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NEW INTERMEDIATES OF PHAEOMELANOGENESIS IN VITRO BEYOND THE 1,4-BENZOTHIAZINE STAGE.

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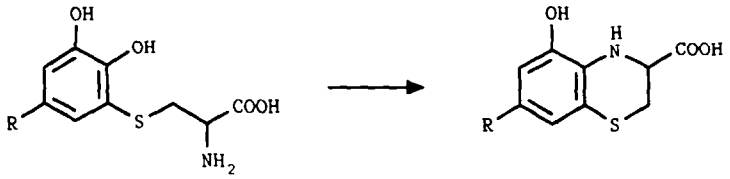
Abstract: Enzymic oxidation of 5-cystein-S-yldopa (**1**) leads to a complex mixture of oligomeric products, two of which were isolated by preparative HPLC and identified as diastereomers corresponding to the gross structure **5**. Under acidic conditions, both compounds are rapidly converted into trichochrome F (**11**), a $\Delta^{2,2'}$-bi-(2*H*-1,4-benzothiazine) pigment previously isolated by extraction of red hair and feathers.

Mammalian colouring is mainly due to two major classes of pigments: the dark brown to black, insoluble eumelanins,[1,2] which arise by tyrosinase-catalysed oxidation of tyrosine, and the yellow to reddish brown, alkali-soluble phaeomelanins,[3] biogenetically derived by a deviation from the eumelanin pathway through the intervention of cysteine and related sulphydryl compounds. Despite extensive investigations carried out over the years, knowledge of the structure of melanin pigments is still rather limited, owing to the unusual complexity of the chemistry involved. Some insight into the structure of eumelanins has recently been gained through investigation of the mode of polymerisation of 5,6-dihydroxyindole[4] and its 2-carboxy derivative,[5] which are the last monomeric precursors of these pigments.[6] Much less is known at present about the chemical nature of phaeomelanins. Extensive studies carried out in the late sixties provided evidence that the principal phaeomelanin precursor is 5-cystein-S-yldopa (**1**),[7] which arises by 1,6-addition of cysteine to dopaquinone produced by enzymic oxidation of tyrosine. Under biomimetic conditions, **1** is rapidly converted into the dihydro-1,4-benzothiazine **2**. However, no stage between

2 and the pigment has been adequately characterised; the polymerisation process is generally rapid and the intermediates are fugitive.



1 R-$CH_2CH(NH_2)COOH$
3 R-CH_3

2 R-$CH_2CH(NH_2)COOH$
4 R-CH_3

In an attempt to fill this gap, we have re-examined the tyrosinase-catalysed oxidation of 5-cystein-S-yldopa (**1**) in phosphate buffer at pH 6.8. Periodical HPLC analyses of the mixture (fig. 1.) showed the rapid and almost quantitative conversion of **1** to **2**, after which the reaction proceeds smoothly to give eventually, besides a considerable amount of chromatographically ill-defined polymeric material, a number of oligomeric products, two of which, present in relatively greater amounts, could be isolated by preparative HPLC.

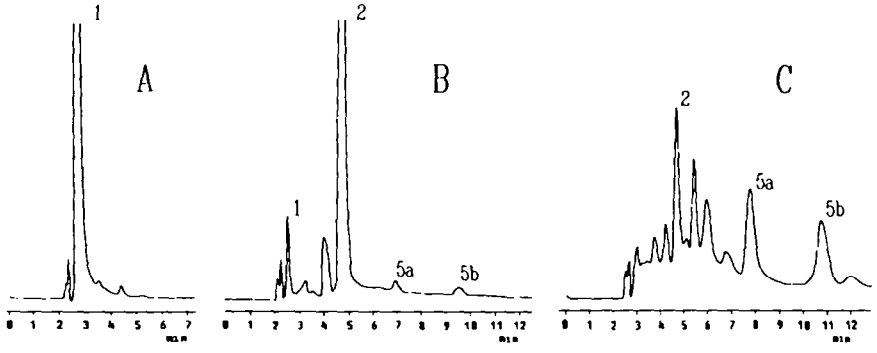
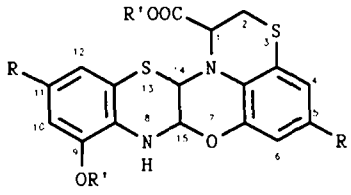


Fig 1. Chromatographic course of the tyrosinase-catalyzed oxidation of 5-cystein-S-yldopa: A, 5 min, B, 15 min; C, 120 min

The faster-moving compound exhibits a UV maximum at 304 nm, reminiscent of the dihydro-1,4-benzothiazine chromophore.[8] In the FAB-MS spectrum, the pseudomolecular ion appears at m/z 549, suggesting a dimeric structure arising from **2**. The ^{1}H-NMR spectrum shows, besides four signals in the aromatic region, two doublets at δ 5.72 and 5.25 ppm (J = 1.8 Hz), attributable to adjacent methine groups attached to heteroatoms, an ABX system at δ 4.40, 3.30 and 3.00 ppm for the cyclic CH_2-CH of a 3-substituted dihydro-1,4-benzothiazine moiety, and signals for two alanine

side chains.

