A New Insight in the Biosynthesis of Pheomelanins: Characterization of a Labile 1,4-Benzothiazine Intermediate

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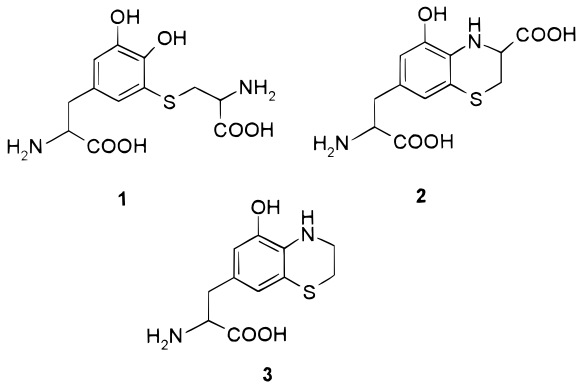
It has long been known that pheomelanins, the distinctive pigments of red hair and celtic skin, arise by the oxidative cyclization of cysteinyldopas, mainly the 5-*S*-isomer **1**, via 1,4-benzothiazines. However, the nature and reactivity of these intermediates have remained poorly defined. In an reexamination of the oxidation of **1**, in aqueous buffers at physiological pHs, a hitherto unknown labile intermediate was identified and formulated as the 3-hydroxy-3,4-dihydro-1,4-benzothiazine **5** based on direct NMR analysis of the reaction mixture and conversion to the stable benzothiazines **3** and **4** under different conditions. Structure **5** was further supported by oxidation of the model compound **6** leading to the analogous more stable 2,2-dimethyldihydro-1,4-benzothiazine **9**.

Introduction

Pheomelanins, the distinctive pigments of red human hair, [1−3] have become in recent years the focus of renewed interest because of their implication as major contributory factors of the abnormal susceptibility of red haired, fair skinned Caucasians to acute and chronic UV damage.[4] Interpretation of the postulated effects on molecular basis has greatly been hampered by the poor knowledge of the chemistry of these pigments.[5]

Biosynthetic studies provided evidence that pigment formation involves oxidative polymerization of cysteinyldopas, mainly the 5-*S*-isomer **1**.[3,6] Under biomimetic conditions, oxidation of **1** was shown to proceed through the 3,4-dihydro-1,4-benzothiazine-3-carboxylic acid **2**.[7] Further investigations led to formulation of more complex oxidation pathways involving the intermediacy of a 1,4-benzothiazine non-carboxylated at the 2-position, isolated after reduction as **3**.[8−10]



In a reexamination of the oxidation of **1** in aqueous buffer at physiological pHs, we have now identified in the early stages of the reaction a new transient 1,4-benzothiazine intermediate which has been characterized by analysis of the spectral features and chemical reactivity, as well as by use of model compounds.

Results and Discussion

Oxidation of **1** was initially performed by potassium ferricyanide at pH 7.2, and the mixture was examined by HPLC with or without reductive treatment in the first few minutes of the reaction. The dihydrobenzothiazine acid **2** was present in both mixtures, but the untreated one contained another significant species whose chromatographic properties did not match those of any of the known intermediates of in vitro pheomelanogenesis.[3,9] Moreover, it was not present in the reduced mixture which showed the dihydrobenzothiazine **3** as the major component. Similar reaction patterns were obtained using other oxidizing agents including sodium periodate, the Fenton reagent,[11] and the enzymatic system peroxidase/H_2O_2. The HPLC fraction of the untreated oxidation mixture corresponding to the unknown peak showed an absorption maximum at 302 nm, which, on acidification, was rapidly converted to that of tricochrome F (587 nm), exhibiting the characteristic reversible pH-dependent shift.[12] Alkalinization resulted in a slow batochromic shift of the absorption maximum to 320 nm, corresponding to

