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A BIOSYNTHETIC APPROACH TO THE STRUCTURE OF EUMELANINS. THE ISOLATION OF OLIGOMERS FROM 5,6-DIHYDROXY-1-METHYLINDOLE.

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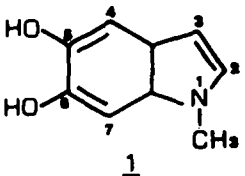
SUMMARY: Enzymic oxidation, or autooxidation at pH 9, of 1 led to a mixture of fluorescent products the major of which could be isolated, as the acetyl derivative, and identified as 5,6,5',6'-tetraacetoxy-1,1'-dimethyl-2,4'-biindolyl (6). In the presence of metal cations, e.g. Ni^{2+}, autooxidation of 1, at pH 7.5, led to the isolation, besides the dimer 6 in lower amounts, of two more oligomers which were formulated as 5,6,5',6'-tetracetoxy-1,1'-dimethyl-2,2'-biindolyl (7) and 5,6-diacetoxy-1-methyl-2,4-di-[5',6'-diacetoxy-1'-methyl-2'-indolyl]-indole (8). The unexpected reactivity of 1 to undergo oxidative coupling at 2- and 4- position rather than at 3- and 7- position, as previously believed, is discussed in relation to the current views on the structure and biosynthesis of eumelanins.

In recent years, whereas knowledge on the chemistry of phaeomelanins[1,2] has been considerably added to, little progress has been made in the investigation of the structure and biosynthesis of eumelanins.[3,4] This latter group of melanins is of the greatest biological interest since it includes the brown to black pigments found in skin, hair and melanomas, as well as those of the eye and the substantia nigra (neuromelanin).[5]

Early studies[6] on the biosynthesis of eumelanins suggested that they arise by oxidative polymerization of 5,6-dihydroxyindoles derived from dopa[7] and related metabolites such as dopamine[8] and epinephrine.[9] However, the proposed mechanism of polymerization as well as the positions of the indole ring involved, remain elusive in spite of a large amount of experimental work.

Extensive studies[10,11] on the oxidative behaviour of variously substituted 5,6-dihydroxyindoles, led to the view that the 3- and 7- position were essential in the formation of eumelanin, but that the 2- and 4- position could be also concerned to some extent. In another study on melanin formation from adrenochrome, Bu'Lock[12,13] proposed that the process involves the intermediacy of 5,6-dihydroxy-1-methylindole (1) subsequently converted to the unstable quinone 2, which could be trapped with 1,4-naphthaquinone to give a purple pigment tentatively identified as 3. From this and other model experiments on the reactions between indoles and quinones, it was proposed that the formation of melanin could proceed by self-condensation of 2 to give 4, followed by coupling of the dihydroxyindolyl group of the dimer with a indolquinone group at 3- or 7- position. Since then, this mode of polymerization of 5,6-dihydroxyindoles has been assumed as a chemical background[3,4] for most speculations concerning the structure of natural and biosynthetic melanins. In contrast with this view, we have recently found[14] that, under biomimetic conditions, oxidation of 5,6-dihydroxyindole leads to a mixture of oligomers, the major of which



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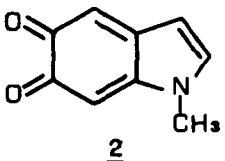

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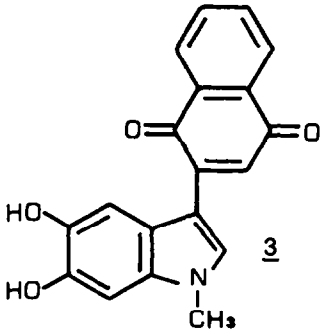

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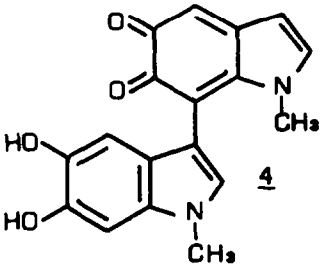