The second compound shows virtually identical UV and FAB-MS spectra, and a similar set of signals in the ^{1}H-NMR spectrum, with only minor differences in the chemical shifts, suggesting a close structural relationship with the other one: on these grounds, both compounds were tentatively identified as two diastereomers corresponding to the gross structure **5**.



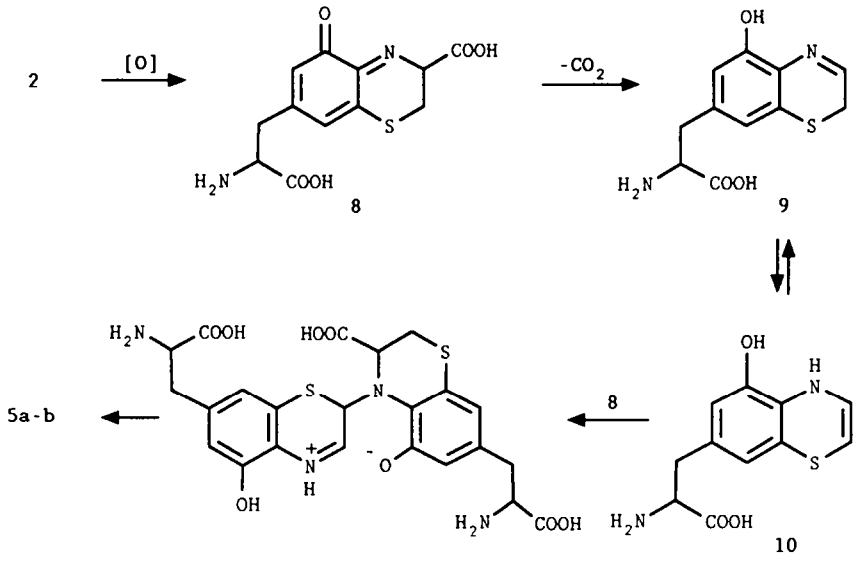
5a-b R=$CH_2CH(NH_2)COOH$, R'=H
6a-b R=CH_3, R'=H
7a-b R=CH_3, R'=CH_3

Supporting evidence for these structures was gained by analysis of the spectral features (^{1}H- and ^{13}C-NMR) of the analogues **6a-b**, obtained by enzymic oxidation of the model compound **3**, in which the alanine side chain was replaced by a methyl group. In particular, in both compounds the H-1 protons gave significant nOe's with the protons at δ around 5.3 ppm, but not with the protons at δ around 5.5 ppm, characterised by vicinal coupling with the NH protons. Moreover, the ^{13}C-NMR spectra of **6a** and **6b** showed two strongly deshielded aliphatic doublets (**6a**: δ 62.87 and 76.64 ppm; **6b**: δ 61.87 and 75.49 ppm), for the C-14 and C-15, respectively. These data provide unambiguous evidence for the proposed relative arrangement of the two benzothiazine moieties.

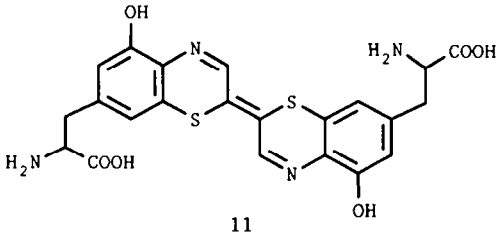
As expected, treatment of **6a** and **6b** with diazomethane afforded the corresponding dimethyl derivatives, $C_{21}H_{22}N_2O_4S_2$ (HRMS), **7a** and **7b**.

Mechanistically, compounds **5a-b** can be envisaged as arising from cycloaddition of the quinonimine **8**, formed by oxidation of the dihydrobenzothiazine **2**, to the 4*H*-1,4-benzothiazine **10**, derived from decarboxylation of **8**. Support to this view can be found in the cycloaddition of the morpholine enamine of cyclohexanone to *o*-quinone monoimides.[9] Alternatively, the formation of **5a-b** could proceed by coupling of semiquinonimine radicals arising from **2** and **10**. The facile generation of such radicals has been demonstrated by ESR spectroscopy.[10] Which of these two mechanisms is actually operative is difficult to assess, owing to the

intrinsic complexity of the chemistry of benzothiazines, and the relatively low yield of **5a-b**.



