Oxidative Polymerization of the Pheomelanin Precursor 5-Hydroxy-1,4-benzothiazinylalanine: A New Hint to the Pigment Structure

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Biosynthetic studies have shown that pheomelanins, the distinctive pigments of red human hair, arise from oxidative polymerization of cysteinyldopas *via* 1,4-benzothiazinylalanine intermediates. However, the mode of formation of the pigment polymer remains controversial. To address this point, we have investigated the conversion of the major biosynthetic precursor 5-*S*-cysteinyldopa (**2a**) to pheomelanin under biomimetic conditions. Peroxidase/H_2O_2 oxidation of **2a** was shown to lead in the early stages to the 1,4-benzothiazinylalanine **8a**, which rapidly declines with concomitant formation of a distinct pattern of oligomeric products. Reduction of the reaction mixture at this stage allowed the isolation of dimer **17** in 10% yield, along with trimers **18** and **19** in smaller amounts. A restricted rotation about the ethereal C−O bond of **17** was apparent by the presence of two NMR-detectable conformational isomers, separated by an activation energy barrier of 17.83 ± 0.03 kcal mol^{-1}. Under similar oxidation conditions, the model catechol **2b** gave the related dimers **15** and **16**. The structure of oligomers **17**−**19**, all characterized by C−C and C−O bonds between the benzothiazine units, would suggest that the peroxidase-promoted polymerization proceeds by phenol-type coupling of an aryloxy radical generated by initial one-electron oxidation of **8a**. Overall, these results point to a structural model for the pheomelanin polymer which is basically different from that proposed on the basis of degradative studies.

Introduction

Melanin pigmentation has many facets, both scientific and sociologic, that support the intense interest and considerable efforts of the numerous studies on the underlying biological and chemical mechanisms.[1] The last decade has witnessed a renewed impetus in melanin research that has significantly expanded our knowledge and appreciation of the field.[2] Considerable steps forward have been made toward the understanding of the structure and biosynthesis of the dark-brown pigments, eumelanins, the main determinants of skin color differences in man.[1c,3] A major gap which remains concerns the chemistry of the distinctive pigments of carroty-red human hair commonly referred to as pheomelanins.[2,4] Mounting evidence indicates that these pigments are also present in the epidermis of celtic-type, fair-skinned people and that their levels correlate with the high susceptibility of these individuals to actinic damage, skin cancer, and melanoma.[5]

Extensive studies carried out in the 1960s showed that pheomelanins are highly heterogeneous polymers or mixtures of polymers, in keeping with their biogenetic origin from isomeric precursors.[6] They also lack any well-defined spectral or other physical characteristics, which makes their structural investigation especially challenging.

Using the classical degradative approach, Nicolaus and colleagues[4,6,7] obtained evidence suggesting that the pigment backbone consists mainly of benzothiazole and tetrahydroisoquinoline units linked as in the partial structure **1**.[7a,c] Moreover, it was proposed that 1,4-benzothiazine units at various oxidation levels could partake to some extent in the building up of the polymer and that uncyclized cysteine residues were present as well.[6d] Such a structural model was challenged by subsequent investigations[8] suggesting that most of the degradation products might be artifacts, due to the harshness of the conditions used. However, in these studies no alternative structure for the pigment polymer was proposed.

Some more insight into the structure of pheomelanins followed from biosynthetic studies showing that pigment formation involves oxidative polymerization of cysteinyldopas, namely 5-*S*-, 2-*S*-, 6-*S*-cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa (**2a**, **3**−**5**), which are formed by nucleophilic addition of cysteine to dopaquinone[2] in yields of ca. 74, 14, 1, and 5%, respectively.[9] Under physiologically relevant conditions, the major pheomelanin precursor **2a**, as well as the model catechol **2b**, undergo 1,2 addition and dehydration of the transient *o*-quinones (**6a**,**b**) to

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(2) Prota, G. *Melanins and Melanogenesis*; Academic Press: San Diego, 1992.