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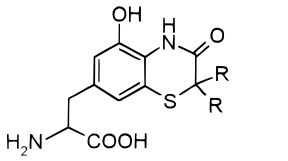
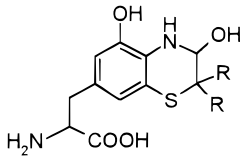
another species identified as the 7-(2-amino-2-carboxyethyl)-5-hydroxy-3-oxo-3,4-dihydro-2*H*-1,4-benzothiazine (**4**),[13] while sodium borohydride reduction led to the dihydrobenzothiazine **3**.[8]

Attempts to isolate the major component of the nonreduced mixture met invariably with failure despite very careful experiments, owing to its marked instability. To circumvent these difficulties, oxidation of **1** was run in phosphate buffer in D_2O at neutral pHs and monitored by ^1H NMR at 400 MHz. At the addition of the oxidant, the resonances of **1** progressively diminished, being replaced by new sets of signals, among which those attributable to a major component could be readily recognized. The most prominent feature of the spectrum was a downfield multiplet at δ 5.44 which showed coupling with CH_2 alanyl chain resonances at around 3 ppm (COSY evidence) and direct CH bond correlation with a signal at δ 73.20. Coupled with consideration of the reactivity, spectral analysis led to formulation of the intermediate as the 3-hydroxy-3,4-dihydro-1,4-benzothiazine **5**. Unfortunately, all efforts toward a more complete characterization were thwarted by the low concentrations which could be generated in the NMR tube and by its relatively short half-life (about 30 min).

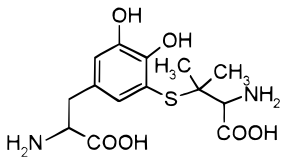
Further support of structure **5** for the labile intermediate was gained by oxidation of the model thioalkylcatechol **6**. The major component of the oxidation mixture exhibited an absorption maximum at 302 nm closely resembling that of **5**. On reduction with $NaBH_4$, it was rapidly converted into the dihydro-1,4-benzothiazine **7**, while exposure to basic or acidic media led to smooth formation of the 3-oxo-dihydro-1,4-benzothiazine **8**.



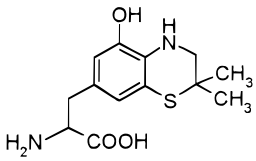
4: R=H
8: R=CH_3



5: R=H
9: R=CH_3



6



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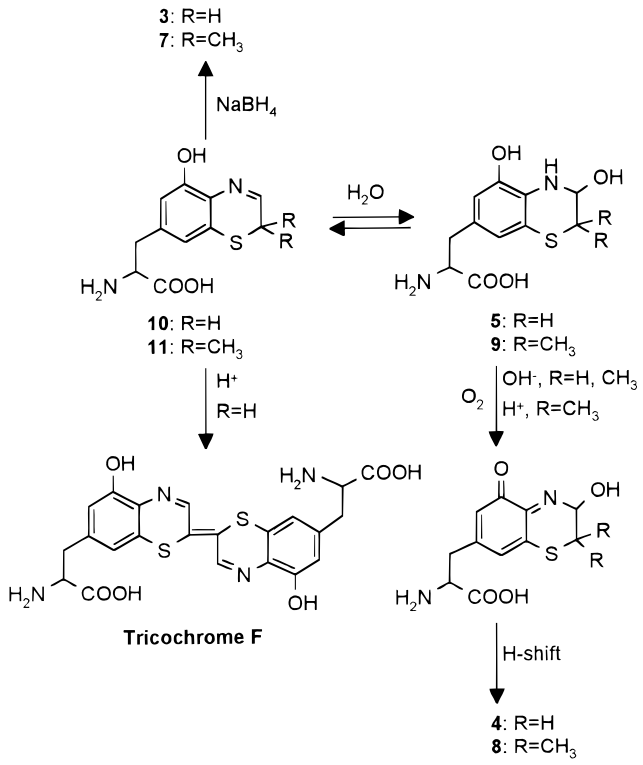
HPLC purification of the mixture obtained by oxidation of **6**, carefully avoiding strong acid or alkaline conditions, afforded eventually a fraction corresponding to the major component, which was characterized as the carbinolamine **9**, analogous to **5** obtained from **1**, on the basis of spectrometric and NMR analysis.

