoxy--2,2'-dicarbomethoxy-4,7'-biindolyl (5) and 5,6,5',6',5",6"-esaacetoxy- 2,2',2" -tricarbomethoxy--4,4':7',4"-terindolyl (6) on the following grounds.

The mass spectrum of 5 exhibits the molecular ion peak at m/e 580 and fragment peaks at m/e 538, 496, 454, 412 corresponding to subsequent losses of four acetyl groups. The ^{1}H-NMR spectrum shows in the aromatic region an H-4 singlet at δ 7.60, a signal at δ 7.34 due to an H-7 proton and two H-3 doublets at δ 7.28 and 6.67, this latter being further splitted (0.8 Hz) by long range coupling with the H-7 proton[16]. Consistent with the proposed structure, in the ^{13}C-NMR spectrum the C-7 and C-4' carbons appear as doublets at δ 107.89 and 115.96, respectively, while the C-7' and C-4 carbons give singlets at δ 112.92 and 119.43, respectively.



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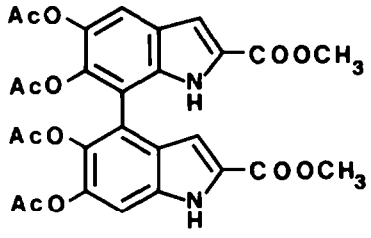

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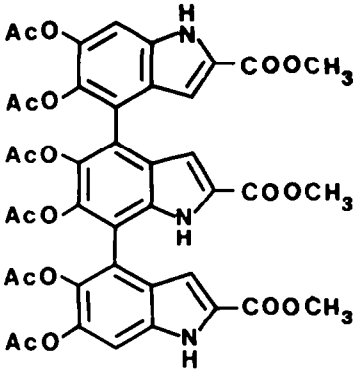

6

The mass spectrum of 6 exhibits the molecular ion peak at m/e 869 and fragment peaks at m/e 827, 785, 743, 701, 659 and 617, due to losses of six acetyl groups. The proposed linkage of the indole units through the 4- and 7-positions followed by the ^{1}H-NMR spectrum, providing evidence for two H-7 protons at δ 7.45 and 7.46 and three H-3 protons, two of which (δ 6.48 and 6.58) long range coupled with the H-7 protons. These assignments were substantiated by the ^{13}C-NMR spectrum indicating that one of the 7-positions and all the 4-positions are substituted.

Taken together the results reported in this study provide evidence that enzymic oxidation of 2 under biomimetic conditions leads to a mixture of oligomers by coupling at the 4- and 7-positions. Of these, the former appears to be involved to a considerably higher extent into the polymerization process, as indicated by the prevailing presence of C_4-C_4 linkages in the oligomers so far identified. Such a symmetrical mode of coupling would be in favour of a radical type of polymerization, rather than the ionic quinone-quinone condensation previously reported[11].

EXPERIMENTAL

M.ps. were determined with a Koffler hot stage apparatus and are uncorrected. U.V. spectra were recorded with a Perkin Elmer 550 S spectrophotometer. ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) spectra were recorded on a Varian XL 200 spectrometer (δ values are referred to TMS as the internal standard). Electron impact mass spectra were determined with a Kratos MS-50 mass spectrometer. 5,6-dihydroxyindole-2-carboxylic acid (2) was prepared according to Benigni and Minnis[17]. All solvents were from Carlo Erba and were of the highest purity available. Dry methyl alcohol was obtained by treatment with magnesium activated by iodine. Analytical and preparative TLC were carried out on precoated silica gel F-254 plates from Merck (0.25 and 0.5 mm), proportions given for mixed solvents are by volume. The chromatograms were examined by UV irradiation at λ 366 nm and 254 nm. Mushroom tyrosinase (3300 units/mg) was purchased as a lyophilized powder from Sigma Chemical Co.(St. Louis MO U.S.A.).

Oxidation of 5,6-dihydroxyindole-2-carboxylic acid.

a) by mushroom tyrosinase.

To a stirred solution of 2 (200 mg) in methanol (12 ml) a solution of L-dopa (10.2 mg) in 0.05 M phosphate buffer (68 ml), pH 6.8, and then mushroom tyrosinase (16500 units) in the same buffer (3 ml) were added under a stream of oxygen. After 8 min the reaction was stopped by addition of $Na_2S_2O_4$, acidified to pH 3 and repeatedly extracted with ethyl acetate. The organic layers were

washed with water and dried over Na_2SO_4. The brown residue thus obtained (170 mg) was first esterified with anhydrous methanol saturated with dry hydrogen chloride and then acetylated with Ac_2O-Pyr, at room temperature for 12 h. After evaporation to dryness, the reaction mixture was thin layer chromatographed on silica with benzene-AcOEt (45:55) to give two bands at Rf 0.5 and 0.3.
The first band was further chromatographed on silica gel plates ($CHCl_3$:MeOH-95:5) to give 4 (18 mg, Rf 0.4), identical in all respects with an authentic specimen (TLC, UV, MS, ^{1}H-NMR, ^{13}C-NMR), and 5 (8 mg, Rf 0.5) as white crystals from MeOH.
The band at Rf 0.3, containing mainly 6, was purified by crystallization from methanol to give 6 (3 mg).

b) by cupric ions.
To a stirred solution of $CuSO_4$ (2.6 mmol) in 0.5 M Tris buffer (140 ml), pH 7.5, a solution of 2 (500 mg, 2.6 mmol) in methanol (30 ml) was added under a stream of oxygen. After 3 min the reaction mixture was reduced with $Na_2S_2O_4$ and acidified to pH 3. After workup as in a), the residue was crystallized from methanol and then re-crystallized from ethyl acetate to give 80 mg of 4. Fractionation of mother liquors on silica gel, as above, led to the isolation, besides additional 30 mg of 4, of 5 (3 mg) and 6 (10 mg).