(3) Prota, G. *Fortsch. Chem Org. Naturst.* **1995**, *64*, 94.

(4) Thomson, R. H. *Angew. Chem., Int. Ed. Engl.* **1974**, *13*, 305.

(5) Thody, A. J.; Higgins, E. M.; Wakamatsu, K.; Ito, S.; Burchill, S. A.; Marks, J. M. *J. Invest. Dermatol.* **1991**, *97*, 340.

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(9) (a) Ito, S.; Prota, G. *Experientia* **1977**, *33*, 1118. (b) Morishima, T. F.; Tatsumi, F.; Fukada, E.; Saito, M.; Fuijta, M.; Nagashima, N.; Hanawa, S. *Arch. Dermatol. Res.* **1983**, *275*, 76.

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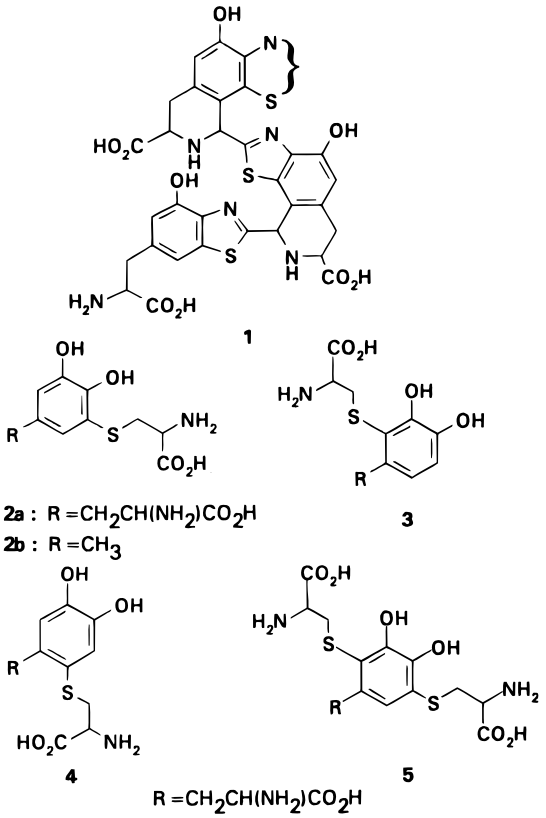
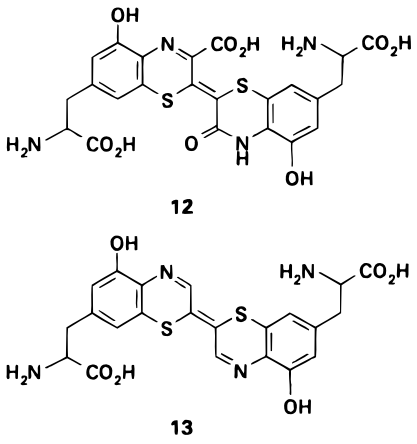


give, depending on the reaction conditions, the 1,4-benzothiazines **8a**,**b**−**9a**,**b**[10] and/or the 2*H*-dihydro-1,4-benzothiazines **10a**,**b**[11] *via* the common quinonimine intermediates **7a**,**b**; in neither case does the amino group cyclize by 1,4-addition to give aminoquinones (**11a,b**) (Scheme 1).[4]

The observed tendency of the major biosynthetic precursor to give on oxidation 1,4-benzothiazine species is well in line with the structure of trichochromes, a variety of low molecular weight pheomelanins featuring the $\Delta^{2,2'}$-bi-2*H*-1,4-benzothiazine skeleton, e.g., **12** and **13**.[3,4] The occurrence of 1,4-benzothiazine units in the



pheomelanin polymer is also supported by isolation of 7-(2-amino-2-carboxyethyl)-5-hydroxy-3-oxy-dihydro-1,4-benzothiazine and 4-amino-3-hydroxyphenylalanine by degradation with hydroiodic acid.[6d]

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(11) (a) Prota, G.; Crescenzi, S.; Misuraca, G.; Nicolaus, R. A. *Experientia* **1970**, *26*, 1508−1509. (b) Costantini, C.; Crescenzi, O., Prota, G.; Palumbo, A. *Tetrahedron* **1990**, *46*, 6831.