The several facets of the reactivity of the benzothiazine intermediate evidenced by HPLC analysis of the oxidation mixture of **1** at the early stages may be reconciled in terms of an equilibrium between the 3-hydroxy-3,4-dihydro-1,4-benzothiazine **5** and the 2*H*-1,4-benzothiazine **10** as a result of reversible addition of water to the imine function (Scheme 1). A similar behavior is observed for the benzothiazine **11** generated from the model

Scheme 1



catechol **6**, which readily undergoes reduction to the dihydrobenzothiazine **7**. The route depicted in Scheme 1 to the 1,4-benzothiazin-3-one **4/8** by aerial oxidation in acidic or alkaline media of the 3-hydroxy-3,4-dihydro-1,4-benzothiazine **5**/**9**, followed by rearrangement with H-shift and tautomerization, accounts for the reactivity observed for this labile intermediate and represents an alternative to that proposed previously in the literature.[14] In the case of the benzothiazine intermediate **10** derived from **1**, such a reaction path is superseded by the acid dimerization to trichochrome F.[12]

Experimental Section

General. Low- and high-resolution fast atom bombardment mass spectrometry (FAB MS) was carried out with a double focusing spectrometer equipped with a cesium gun operating at 25 kV using a glycerol/thioglycerol matrix. ^1H (^{13}C) NMR spectra were recorded at 400.1 (100.6) or 270.1 (67.9) MHz, using *tert*-butyl alcohol or dioxane as internal standards. COSY experiments were run using a standard Bruker pulse program. 2D proton−carbon shift correlation experiments were performed with gradient pulse using a delay corresponding to *J* values of 140 Hz. HPLC analyses were run under conditions previously described.[15] Ion exchange chromatography was carried out using a Dowex 50W-X2 (H^+ form, 200−400 mesh) resin. Horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) type II (167 U/mg, RZ $E_{430}/E_{275} = 2.0$) was used. 3-[(*R*)-2-Amino-2-carboxyethylthio]-5-[(*S*)-2-amino-2-carboxyethyl]-1,2-dihydroxybenzene (**1**) was prepared as previously described.[16]

3-[(*S*)-2-Amino-2-carboxy-1,1-dimethylethylthio]-5-[(*S*)-2-amino-2-carboxyethyl]-1,2-dihydroxybenzene (6). A solution of L-dopa (1.97 g, 10 mmol) in 2 M sulfuric acid (50 mL)

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was treated with cerium ammonium nitrate (10.96 g, 20 mmol) in 2 M sulfuric acid (100 mL) under stirring. The resultant yellow-orange mixture was added to a solution of D-penicillamine (6.0 g, 40 mmol) in 2 M sulfuric acid (50 mL). After 10 min, the reaction mixture was purified by ion-exchange chromatography (2 × 60 cm column), using water (200 mL), 0.5 M HCl (1.5 L), and 3 M HCl (2.0 L) as the eluant. Fractions from 3 M HCl showing an absorption maximum at 295 nm were collected and evaporated to dryness under reduced pressure to afford **6** dihydrochloride (2.10 g, 50% yield) as a yellow glassy oil, homogeneous to HPLC (0.1 M phosphoric acid, 0.1 M methanesulfonic acid, 0.1 mM EDTA, pH 3.1, eluant A; 0.05 M sodium citrate pH 4.0/methanol 85:15, eluant B): UV λ_{max} (0.1 M HCl) 295 nm; ^{1}H NMR (D_2O) δ 1.32 (s, 3H), 1.36 (s, 3H), 3.08 (dd, 2H, J = 12.2, 6.6 Hz), 3.68 (s, 1H),4.20 (t, 1H, J = 6.6 Hz), 6.88 (s, 1H × 2); ^{13}C NMR (D_2O) δ 18.76 (CH_3), 23.26 (CH_3), 31.32 (CH_2), 46.56 (C), 50.63 (CH), 56.85 (CH), 111.94 (C), 115.85 (CH), 122.83 (C), 127.92 (CH), 141.27 (C), 143.65 (C), 166.18 (C), 167.84 (C); FAB MS *m*/*z* 345 (MH^+, 100); exact mass calcd for $C_{14}H_{21}N_2O_6S$ 345.1120, found 345.1115.