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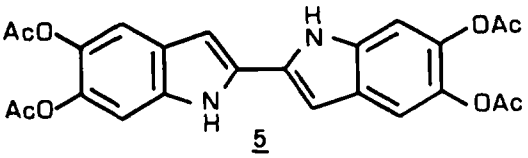

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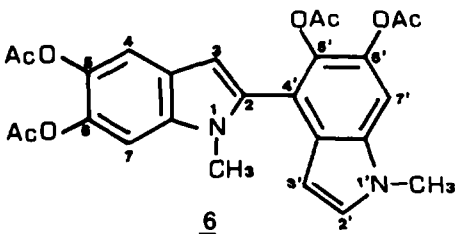
was isolated, as the acetyl derivative, and identified as 5,6,5',6'-tetraacetoxy-2,2'-biindolyl (5). On mild hydrolysis under reductive conditions, this was converted to the parent blue fluorescent tetrahydroxybiindolyl corresponding probably to the unidentified dimer(s) detected by Bu'Lock[15] in the early stage of the enzymic oxidation of 5,6-dihydroxyindole into melanin. This result prompted us to extend the investigation to the oxidation products derived from 1 exhibiting more favourable solubility and analytical properties.

Initially, the oxidation behaviour of 1 was investigated under the usual conditions of melanogenesis in vitro involving the use of the copper containing enzyme tyrosinase. When a solution of the enzyme in 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 6.8 was added to the indole 1 in the same buffer, a purple colour developed which reached the maximum intensity in approximately 20 minutes. At this stage the oxidation was stopped by addition of $Na_2S_2O_4$ and the ethyl acetate extractable fraction was acetylated overnight at room temperature with Ac_2O-pyridine.

TLC and HPLC analysis of the mixture revealed the presence of a complex pattern of blue fluorescent products, the major of which could be isolated in crystalline form and identified as 5,6,5',6'-tetraacetoxy-1,1'-dimethyl-2,4'-biindolyl (6) on the basis of the following evidence,



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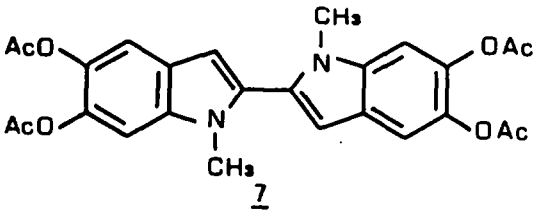
The mass spectrum showed the molecular ion peak at m/e 492 corresponding to the formula $C_{26}H_{24}N_2O_8$ (requires 492.1533, found 492.1536) and diagnostic fragments at m/e 450,408,366 and 324 (base peak), due to subsequent losses of the four acetyl groups.

The ^{1}H-NMR spectrum exibited, in addition to the aliphatic signals of acetyl and methyl groups, two doublets (J=3.68 Hz) at δ6.20 and δ7.08 for the H-3' and H-2' protons, a singlet at δ6.54 due to H-3,and three singlets in the aromatic region at δ 7.19, δ7.26 and δ 7.38 attributable to the H-7, H-7' and H-4,respectively. Significant nuclear Overhauser enhancement difference (NOED) effects were measured between the signal at δ 6.54 (H-3) and the H-4 signal at δ 7.38, between the N-CH_3 resonance at δ3.50 and that of the H-7 proton at δ7.19, and between the N'-CH_3 signal at δ 3.79 and that of H-2' and H-7' protons at δ 7.08 and δ 7.26, respectively. These data provided evidence that the linkage between the two indole moieties involved the 2- and 4- position. Consistent with this view, in the ^{13}C-NMR spectrum, the C-4 and C-2' appeared as doublets at δ 113.91 and δ130.90, respectively, while the C-4' and C-2 gave singlets at δ118.05 and δ133.56, respectively.

In further experiments, the reaction behaviour of 1 was studied in HEPES buffer at pH 9.0 without enzymic catalysis. Under these conditions the reaction proceeded similarly as in the case of the enzymic oxidation to give a mixture of fluorescent products, the major of which proved to be identical to the dimer 6.