However, while conversion of 1,4-benzothiazine intermediates to trichochromes is fairly well understood,[4,12] the mechanism by which they may form the pigment polymer remains elusive. To address this point, we have examined the oxidative polymerization of **2a** under conditions which mimic the biosynthetic process. As the oxidizing agent, we chose the peroxidase/H_2O_2 system instead of the tyrosinase/O_2 couple,[13] as the former was recently shown to be more effective for the conversion of cysteinyldopas to pheomelanins.[10]

Results and Discussion

Peroxidase/H_2O_2 oxidation of the model compound **2b**, lacking the alanyl side chain, was first examined in view of the more favorable analytical properties of the compound and expectedly of the reaction products compared to those of **2a**. Direct analysis of the oxidation mixture proved difficult owing to the marked instability of the species involved. However, after reduction of the oxidation mixture with $NaBH_4$ according to the procedure adopted in a previous study,[10] a well-defined pattern of products was obtained as evidenced by HPLC analysis. This consisted, in the very first minutes of the oxidation, of two components, which in the further stages of the reaction decrease, while two additional species are formed. Preparative HPLC fractionation of the reaction mixture allowed isolation of the major reaction products, which were identified as the 3,4-dihydro-2*H*-1,4-benzothiazines **10b**[11a] and **14b**[14] and the isomeric dimers **15** and **16** arising *presumably* by C−C coupling at the positions *ortho* and *para* to the hydroxyl group of the 2*H*-1,4-benzothiazine intermediate **8b**.

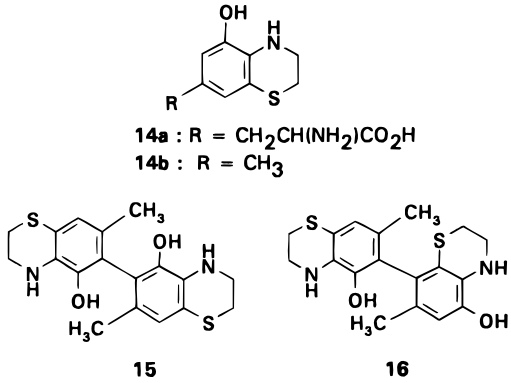


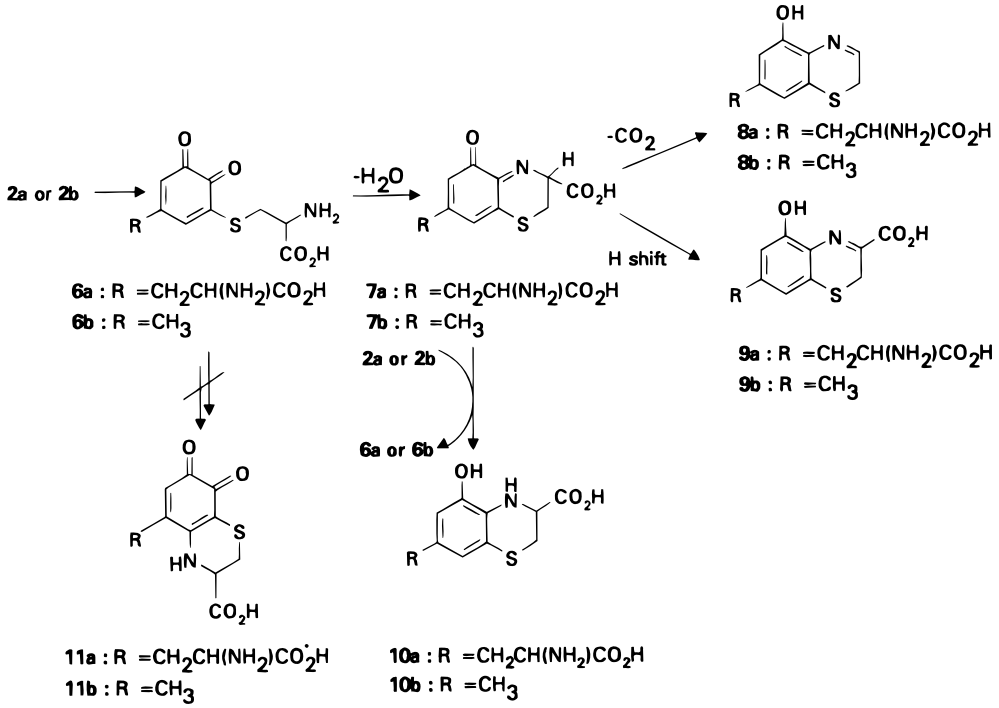
Figure 1 shows a series of reversed-phase HPLC profiles recorded throughout the peroxidase/H_2O_2 oxidation of **2a**, following $NaBH_4$ treatment of the periodically withdrawn aliquots. Within the first 2 min, the dihydro-1,4-benzothiazine **14a**[10] (peak B in elutogram I) is observed as the major reaction product, along with the 3-carboxy analogue **10a**[11a] (peak A). Only the former is significantly consumed in the subsequent minutes with concomitant increase of a series of other products, the major of which are designated as C, D, and E in elutogram II. At 2 h (elutogram III), these intermediates, presumably oligomeric in nature, have mostly declined,