A characteristic feature of the chemistry of **5a-b** is the marked instability to acids: when an aqueous solution of **5a** or **5b** is treated with hydrochloric acid, a deep violet colour develops in few minutes (λ_{max} ca. 590 nm). The product responsible for this absorption was identified as trichochrome F[11,12] (**11**) by the characteristic pH-dependent chromophore ($\lambda_{max}^{2M\ HCl}$ = 585 nm; $\lambda_{max}^{0.1M\ NaOH}$ = 486 nm), typical of $\Delta^{2,2'}$-bi-(2*H*-1,4-benzothiazines), as well as by comparison of its paper chromatographic behaviour with that of an authentic sample.[11] The formation of **11** involves probably acid-catalysed ring-opening of **5a-b** to give the 1,4-benzothiazine **9**, which, under acidic conditions, is highly unstable,[13] and readily undergoes oxidative coupling at the 2 position.



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Noteworthy, in their extensive studies on the biosynthesis of phaeomelanins Nicolaus and his associates observed that when the enzymic

oxidation of 5-cystein-S-yldopa (**1**) is stopped in the early stages by acidification of the mixture, a small amount of trichochrome F is formed.[14] At that time, this behaviour was ascribed to an acid-unstable benzothiazine intermediate. However, in the light of our findings, it is clear that formation of trichochrome F is due to the acid decomposition of the isomeric dimers **5a-b**.

Compounds **5a-b** are the first products beyond the benzothiazine stage that have been isolated from the biomimetic oxidation of 5-cystein-S-yldopa. Although it is not clear whether they play a role in the biosynthesis of phaeomelanins, or merely represent a side branch of the pathway, their marked instability to acids is of interest in connection with the occurrence of trichochrome F in hair and feathers. To our knowledge, all reported isolation procedures of these pigments from natural sources invariably involve more or less drastic acid treatments.[11,15] This suggests that trichochrome F (and probably also the isomeric trichochrome E[12]) is an artifact arising from **5a-b** (and analogous compounds deriving from 2-cystein-S-yldopa) during the extraction and work-up procedures. Studies are currently underway to assess whether **5a-b** occur in the natural sources from which trichochromes have been isolated.

Experimental

UV spectra were determined with a Perkin Elmer Lambda 7 spectrophotometer. ^{1}H-NMR (270 MHz) and ^{13}C-NMR (67.9 MHz) were performed on a Bruker AC 270 spectrometer (tetramethylsilane or *t*-BuOH as internal standard). Electron impact and fast atom bombardment mass spectra were determined with a Kratos MS 50 spectrometer. IR spectra were taken on a Perkin Elmer Mod. 1760-X. Analytical and preparative TLC were carried out on precoated silica gel F-254 plates (0.25 and 0.50 mm layer thickness, E. Merck). Proportions for mixed solvents are by volume. Analytical and preparative HPLC were carried out on a Waters model 6000A instrument, using a 4 x 250 mm RP18 Lichrochart and a 10 x 250 mm RP18 Lichrosorb Hibar column, respectively. The flow rate was maintained at 1 ml/min with the analytical column and at 6 ml/min with the preparative column. Detection was carried out with a Waters model 440 UV spectrophotometer (λ=280 nm). Mushroom tyrosinase was purchased as a lyophilised powder (2200 units/mg) from Sigma Chemical Co. (St. Louis, MO, USA). 5-Cystein-S-yldopa was prepared as described by Chioccara *et al.*[16] 3-Cystein-S-yl-5-methylcatechol was prepared as described by Prota *et al.*[7]

Tyrosinase-catalysed oxidation of 5-cystein-S-yldopa (1). Isolation of 5a-b.

To a solution of **1** (500 mg) in 0.05 *M* phosphate buffer, pH 6.8 (500 ml), mushroom tyrosinase (50 mg) and L-dopa (10 mg) were added and the mixture was stirred under a stream of air for four hours.

Evaporation to dryness *in vacuo* at 35 °C afforded a residue which was cromatographed on Sephadex G-10 (90 x 1.5 cm column; eluant: water). Fractions of 10 ml were collected and monitored by HPLC (eluant: 0.05 *M*

formic acid-methanol, 65:35). Fractions 25-50 were evaporated to dryness at 35°C under reduced pressure to give a mixture of **5a** and **5b** (retention times 8 and 12 min, respectively). The mixture was fractionated by preparative HPLC (eluant as above) to give **5a** (35 mg) and **5b** (35 mg).