A different pattern of oxidation products was observed when the autooxidation of 1 was carried out at lower pH, e.g. pH 7.5, in the presence of metal ions (Ni^{2+}) which, as shown in separate experiments,[14] have a profound effect on the rate and mode of polymerization of 5,6-dihydroxyindoles. After the usual work up ,fractionation of the reaction mixture by preparative TLC led to the isolation, besides the dimer 6, of two more oligomers which were identified as 5,6,5',6'-tetraacetoxy-1,1'-dimethyl-2,2'-biindolyl (7) and 5,6-diacetoxy-1-methyl-2,4-di-[5',6'-diacetoxy-1'-methyl-2'-indolyl]-indole (8) .



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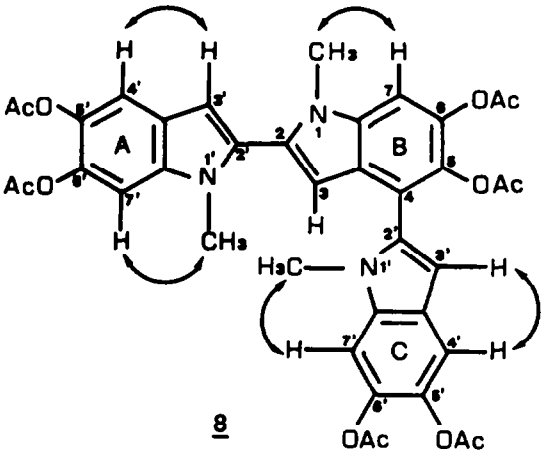
The stucture of the symmetrical dimer 7, $C_{26}H_{24}N_2O_8$ (requires 492.1533, found 492.1533) followed by straightforward analysis of the ^{1}H-NMR spectrum, characterized by two singlets (1Hx2 each) at δ7.21 and δ7.42 for the aromatic protons H-7 and H-4, respectively, and a singlet at δ6.60 due to the H-3 protons. That the coupling between the two indole moieties involved position 2 was substantiated by the ^{13}C-NMR spectrum showing a doublet at δ104.83 attributable[16] to the C-3 and a singlet at δ 132.53, typical of the C-2 of the indole ring. The mass spectrum of 8 showed the molecular ion peak at m/e 737 and the diagnostic fragments at m/e 695, 653, 611,569, 527 and 485. The ^{1}H-NMR spectrum exibited six singlets, two of which at δ 6.39 and δ 6.59 (1Hx2) due to the H-3 and H-3', respectively, and the remaining four in the aromatic region at δ 7.19 (1Hx2), δ 7.34, δ 7.39 and δ7.41, attributable to the H-7' and H-7 and the two H-4' protons,respectively.



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These assigments were substantiated by the result of the NOED experiments, as illustrated in 8. Notably, significant NOED effects were measured between the N-methyl groups and the proximal H-7 protons showing that 7-positions were not involved in the bonding with the other indole nuclei. The structure and characterization of the trimer 8, as well as of the dimers 6 and 7, is of chemical interest since it reveals for the first time an unexpected tendency of 1 to undergo oxidative coupling at 2- and 4- position rather than at 3- and 7- position, as previously suggested.[10,12,13] Although the involvement of these latter positions in the polymerization process cannot be definitively ruled out until a more complete analysis of the oxidation mixture is carried out, the results of the present study provide a new chemical background to look into the structure of natural and biosynthetic eumelanins.

