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Characterisation of 1,4-Benzothiazine Intermediates in the Oxidative Conversion of 5-*S*-Cysteinyldopa to Pheomelanins.

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Abstract: Under biologically relevant conditions, oxidation of 5-*S*-cysteinyldopa (1) to pheomelanins proceeds through the formation of the 1,4-benzothiazine 7 along with the 3-carboxy analogue 6 in much smaller amounts, as evidenced by isolation of the reduced forms 5 and 4 and by deuterium labelling experiments.

Pheomelanins are a group of sulphur-containing pigments ranging from yellow to reddish brown, which characterise certain types of mammalian hair and avian feathers, wherein they usually occur together with the biogenetically related trichochromes.[1,2] Recent studies have shown that these pigments are also present in the epidermis of white caucasians of skin type I, II and III and their levels correlate with the susceptibility of these individuals to UV induced skin cancer and melanoma.[3]

Despite extensive investigation carried out in the sixties, the structure of pheomelanins is still poorly defined, owing to the unusual complexity of the chemistry involved. It is known, however, that these pigments derive biogenetically by oxidative polymerisation of cysteinyldopas, mainly the 5-*S* isomer (1), arising from 1,6 addition of cysteine to dopaquinone.[4] On the basis of model experiments[5-7] it was proposed[1,2] that the early stages of pheomelanogenesis (see scheme) involve the oxidation of 1 to cysteinyldopaquinone 2, followed by ring closure of the cysteine side chain leading to the *o*-quinonimmine 3; however, the subsequent fate of this labile intermediate has so far remained elusive.

In connection with our studies on the chemistry of melanin biosynthesis, we have reexamined the oxidation chemistry of 1 under biomimetic conditions. Preliminary kinetic experiments provided evidence that the peroxidase/H_2O_2 system is much more effective than tyrosinase, generally regarded as the major enzyme involved in melanogenesis,[8] in bringing about the oxidative conversion of 1 to pheomelanin pigments. Attempts to detect intermediates were unsuccesful owing to the marked instability of the species involved. However, after reduction of the oxidation mixture with sodium borohydride a simple and well defined pattern of products could be observed. This was found to consist, in the very first minutes of the reaction, of a major component designated as I in the elutogram of Fig. 1A, which then decreased with concomitant appearance of another compound (II, Fig. 1B). Fractionation of the reaction mixture at this stage by preparative HPLC allowed isolation of the two reaction products, I and II. The faster moving one was identified as 4 by comparison of its spectral properties with those of an authentic sample,[5] whereas the other one was assigned

the dihydrobenzothiazine structure 5 by NMR ^{13}C-^{1}H correlation and selective decoupling experiments.[9,10]

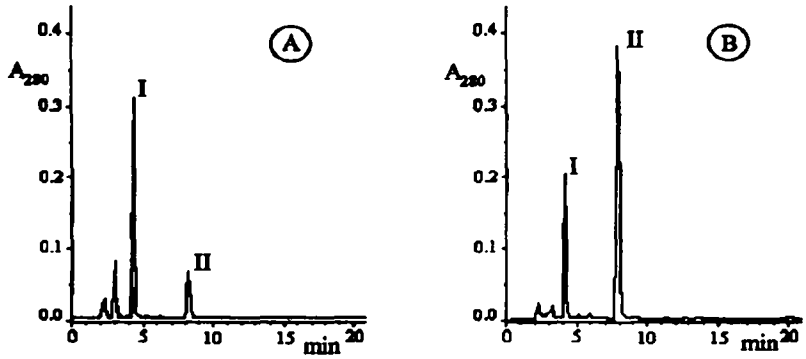


Fig. 1. HPLC elution profile of the mixture obtained by oxidation of **1** (3 mM) with peroxidase (9.0 purpurogallin U/ml) and hydrogen peroxide (2.4 mM) in 0.1 M phosphate buffer, pH 7.0, after $NaBH_4$ reduction: **A**, 3 min, **B**, 10 min reaction time. (Spherisorb S5-ODS2 column, 250x4mm; mobile phase: 0.05 M citrate buffer, pH 4.0 - MeOH 8:2 v/v, 1 ml/min).