5,6,5',6'-tetraacetoxy-2,2'-dicarbomethoxy-4,7'-biindolyl (5): m.p. 200 °C (dec); λ_{max} 302 nm (log ε 4.57); m/e 580 (M+), 538, 496, 454, 412; ^{1}H-NMR ($CDCl_3$): δ 2.01 (3Hx2, s, CH_3CO), 2.30 (3H, s, CH_3CO), 2.31 (3H, s, CH_3CO), 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.67 (1H, dd, J=1.8, 0.7 Hz, H-3), 7.28 (1H, d, J=1.8 Hz,H-3'), 7.34 (1H, d, J=0.7 Hz, H-7), 7.60 (1H, s, H-4'),8.80 (1H, bs, NH), 9.30 (1H,bs, NH); ^{13}C-NMR: δ 20.16 ($\underline{C}H_3CO$), 20.21 ($\underline{C}H_3CO$), 20.72 ($\underline{C}H_3CO$), 29.67 ($\underline{C}H_3CO$), 52.19 (OCH_3), 52.28 (CH_3CO), 106.88 (C-7), 107.89 (C-3'), 109.30 (C-3), 112.92 (C-7'), 115.96 (C-4'), 119.43 (C-4), 124.69 (C-9 or C-9'), 124.76 (C-9' or C-9), 128.89 (C-2 or C-2'), 129.08 (C-2' or C-2), 133.71 (C-5 or C-5'), 133.96 (C-5'or C-5), 135.75 (C-6), 137.87 (C-8'), 138.82 (C-6'), 141.32 (C-8), 161.84 ($\underline{C}OOCH_3$), 162.32 ($\underline{C}OOCH_3$), 168.34 ($CH_3\underline{C}O$), 168.65 ($CH_3\underline{C}O$), 168.86 ($CH_3\underline{C}O$), 168.97 ($CH_3\underline{C}O$).

5,6,5',6',5",6"-esaacetoxy-2,2',2"-tricarbomethoxy-4,4':7',4"-terindolyl (6): m.p. 270°C (dec); λ_{max}306 nm (log ε 4.47); m/e 869 (M+), 827, 785, 743, 701, 659, 617; ^{1}H-NMR (DMSO-d_6): δ 1.92 (3H, s, CH_3CO), 1.99 (3H, s, CH_3CO), 2.01 (3H, s, CH_3CO), 2.05 (3H, s, CH_3CO), 2.35 (3H, s, CH_3CO), 2.36 (3H, s, CH_3CO), 3.72 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 6.48 (1H, dd, J= 2.1, 1.0 Hz, H-3), 6.51 (1H, d, J= 2.1 Hz, H-3'), 6.58 (1H, dd, J= 2.1, 1.0 Hz, H-3"), 7.45 (1H, d, J= 1.0 Hz, H-7"), 7.46 (1H, d, J= 1.0 Hz, H-7), 11.70 (1H, bs, N-H), 12.20(1H, bs, N-H), 12.32 (1H, bs, N-H); ^{13}C-NMR (DMSO-d_6): δ 19.57($\underline{C}H_3CO$), 19.66 ($\underline{C}H_3CO$), 19.75 ($\underline{C}H_3CO$), 19.79 ($\underline{C}H_3CO$), 20.38 ($\underline{C}H_3CO$), 20.55 ($\underline{C}H_3CO$), 51.66 (OCH_3), 51.78 (OCH_3), 51.87 (OCH_3), 106.90 (C-7 or C-7"), 107.18 (C-7" or C-7), 107.25 (C-3), 107.30 (C-3'), 107.98 (C-3"), 113.41 (C-7'), 118.31 (C-4"), 120.33 (C-4'), 120.71 (C-4), 123.87 (C-9), 124.07 (C-9'), 124.69 (C-9"), 128.07 (C-2' or C-2"), 128.45 (C-2), 129.06 (C-2" or C-2'),133.43 (C-5"), 134.08 (C-5), 134.34 (C-5'), 134.81 (C-6), 135.10 (C-6"), 135.10 (C-8'), 138.77 (C-6'),140.57 (C-8" or C-8), 140.68 (C-8 or C-8"), 160.79 ($\underline{C}OOCH_3$), 161.11 ($\underline{C}OOCH_3$), 161.30 ($\underline{C}OOCH_3$), 167.81 ($CH_3\underline{C}O$), 167.86 ($CH_3\underline{C}O$), 167.91 ($CH_3\underline{C}O$), 168.13 ($CH_3\underline{C}O$), 168.27 ($CH_3\underline{C}O$), 168.62 ($CH_3\underline{C}O$).

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