5a: $\lambda_{max}(H_2O)$ 304 nm; FAB-MS (matrix: glycerol): m/z 549 $(M+H)^+$; ^{1}H-NMR (D_2O), δ (ppm): 6.67 (1H, d, J=1.9 Hz, aromatic proton), 6.53 (1H, d, J=1.5 Hz, aromatic proton), 6.50 (1H, d, J=1.9 Hz, aromatic proton), 6.45 (1H, d, J=1.5 Hz, aromatic proton), 5.72 (1H, d, J=1.8 Hz, H-15), 5.25 (1H, d, J= 1.8 Hz, H-14), 4.40 (1H, t, J=3.5 Hz, H-1), 3.85 (2H, m, 2xCH_2-C<u>H</u>), 3.30 (1H, dd, J=12.8, 4.0 Hz, H-2a), 3.11 (1H, dd, J=13.0, 4.7 Hz, C<u>H</u>(H)-CH), 3.06 (1H, dd, J=12.8, 4.3 Hz, C<u>H</u>(H)-CH), 3.00 (1H, dd, J=12.8, 3.4 Hz, H-2b), 2.86 (2H, m, 2xC<u>H</u>(H)-CH).

5b: $\lambda_{max}(H_2O)$ 304 nm; FAB-MS (matrix: glycerol): m/z 549 $(M+H)^+$; ^{1}H-NMR (D_2O), δ (ppm): 6.67 (1H, d, J=1.8 Hz, aromatic proton), 6.54 (1H, d, J=1.5 Hz, aromatic proton), 6.47 (2H, m, aromatic protons), 5.44 (1H, bs, H-15), 5.15 (1H, bs, H-14), 4.40 (1H, bt, J=3.0 Hz, H-1), 3.86 (2H, m, 2xCH_2-C<u>H</u>), 3.05 (6H, m, H-2a, H-2b and 2xC<u>H_2</u>-CH).

<u>Tyrosinase-catalysed oxidation of 3-cystein-S-yl-5-methylcatechol (**3**). Isolation of **6a-b**.</u>

A solution of **3** (500 mg), mushroom tyrosinase (50 mg) and L-dopa (10 mg) in 0.05 *M* phosphate buffer (500 ml), pH 6.8, was stirred under a stream of air for 90 minutes.

The mixture was repeatedly extracted with ethyl acetate, the combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to dryness.

The residue was taken up in methanol and fractionated by preparative HPLC (0.025 *M* phosphate buffer, pH 6.0 - CH_3CN, 65:35) to give two main fractions (retention times 4 and 8 min). Each fraction was concentrated in a rotary evaporator and extracted with ethyl acetate; the organic phase was dried over Na_2SO_4 and evaporated to dryness to give **6a** (24 mg) and **6b** (27 mg), respectively.

6a: $\lambda_{max}(CH_3OH)$ 305 nm; IR ($CHCl_3$): ν_{max} 3400 (b), 1726 cm^{-1}; FAB-MS (matrix: glycerol): m/z 403 $(M+H)^+$; ^{1}H-NMR (acetone-d_6), δ (ppm): 6.42 (1H, d, J=1.9 Hz, aromatic proton), 6.40 (1H, d, J=1.9 Hz, aromatic proton), 6.37 (1H, d, J=1.9 Hz, aromatic proton), 6.24 (1H, d, J=1.9 Hz, aromatic proton), 6.06 (1H, bd, J=5.4 Hz, N-H), 5.43 (1H, dd, J=5.4, 1.5 Hz, H-15), 5.30 (1H, d, J=1.5 Hz, H-14), 4.70 (1H, t, J=3.3 Hz, H-1), 3.36 (1H, dd, J=13.1, 3.3 Hz, H-2a), 3.22 (1H, dd, J=13.1, 3.3 Hz, H-2b), 2.12 (3H, s, CH_3), 2.09 (3H, s, CH_3); ^{13}C-NMR (acetone-d_6), δ (ppm): 172.02 (s), 145.02 (s), 143.46 (s), 129.34 (s), 128.99 (s), 124.99 (s), 124.01 (s), 121.73 (d), 119.32 (d), 118.87 (s), 118.54 (s), 116.03 (d), 112.72 (d), 76.64 (d), 62.87 (d), 62.80 (d), 28.82 (t), 21.06 (q), 20.74 (q).