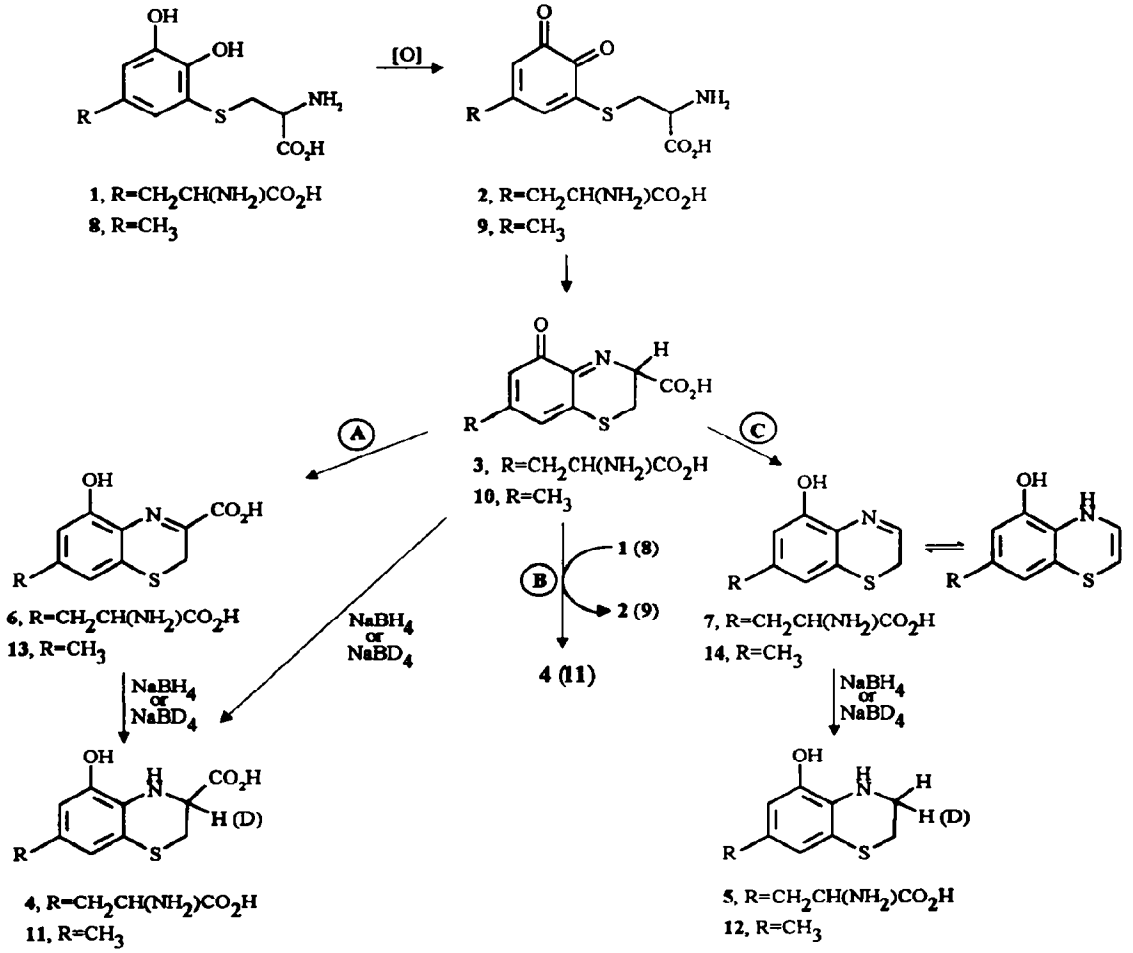
From consideration of the oxidation pathway of **1** as outlined in the scheme, different routes may be envisaged to account for the formation of **4**, primarily the reduction of the cyclic *o*-quinonimine **3** and/or the 3-carboxybenzothiazine **6**, which is likely to arise by rearrangement of **3** (path A). In addition, the possibility that the dihydrobenzothiazine **4** may be present in the reaction mixture, as a result of a redox exchange of **3** with the starting catechol **1** (path B), in analogy to that reported in the case of the tyrosinase catalysed process,[5] should be considered. Conversely, reduction of the 1,4-benzothiazine **7** generated by decarboxylative rearrangement of **3** (path C) is conceivably the sole origin of **5**.