Experimental

M.ps. were determined with a Kofler-hot stage apparatus and are uncorrected. UV spectra were recorded with a Perkin Elmer Mod. 141 spectrometer. ^{1}H-NMR (270 MHz) and ^{13}C-NMR (67.88 MHz) spectra were recorded on a Fourier transform Bruker WH 270 spectrometer with ASPECT 2000 computer 48 K memory. The nuclear Overhauser effect difference FID's were obtained by gated decoupling with a microprogram virtually identical with one decribed in the Bruker Aspect 2000 NMR Software Manual 1. For each measurement, 80 scans with irradiation were subtracted from those with irradiation on resonance. A decoupler amplituder up to 15 Hz was utilized. A flip angle of about 50° was applied. The sample concentration was 10÷15 mg in 0.5 ml of $CDCl_3$ with TMS as the internal reference. Electron impact mass spectrometry was determined with a Kratos MS-50 mass spectrometer. Analytical and preparative TLC were carried out on precoated silica gel F-254 plates (E. Merck), proportion given for mixed solvents are by volume. The chromatograms were examined by UV irradiation at λ 366 nm and λ254 nm. Mushroom tyrosinase was purchased as a lyophilized powder (4800 enzyme units/mg) from Sigma Chemical Co. (St. Louis MO U.S.A.)

5,6-Dihydroxy-1-methylindole (1)

1 was prepared by a procedure similar to that described by Mattok and Heacock.[17] In a typical experiment, to a solution of 1.37 g of epinephrine and 2.24 g of tartaric acid in 25 ml of water, a solution of potassium ferricyanide (13 g) and sodium bicarbonate (4.2 g) in 50 ml of water was added under vigorous stirring. After 5 minutes peroxide free ethyl ether (150 ml) was added to the reaction mixture, followed by addition of ascorbic acid until the red colour was discharged. The aqueous layer was extracted with ethyl ether (3x100 ml) and, after washing with water,the organic layers were dried over sodium sulphate and evaporated to dryness under nitrogen. The yellow residue was crystallized from ether-benzene to give 1 (700 mg) in 57 % yield,as pale yellow needles, m.p.136°C, which darken on exposure to air.

Enzymic oxidation of 1.

To a stirred solution of 1 (100 mg) in 80 ml of phosphate buffer (0.1 M) at pH 6.8 , a solution of mushroom tyrosinase (48000 units) in 20 ml of the same buffer was added. After 20'the reaction was stopped by addition of $Na_2S_2O_4$ and the mixture was repeatedly extracted with ethyl acetate. The organic layers were washed with water and dried over Na_2SO_4. The brown residue so obtained was acetylated with acetic anhydride (2 ml) and pyridine (100 μl) at room temperature for 12 h. After evaporation to dryness, the reaction mixture was chromatographed on preparative TLC with benzene-AcOEt (6:4) to give 27 mg of 6 (Rf 0.66), yellow prisms from $CHCl_3$-petroleum ether,m.p. 105-107°C (dec) ; λ_{max} 303 nm (log ε 4.20); m/e 492 (M^+,$C_{26}H_{24}N_2O_8$: found 492.1536, requires 492.1533), 450, 408, 366, and 324 (base peak);^{1}H-NMR ($CDCl_3$): δ2.06 (3H,s,acetyl group), δ2.33 (3Hx3,s,acetyl groups), δ3.50 (3H,s,N-CH_3),δ3.79 (3H,s,N'-CH_3), δ6.20 (1H,d,J=3.68 Hz,H-3'),δ6.54 (1H,s,H-3), δ7.08 (1H,d,J=3.68 Hz,H-2'), δ7.19 (1H,s,H-7), δ 7.26 (1H,s,H-7'), δ7.38 (1H,s,H-4); ^{13}C-NMR ($CDCl_3$): δ20.55 (q), δ20.62 (q), δ31.08 (q), δ33.07 (q), δ101.19 (d),δ103.13 (d), δ103.82 δ 103.82 (d), δ104.38 (d), δ113.91 (d), δ 118.05 (s), δ 125.70 (s), δ 127.13 (s), δ 130.90 (d), δ 133.56 (s), δ135.36 (s), δ 135.94 (s), δ 136.21 (s), δ137.74 (s), δ 138.36 (s), δ 168.85 (s), δ 169.03 (s), δ169.16 (s), δ169.30 (s).

Autooxidation of 1.