(12) Brown, C.; Davidson, R. M. In *Advances in Heterocyclic Chemistry*; Katritsky, A. R., Ed.; Academic Press: New York, 1985; Vol. 38, p 135.

(13) (a) Fattorusso, E.; Minale, S.; De Stefano, S.; Cimino, G.; Nicolaus, R. A. *Gazz. Chim. Ital.* **1969**, *99*, 969. (b) Ito, S. *Pigment Cell Res.* **1989**, *2*, 53.

(14) Prota, G.; Petrillo, O.; Santacroce, C.; Sica, D. *J. Heterocycl. Chem.* **1970**, *7*, 555.

Scheme 1



while some 3-carboxydihydro-1,4-benzothiazine **10a**, peak A, is still present in the mixture. The pigment formed at this stage of the oxidation exhibited an absorption spectrum and chromatographic and solubility properties closely similar to those of native and synthetic pheomelanins.[7d,13] Combined ion exchange chromatography and preparative HPLC of the mixture at 10 min reaction time, after $NaBH_4$ reduction, allowed isolation of three products corresponding to peaks C, D, and E of chromatogram II.

The most abundant of these, E, obtained in 10% yield, exhibited in the MALDI mass spectrum a pseudomolecular ion peak at m/z 507 suggesting a tetrahydrobibenzothiazinyl structure. An intriguing feature of the proton and carbon spectra was the presence of a number of signals doubled with respect to those expected for a dihydrobenzothiazine dimer. The proton signals could be clearly divided in two closely related but distinct sets, with areas approximately in a 1:0.9 ratio. However, a single set of peaks was observed when the spectrum was taken at 343 K, the effect being reversed as the sample was cooled down to room temperature. Such a temperature dependence coupled with consideration of the chromatographic behavior of the product, which proved homogeneous to HPLC under a variety of conditions, would suggest a slow interconversion of two conformational isomers. Line shape analysis at several temperatures, around the coalescence temperature, allowed calculation of the mean lifetimes, from which a free energy of activation ($\Delta G^{\ddagger}$) of 17.83 ± 0.03 kcal mol^{-1} was determined by straightforward application of the Eyring equation.[15]