6b: $\lambda_{max}(CH_3OH)$ 308 nm; IR ($CHCl_3$): ν_{max} 3400 (b), 1723 cm^{-1}; FAB-MS (matrix: glycerol): m/z 403 $(M+H)^+$; ^{1}H-NMR (acetone-d_6), δ (ppm): 6.41 (2H, m, aromatic protons), 6.38 (1H, m, aromatic proton), 6.25 (1H, m, aromatic proton), 6.14 (1H, bd, J=5.4 Hz, N-H), 5.64 (1H, dd, J=5.4, 1.7 Hz, H-15), 5.36 (1H, d, J=1.7 Hz, H-14), 4.72 (1H, t, J=3.6 Hz, H-1), 3.40 (1H, dd, J=12.9, 3.6 Hz, H-2a), 3.14 (1H, dd, J=12.9, 3.6 Hz, H-2b), 2.11 (3H, s, CH_3), 2.09 (3H, s, CH_3); ^{13}C-NMR (acetone-d_6), δ (ppm): 171.83 (s), 144.98 (s), 142.95 (s), 129.22 (2xs), 124.69 (s), 122.64 (s), 121.16 (d), 118.78 (d), 117.34 (s), 116.60 (s), 115.68 (d), 112.75 (d), 75.49 (d), 61.87 (d), 61.01 (d), 26.86 (t), 20.64 (q), 20.38 (q).

Treatment of a methanolic solution of **6a** (or **6b**) with ethereal diazomethane followed by TLC purification (C_6H_6-Et_2O, 95:5) afforded **7a** (or **7b**) as a colourless oil.

7a: $\lambda_{max}(CH_3OH)$: 305 nm; IR (CCl_4): ν_{max} 3452, 1737 cm^{-1}; HRMS: m/z 430.1010 (M^+) (calc. for $C_{21}H_{22}N_2O_4S_2$ 430.1014); ^{1}H-NMR ($CDCl_3$), δ (ppm):

6.51 (2H, bs, aromatic protons), 6.44 (1H, m, aromatic proton), 6.40 (1H, m, aromatic proton), 5.36 (1H, d, J=1.3 Hz, H-15), 5.06 (1H, d, J=1.3 Hz, H-14), 4.42 (1H, t, J=3.4 Hz, H-1), 3.83 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.29 (1H, dd, J=13.0, 3.4 Hz, H-2a), 3.21 (1H, dd, J=13.0, 3.4 Hz, H-2b), 2.18 (3H, s, CH_3), 2.17 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$), δ (ppm): 170.51 (s), 146.53 (s), 141.36 (s), 129.24 (s), 129.13 (s), 123.84 (s), 121.33 (d), 119.61 (d), 117.38 (s), 115.93 (s), 115.61 (d), 108.35 (s), 108.01 (d), 75.83 (d), 62.10 (d), 61.56 (d), 55.55 (q), 52.58 (q), 28.05 (t), 20.94 (q), 20.34 (q).

7b: λ_{max}(CH_3OH) 305 nm; IR (CCl_4): ν_{max} 3451, 1752 cm^{-1}; HRMS: m/z 430.1006 (M^+) (calc. for $C_{21}H_{22}N_2O_4S_2$ 430.1014); ^{1}H-NMR ($CDCl_3$), δ (ppm): 6.51 (2H, bs, aromatic protons), 6.41 (2H, bs, aromatic protons), 5.60 (1H, bs, H-15), 5.08 (1H, bs, H-14), 4.42 (1H, t, J=3.7 Hz, H-1), 3.84 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.32 (1H, dd, J=13.0, 3.7 Hz, H-2a), 3.07 (1H, dd, J=13.0, 3.7 Hz, H-2b), 2.21 (3H, s, CH_3), 2.15 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$), δ (ppm): 170.30 (s), 146.67 (s), 141.91 (s), 129.35 (s), 129.20 (s), 124.24 (s), 121.51 (d), 119.49 (d), 116.06 (s), 115.76 (d), 114.25 (s), 108.38 (s), 108.35 (d), 75.03 (d), 61.03 (d), 60.52 (d), 55.62 (q), 52.88 (q), 26.30 (t), 20.91 (q), 20.30 (q).

Acid degradation of **5a**; identification of trichochrome F (**11**).

A stirred solution of **5a** (1.9 mg, 3.5 μmol) in 300 μl of water was treated with 150 μl of 6*M* HCl. After about 5 min the solution was diluted with 2*M* HCl and the concentration of trichochrome F was determined spectrophotometrically at 585 nm. The yield of trichochrome F was 0.43 μmol. The identity of the product was confirmed by paper chromatography in comparison with an authentic sample (eluant: butanol - acetic acid - water - conc. HCl, 20:30:50:1 or isopropanol - formic acid - conc. HCl, 70:30:1).

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