A solution of 1 (400 mg) in 720 ml of HEPES buffer (0.1 M, pH 9.0) was stirred for 30' and the reaction was stopped by addition of $Na_2S_2O_4$. The mixture was extracted with ethyl acetate and the organic layer was washed with water, dried over Na_2SO_4 and evaporated to dryness. The brown residue so obtained was acetylated with Ac_2O-Pyr at room temperature for 12 h. The reaction mixture was chromatographed on preparative TLC with benzene-AcOEt (6:4) yielding 6 (35 mg) as yellow prisms from $CHCl_3$ -petroleum ether.

Autooxidation of 1 in the presence of metal cations.

To a stirred solution of 1 (800 mg) in 720 ml of HEPES buffer (0.1 M pH 7.5) a solution of $NiSO_4$ (1 eq) in 720 ml of the same buffer was added. After 20' the deep-violet reaction mixture was treated with $Na_2S_2O_4$, and repeatedly extracted with ethyl acetate. The organic layers were collected, washed with water, dried over Na_2SO_4 and evaporated to dryness. The brown residue so obtained was acetylated with Ac_2O-Pyr at room temperature for 12 h.

The reaction mixture (720 mg) was chromatographed on silica gel using $CHCl_3$:Et_2O (1:1) to give, besides 5,6-diacetoxy-1-methylindole (45 mg), a fraction (372 mg) consisting mainly of oligomers of 5,6-diacetoxy-1-methylindole. Subsequent fractionation by preparative TLC with AcOEt-cyclohexane (7:3) led to the isolation , besides 50 mg of 6, 7 (Rf 0.75 , 57 mg) as yellow crystals (from THF) m.p. 225°C (dec)λ_{max}($CHCl_3$) 302 nm (logε4.31); m/e 492 (M^+, $C_{26}H_{24}N_2O_8$:found 492.1533, requires 492.1533), 450, 408, 366, 324,162 (base peak);^{1}H-NMR ($CDCl_3$): δ2.33 (3Hx2, s, acetyl groups), δ 2.34 (3Hx2,s,acetyl groups), δ 3.62 (3Hx2,s,N-CH_3), δ 6.60 (1Hx2,s,H-3,H-3'),δ7.21 (1Hx2,s,H-7,H-7'), δ7.42 (1Hx2,s,H-4,H-4'); ^{13}C-NMR ($CDCl_3$): δ20.58 (q), δ 20.65 (q), δ 30.96 (q), δ104.12 (d), δ104.83 (d), δ114.18 (d), δ 125.05 (s), δ 132.53 (s), δ135.34 (s), δ 136.60 (s), δ138.43 (s), δ169.07 (s), δ169.22 (s); 8 (Rf 0.59, 30 mg) as needles from MeOH m.p, 203°C; λ_{max}310 (log ε 4.38); m/e 737 (M^+), 695, 653, 611, 569, 527, 485; ^{1}H-NMR ($CDCl_3$): δ 2,04 (3H,s, acetyl group), δ 2.09 (3H,s,acetyl groups), δ2.33 (3Hx3,s,acetyl groups), δ2.36 (3H,s,acetyl group), δ 3.55 (3H,s,N-CH_3 (C)), δ 3.61 (3H,s,N-CH_3(A)), δ 3.71 (3H,s,N-CH_3 (B)), δ 6.39 (1H,s,H-3), δ 6.59 (1Hx2,s,H-3'), δ7.19 (1Hx2,s,H-7'), δ7.34 (1H,s,H-7), δ7.39 (1H,s,H-4'),δ 7.41 (1H,s, H-4');^{13}C-NMR($CDCl_3$): δ 20.26 (q), δ 20.53 (q), δ 31.19 (q), δ 103.44 (d), δ103.93 (d),δ104.20 (d), δ 104.38 (d), δ 104.96 (d), δ105.01 (d), δ114.00 (d), δ114.23 (d), δ118.27 (s), δ125.11 (s),δ125.73 (s), δ 126.27 (s), δ 132.16 (s), δ 133.33 (s), δ134.95 (s), δ135.49 (s), δ 135.63 (s), δ 136.40 (s), δ 136.84 (s), δ137.96 (s), δ 138.73 (s), δ 139.13 (s), δ168.71 (s), δ 168.99 (s), δ 169.07 (s), δ 169.25 (s).

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