The proton spectrum of compound E run at 298 K displayed for each conformer, beside the resonances of two alanyl side chains, four multiplets at δ 3.13, 3.64, and 3.29, 3.75 attributable to the AA′BB′ systems of two dihydrothiazine rings. In the sp^2 region two pairs of doublets centered at δ 6.11 and 6.83 (J = 1.6 Hz) and two singlets at δ 6.73 and 6.78 for the major and minor conformer, respectively, could be distinguished. 2D carbon−proton shift correlation experiments optimized for different J values showed direct coupling between the δ 6.11 protons and CH carbons at δ 110.65/110.75 and between the 6.83 protons and the CH carbons at δ 123.68. Moreover, the δ 6.11 protons exhibited ^2J coupling with the C-5 oxygen bearing carbons at δ 150.09/150.48 and ^3J couplings with the quaternary carbons at δ 118.10/118.74 and with the CH carbons at δ 123.68 (Table 1). These data, coupled with analysis of the one-bond and long-range couplings of the aliphatic protons with aliphatic/aromatic carbons, allowed us to build up the skeleton of a dihydro-1,4-benzothiazine ring system unsubstituted at both C-6 and C-8 aromatic positions, suggesting that coupling between the two moieties in the dimer may involve an heteroatom bridge. Support for this view was obtained by analysis of the connectivities of the remaining aromatic protons. In addition to one-bond correlation with CH carbons at δ 114.23 and 114.45, the singlets at δ 6.73/6.78 ppm exhibited ^2J couplings with C-5 carbons at δ 149.60/149.68 and ^3J coupling with quaternary carbon pairs at δ 117.87/117.60 ppm and notably with a deshielded carbon pair at δ 140.07/140.24, providing evidence for a dihydrobenzothiazine ring system, where the C-8 position is shifted downfield by direct attachment to a heteroatom. This is likely the C-5 oxygen based on consideration of the chemical shift values as well as the lack of C,H couplings between the two dihydrobenzothiazine rings. Taken together, these elements led to formulation of compound E as the dimer **17**.

From consideration of structure **17** it seems that the observed conformational isomerism may be ascribed to a restricted rotation about the ethereal C−O bond dictated by the steric hindrance of the substituents at the *ortho* positions. Slow rotations around benzene−oxygen bonds have been observed in some *ortho* di- and

(15) Oki, M. *Applications of Dynamic NMR Spectroscopy to Organic Chemistry*; VCH, Inc.: Weinheim, 1985.

