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A New Benzothiazole Derivative by Degradation of Pheomelanins with Alkaline Hydrogen Peroxide.

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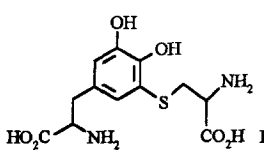
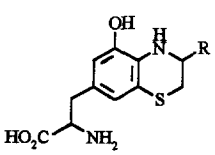
Abstract: Oxidation of natural and synthetic pheomelanins with alkaline H_2O_2 at room temperature led to the formation of a major product (up to 25% yield w/w) which was identified as the novel 2-carboxy-4-hydroxy-6-(2-amino-2-carboxyethyl)benzothiazole (**6**).

Traditionally dismissed as secondary members of the family of melanins, the reddish-brown, sulphur-containing pheomelanins have recently been the focus of keen interest following their implication as major determinants of the abnormal susceptibility of red-haired, fair-complexioned individuals to sunburn and skin cancer.[1] In spite of considerable pressure for such studies, the basic structure of pheomelanins still represents an open problem, because of the heterogeneous nature of these pigments and the lack of well defined physico-chemical and spectral properties. Most of what is presently known about them has derived from biosynthetic studies[2] aimed at elucidating the oxidation chemistry of the chief precursor, 5-*S*-cysteinyldopa (**1**). These studies have shown that pigment formation involves the oxidative cyclisation of **1** to give 1,4-benzothiazine intermediates structurally related to **2**[3] and **3**,[4] which subsequently polymerise, probably *via* dimers of the type **4**[4] and/or **5**.[5]



1



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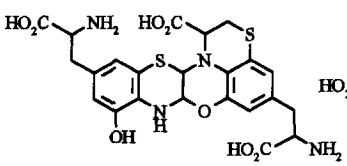
3: R=H



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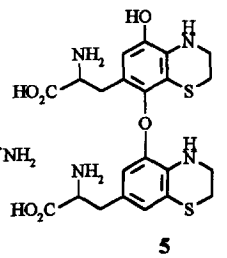

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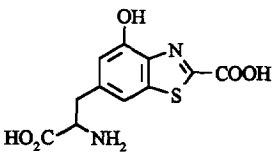
On chemical degradation, natural pheomelanins were found to afford a number of products,[6] most of which in exceedingly low yields. Minute amounts of thiazolecarboxylic acids and pyridinecarboxylic acids were obtained by permanganate oxidation, whereas hydrolyses with boiling HCl or HI gave some 3% of hydroxybenzothiazolylalanine derivatives[7] and an 8.5% yield of isomeric aminohydroxyphenylalanines.[8] Some of these products are currently utilised as markers for the microanalysis of pheomelanins in pigmented

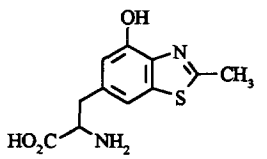
tissues;[9] however their origin is still uncertain, nor has their structural significance been definitively assessed, because of the rather harsh degradation conditions.[6]

In the course of a programme on the origin, structure and chemical properties of pheomelanins, we recently found that treatment of mammalian red hair with hydrogen peroxide in alkaline media at room temperature leads to the formation of a single major product, whose chromatographic properties did not match with those of any known degradation product of melanins. The same product was formed by degradation with 1% hydrogen peroxide in 1 M NaOH of a synthetic pheomelanin sample prepared by enzymatic oxidation of **1** with tyrosinase. Accordingly, a procedure was set out which allowed isolation of the product in sufficient amounts for chemical analysis.

The product, about 25% yield w/w, was positive to the ninhydrin reaction, migrated toward the anode on paper electrophoresis (0.33 mm/min, 0.05 M borate buffer, pH 10, 250 V) and exhibited an absorption maximum at 332 nm, reminiscent of the chromophore of 4-hydroxybenzothiazoles.[10] Both the ^{1}H-NMR and ^{13}C-NMR spectra,[11] integrated by DEPT and 2D carbon-proton-shift correlation experiments, were consistent with the presence of an alanyl chain and a tetra-substituted benzene ring. These data, together with a distinct quaternary carbon at δ 159.78, and a resonance for an aromatic carboxyl group at δ 164.02 in the ^{13}C-NMR spectrum, argued strongly in favour of the novel 2-carboxy-4-hydroxy-6-(2-amino-2-carboxyethyl)benzothiazole (**6**). In accord with this assignment, the product gave a $(M+H)^{+}$ ion peak in the electrospray MS spectrum at m/z 283. [12]



6



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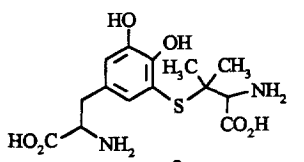


8

Product **6** was unstable to drastic oxidation conditions, e.g. alkaline permanganate as well as acids and heating. The optimal oxidation time, ensuring maximum yield of **6** without concomitant product loss, was about 18 hours. Such prolonged oxidation was especially critical in the case of intact tissues, where an efficient swelling of the keratin matrix and degradation of the pigment granules is normally important. Notably, a substantial yield of the product (about 5% w/w) could be obtained by simply treating synthetic pheomelanin with 1 M NaOH at room temperature under oxygen-free atmosphere. This suggests that a significant proportion of the monomer units in the pheomelanin pigment are present in such a form that they may be readily hydrolysed off as **6** without requiring an oxidative step.