Oxidation of 1 or 6. To a solution of **1** or **6** (1 mM) in phosphate buffer (0.05 M) pH 7.2 at 310 K was added $K_3Fe(CN)_6$ (0.8 molar equiv) under vigorous stirring. The reaction course was monitored by HPLC analysis (eluant B in the case of **1**, 0.05 M sodium citrate pH 4.0/methanol 80:20, eluant D in the case of **6**). Aliquots of the reaction mixture were periodically withdrawn, treated with $NaBH_4$ (0.5 mg/mg substrate) when necessary, and analyzed. Oxidation of **1** by peroxidase/H_2O_2,[9] sodium periodate, and Fe^{2+}/EDTA−H_2O_2[11b] was run under similar conditions.

7-(2-Amino-2-carboxyethyl)-5-hydroxy-3-oxo-3,4-dihydro-2*H*-1,4-benzothiazine (4) and 7-(2-Amino-2-carboxyethyl)-3,5-dihydroxy-3,4-dihydro-2*H*-1,4-benzothiazine (5). The mixture obtained by $K_3Fe(CN)_6$ oxidation of **1** as above was fractionated at 2 min reaction time by preparative HPLC (eluant B, flow rate 10 mL/min). The peak corresponding to the species at t_R 8 min was collected, and the UV spectrum was taken immediately (λ_{max}, HPLC eluant, pH 4: 302 nm). Aliquots of the HPLC fraction were treated with (a) $NaBH_4$, after careful neutralization with 0.1 M NaOH, (b) 1 M NaOH to pH 13, and (c) 1 M HCl to pH 1. The mixture thus obtained was then subjected to HPLC, spectrophotometric, and in the case of the acidified mixture paper chromatographic (eluant: butanol−acetic acid−water−concentrated HCl, 20:30:50:1 or 2-propanol−formic acid−concentrated HCl, 70:30:1) analysis. Identification of **4** in the oxidation mixture after alkalinization as in (b) followed from HPLC analysis (eluants A and B). Preparative HPLC (eluant B) and subsequent desalting by ion-exchange chromatography (eluant HCl gradient) afforded **4** as a glassy oil: UV λ_{max} (0.1 M HCl) 300 nm; (0.1 M NaOH) 320 nm; ^{1}H NMR (D_2O) δ 3.06 (dd, 1H, J = 15.7, 8.0 Hz), 3.19 (dd, 1H, J = 15.7, 6.0 Hz), 3.41 (s, 2H), 4.26 (dd, 1H, J = 8.1, 6.0 Hz), 6.65 (s, 1H), 6.75 (s, 1H), 7.90 (bs, 1H); ^{13}C NMR (D_2O) δ 32.19 (CH_2), 37.65 (CH_2), 56.56 (CH), 116.86 (CH), 122.09 (CH), 124.27 (C), 126.54 (C) 132.98 (C) 147.51 (C), 170.32 (C), 174.01 (C). In other experiments, the oxidation of **1** was repeated in a NMR cuvette: to a solution of **1** (3 mM) in deuterated 0.2 M phosphate buffer pH 7.2 was added $K_3Fe(CN)_6$ (0.8 equiv), and spectra were recorded at 2 min intervals. ^{1}H NMR (D_2O) of **5**: δ 3.00 (m, 4H), 3.88 (m, 1H), 5.44 (m, 1H), 6.56 (s, 1H), 6.59 (s, 1H).