In order to estimate the ratio of formation of the benzothiazine intermediates **6** and **7**, the oxidation of **1** was repeated, but $NaBD_4$ was used as the reducing agent. After the usual work up and fractionation of the reaction mixture, ^{1}H NMR spectral analysis of the 1,4-dihydrobenzothiazine products provided evidence, as expected, for a complete monodeuteration at the 3-position of **5**, whereas a 40% deuterium labelling was observed in the case of the carboxy derivative **4**. These data, coupled with consideration of the yield of formation of **5** with respect to that of **4**, allowed us to assess the relative operation of the rearrangement paths of **3**, at the initial stages of the oxidation process (10 min), in terms of about 85% for path C *vs* 15% for path A. Moreover, the results of the deuterium labelling experiments, which unambiguously demonstrated that the isolated products **4** and **5** actually arise from benzothiazine intermediates, provide additional support to the suitability of the reductive derivatisation procedure adopted in this study.

In further experiments, in which the oxidation of **1** was performed using either tyrosinase or chemical agents, e.g. periodate, the same pattern of reaction products was invariably obtained. Moreover, when the model compound **8** was used in place of the melanogenic precursor **1**, the peroxidase/H_2O_2 promoted oxidation was shown to lead to the corresponding dihydrobenzothiazine **11** and **12**, in similar yields. It seems therefore that the observed course of the oxidation reaction does not depend on the nature of the oxidizing

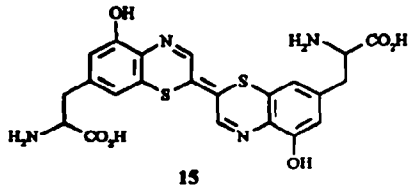
agent, but rather reflects the general reactivity in aqueous solutions at neutral pHs of cyclic *o*-quinonimimes of the type **3** or **10**.

Although the chemistry of a series of natural and synthetic 1,4-benzothiazines has been extensively investigated,[11] not much information is available in the literature on the parent compound unsubstituted at the 3-position, which however has been postulated as a key intermediate in several biological processes including the biosynthesis of trichochromes pigments, featuring the $\Delta^{2,2'}$-bi(2*H*-1,4-benzothiazine) skeleton.[1] Thus, our study is of particular interest in that it provides for the first time direct evidence for the formation of 1,4-benzothiazines such as **7** and **14** by oxidation of the cysteinylcatechols **1** and **8**. In line with this view, HCl treatment of the oxidation mixture of **1** at 10 min reaction time resulted in the formation in 26% yield of trichochrome F,[12] (**15**) arising by the acid catalysed oxidative coupling at the 2-position typical of 3-unsubstituted 1,4-benzothiazine systems.[13]



SCHEME

Apart from the chemical interest, the finding that, under biologically relevant conditions, the rearrangement of the key *o*-quinonimine intermediate **3** proceeds mainly with decarboxylation bears important implications with respect to both the biosynthesis and the structure of pheomelanin pigments. Work is underway to further investigate the course of pheomelanogenesis by characterisation of oligomer intermediates, which are expectedly generated by oxidative coupling of the 1,4-benzothiazine **7** and to a lesser extent the acid **6**.



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9. By measurement of peak areas in the HPLC elutogram and comparison with external calibration curves, the yields of formation of **4** and **5** were estimated as 31 and 54%, respectively.
10. UV λ_{max}(HCl 0.1 M) 293, 285, 254 (shoulder) nm, (log ε = 3.48, 3.48, 3.88); FAB MS (glycerol matrix), m/z 255 $(M+H)^+$; ^{1}H NMR (D_2O+DCl), δ (ppm): 2.88 and 2.98 (1H, dd, J=14.5 Hz, 5.8 Hz and 1H, dd, J=14.5 Hz, 7.5 Hz, -C<u>H</u>$_2$-CH<), 3.15 (2H, m, H-2), 3.59 (2H, m, H-3), 3.94 (1H, dd, J=7.5 Hz, 5.8 Hz, -CH$_2$-C<u>H</u><), 6.49 (1H, d, J= 1.4 Hz, H-8), 6.53 (1H, d, J=1.4 Hz, H-6); ^{13}C NMR (D_2O+DCl), δ (ppm): 23.9 (t, C-2), 36.3 (t, -<u>C</u>H$_2$-CH<), 42.8 (t, C-3), 55.4 (d, -CH$_2$-<u>C</u>H<), 113.4 (d, C-6), 117.0 (s, C-9), 120.2 (d, C-8), 129.0 (s, C-7), 136.4 (s, C-10), 151.1 (s, C-5), 173.0 (s, -COOH).
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