Table 1 reports the yields of benzothiazole **6** obtained by direct hydrogen peroxide degradation of representative pheomelanin-containing tissues, i.e. red mammalian hairs and chicken feathers. Based on an average melanin content of human hair of 2 %,[13] the yield of compound **6** in that case turns out to be about 10%.

The formation of **6** was also investigated using synthetic pigments and a number of model compounds. The results, summarised in Table 2, indicate that **6** is formed both from pheomelanins and benzothiazine

Table 1. Yields of **6** by Oxidative Degradation[a] of Pheomelanic Tissues.

Tissue	*Yield (μg/mg)*[b]
Red human hair	2.2
Yellow mouse hair	2.5
Irish setter hair	0.43
New Hampshire chicken feathers	6.2

[a] 1% H_2O_2 in 1M NaOH, 18 h, at room temperature. [b] Determined by HPLC, average of three experiments, S.D. ≤5%.

Table 2. Yields of **6** by Oxidative Degradation of Synthetic Pheomelanins and Model Compounds.

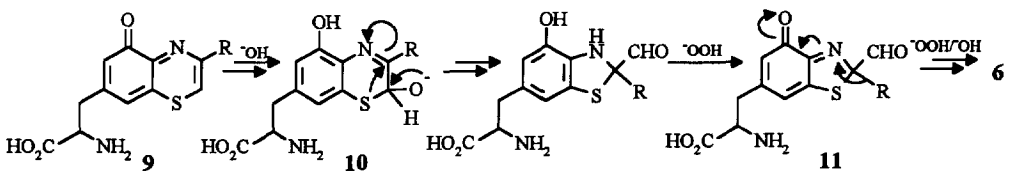
Compound	*Yield (μg/mg)* [a]
Pheomelanin from **1** [b]	280
Pheomelanin from **8** [c]	--
2	175
3	31.5
4	37.9
5	2.20
7	--

[a]Determined by HPLC, average of three experiments, S.D. ≤5%.[b] Prepared by tyrosinase oxidation of **1**, under a stream of oxygen for 4 hrs. [c] Prepared by oxidation of **8** as in a).

precursors, such as **2-5**, but not from the benzothiazole **7**[14] nor from the pheomelanin derived from **8**, which on oxidation cyclises to 2,2-dimethyl-1,4-benzothiazine intermediates.

Although the formation of benzothiazole derivatives by ring contraction of benzothiazine rings is not unprecedented,[14,15] the origin of **6** is intriguing. Besides the mechanism of formation of the carboxyl group, which should expectedly originate from the C-2 carbon of benzothiazine units, a crucial issue is whether the ring contraction process occurs concurrent with pigment synthesis or during the process of oxidative breakdown. Several lines of evidence from previous studies[2] would argue in favour of the latter option, since benzothiazine intermediates formed in the oxidation of **1** appear to be stable enough to survive the biosynthetic process without suffering ring contraction. Moreover, it has been shown that aminohydroxyphenylalanines, the major degradation products of pheomelanins obtained prior to the present study, arise by HI hydrolysis of benzothiazines but not benzothiazoles.[1]

Taking the above for granted, various mechanisms may be envisaged to account for the carboxyl-forming ring-contraction process. A most reasonable one is outlined in the Scheme and would involve formation from pheomelanins or benzothiazine compounds **2-5** of oxidised 1,4-benzothiazine species of the type **9**. The critical step, then, is the nucleophilic attack of the hydroxyl anion at C-2 to afford a transient hemithioketal, *i.e.* **10**, akin to the intermediate invoked in the alkali-induced ring contraction of 2*H*-1,4-benzooxazines.[16] A species like **10** would be amenable to rearrangement and oxidation to give **11**, which could undergo further oxidation and aromatisation to yield eventually **6**.



R= H,CO_2H

Scheme

That the suggested nucleophilic attack occurs prior to, and not after, ring-contraction would be supported by the observed failure of **7** to give **6** on oxidation with alkaline hydrogen peroxide, which clearly rules out a late carboxyl-forming step by oxidation of a methyl group. Attempts to substantiate the proposed mechanism failed to provide conclusive results, because of the inherent complexity of the chemistry involved and the elusive character of the intermediate products, so other degradation pathways can not be excluded.

In conclusion, the results of this study open new perspectives in the chemistry of pheomelanins, as they furnish an improved basis to address the controversial origin of thiazole and benzothiazole degradation products, and bring to light a novel potential marker for microanalysis of pheomelanins in pigmented tissues.

ACKNOWLEDGEMENTS

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11. Compound **6**: UV λ_{max} 250, 289, 332 nm (log ε 3.88, 3.64, 3.19); ^{1}H-NMR (400.1 MHz, D_2O), δ (ppm): 3.12 (1H, dd, J=14.5, 4.1 Hz), 3.37 (1H, dd, J=14.5, 3.0 Hz), 4.35 (1H, dd, J= 4.1, 3.0 Hz), 6.72 (1H, s), 7.18 (1H, s); ^{13}C-NMR (100.1 MHz, D_2O), δ (ppm): 37.28 (CH_2), 55.33 (CH), 114.03 (CH), 115.67 (CH), 137.17 (C), 139.80 (C), 142.63 (C), 152.37 (C),159.78 (C), 164.02 (C),172.55 (C)
12. The compound gave satisfactory microanalysis.
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