7-(2-Amino-2-carboxyethyl)-3,5-dihydroxy-2,2-dimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (9). A solution of **6** dihydrochloride (100 mg, 0.24 mmol) in 0.05 M phosphate buffer pH 7.2 (72 mL), at 310 K, was treated with $K_3Fe(CN)_6$ (0.8 molar equiv). HPLC analysis of the reaction mixture after 2 min was found to consist of a predominant product eluted at t_R 16 min (eluant D). Preparative HPLC fractionation (0.05 M formic acid/methanol 55:45, eluant E, flow rate 10 mL/min) of the mixture after lyophilization afforded **9** (15 mg, 21% yield) as a glassy oil: UV λ_{max} (H_2O) 302 nm; ^{1}H NMR (D_2O) δ 1.33 (s, 3H), 1.45 (s, 3H), 3.02 (m, 1H), 3.17 (m, 1H), 3.98 (m, 1H), 4.85 (s, 1H), 6.65 (s, 2H);^{13}C NMR (D_2O) δ 19.58 (CH_3), 22.85 (CH_3), 31.55 (CH_2), 40.05 (C) 51.96 (CH), 73.78 (CH), 108.10 (CH), 115.77 (CH), 120.90 (C), 140.06 (C), 149.01 (C), 169.94 (C); FAB MS *m*/*z* 299 (MH^+, 100); exact mass calcd for $C_{13}H_{19}N_2O_4S$ 299.1065, found 299.1072.

7-(2-Amino-2-carboxyethyl)-5-hydroxy-2,2-dimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (7). For isolation of **7**, the oxidation of **1** was repeated under the above conditions, and the reaction mixture was treated with sodium borohydride (50 mg) and, after additional 3 min, acidified to pH 2 with 2 M HCl. HPLC fractionation of the reduced mixture (eluant E) yielded **7** (40 mg, 59% yield) as a glassy oil, homogeneous to HPLC (eluant D and E): UV λ_{max} (0.1 M HCl) 288, 292 nm; ^{1}H NMR (0.1 M DCl) δ 1.45 (s, 6H), 3.08 (dd, 1H, J = 6.8, 14.6 Hz), 3.16 (dd, 1H, J = 14.6, 5.8 Hz), 3.52 (s, 2H), 4.22 (dd, 1H, J = 6.8, 5.8 Hz,), 6.65 (s, 1H), 6.66 (s, 1H); ^{13}C NMR (0.1 M DCl) δ 27.42 (CH_3), 36.58 (CH_2), 40.55 (C), 53.87 (CH_2), 55.28 (CH), 114.13 (CH), 115.32 (C), 120.97 (CH), 130.07 (C), 137.39 (C), 152.32 (C), 172.75 (C); FAB MS *m*/*z* 283 (MH^+, 100); exact mass calcd for $C_{13}H_{19}N_2O_3S$ 283.1116, found 283.1110.

7-(2-Amino-2-carboxyethyl)-5-hydroxy-2,2-dimethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzothiazine (8). The oxidation mixture of **6** obtained as for isolation of **9** was acidified to pH 1 with 2 M HCl at 2 min reaction time and allowed to stand at room temperature for additional 15 min. After removal of the solvents under reduced pressure, the mixture was fractionated by preparative HPLC (eluant: 0.05 M formic acid/acetonitrile 75:25, eluant F, flow: 10 mL/min) to give **8** (22 mg, 31% yield) as a glassy oil, homogeneous by HPLC (eluant F and D): UV λ_{max} (0.1 M HCl) 300 nm, (0.1 M NaOH) 320 nm; ^{1}H NMR (0.1 M DCl) δ 1.58 (s, 6H), 3.18 (dd, 1H, J = 14.7, 7.8 Hz), 3.28 (dd, 1H, J = 14.7, 7.4 Hz), 4.35 (t, 1H, J = 7.4 Hz), 6.84 (s, 1H), 6.91 (s, 1H), 7.90 (bs, 1H); ^{13}C NMR (DCl 0.1 M) δ 25.37 (CH_3), 37.75 (CH_2), 41.51 (C), 55.80 (CH), 117.03 (CH), 122.05 (CH), 131.75 (C), 138.76 (C), 141.44 (C), 152.89 (C), 173.15 (C); 175.65 (C); FAB MS *m*/*z* 297 (MH^+, 100); exact mass calcd for $C_{13}H_{17}N_2O_4S$ 297.0909, found 297.0897.

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