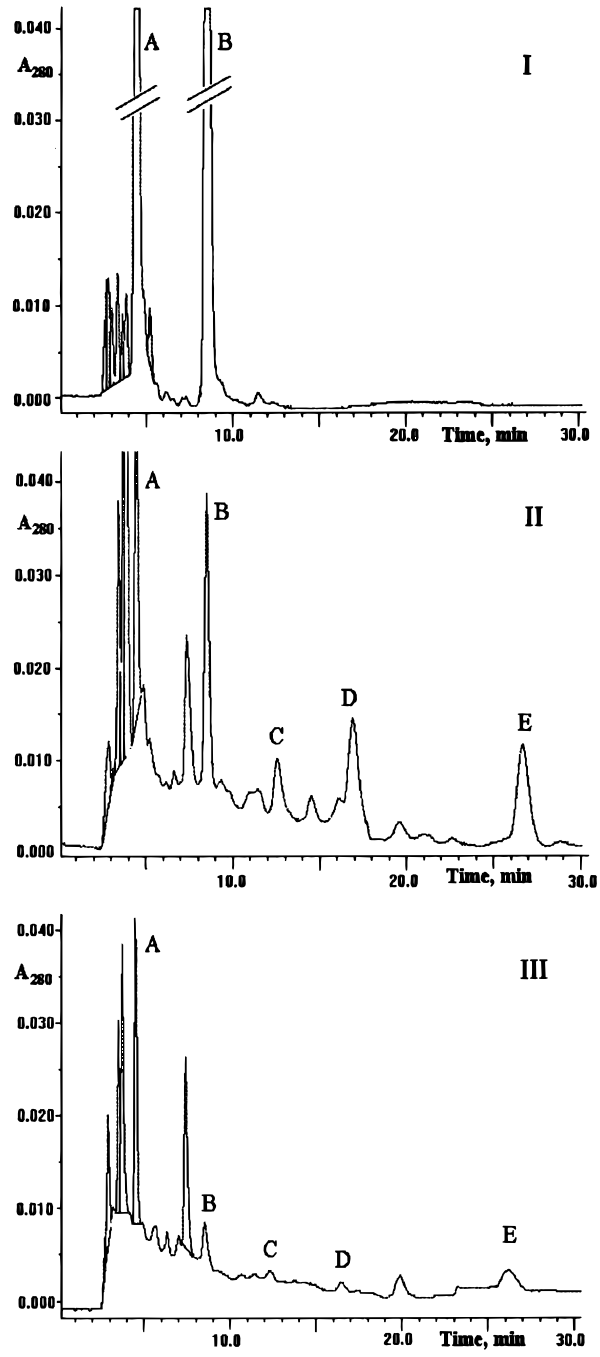


Figure 1. HPLC elution profile of the mixture obtained by peroxidase/H_2O_2 oxidation of **2a** in 0.1 M phosphate buffer, pH 7.0, after $NaBH_4$ reduction: I, 2 min, II, 10 min, III, 120 min reaction time. Eluant A was used as mobile phase; all other experimental and analytical conditions were as detailed in the Experimental Section.

trisubstituted diaryl ethers.[16] In principle, a restricted rotation may be envisaged also in the case of dimers **15** and **16** featuring a hindered biphenyl system. However, in the absence of other asymmetry centers, the atropoisomers would in fact be enantiomers and, therefore, would not give rise to distinct sets of NMR signals.

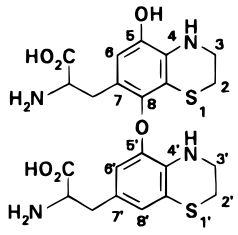
The other two products corresponding to peaks C and D in elutogram II of Figure 1, exhibited virtually identical MALDI mass spectra, with pseudomolecular ion peaks at *m/z* 759, corresponding to a dihydrobenzothiazine trimer. The structural analogy of the compounds was also apparent from comparison of the proton spectra.

(16) Kessler, H.; Rieker, A.; Rundel, W. *J. Chem. Soc., Chem. Commun.* **1968**, 475. Bolon, D. A. *J. Am. Chem. Soc.* **1966**, *88*, 3148.

Table 1. ^{13}C NMR (100.6 MHz) Data for Compound 17[a]

		δ ^1H[c] (ppm) $^{13}C-^1H$ correlation		
	δ ^{13}C (ppm)[b]	one-bond	multiple-bond	selected ^{13}C-^1H coupling constants[d] (Hz)
C-2	23.14 (t)	3.13	3.64	
C-3	42.24 (t)	3.64	3.13	
C-5	149.60		6.73	2.7
	149.68		6.78	2.8
C-6	114.23 (d)	6.78 (minor conformer)		164.0, 4.8, 4.8
	114.45 (d)	6.73 (major conformer)		164.0, 5.9
C-7	129.74[e]		2.92	
	130.11[e]		2.92	
C-8	140.07		6.78, 2.92	11.2, 5.8, 5.8
	140.24		6.73, 2.92	9.6, 6.5, 3.7
C-9	125.18		3.13	3.2, 3.2
C-10	117.60		6.78, 3.64	
	117.77		6.73, 3.64	9.0, 4.4
αCH	54.30 (d)[f]	4.10	2.92	
βCH_2	31.23 (t)	2.92		
	31.46 (t)	2.92		
C-2′	24.01 (t)	3.29	3.75	
	24.13 (t)	3.29	3.75	
C-3′	43.10 (t)	3.75	3.29	
C-5′	150.09		6.11	2.9
	150.48		6.11	2.6
C-6′	110.65 (d)	6.11	6.83	164.0, 13.2, 7.0
	110.75 (d)	6.11	6.83	164.0
C-7′	135.45[e]		2.92, 4.10	10.3, 4.8
	135.94[e]		2.92, 4.21	
C-8′	123.68 (d)	6.83	6.11, 2.92	166.0, 10.8, 4.8
C-9′	130.22		3.29	
	130.58		3.29	3.0, 3.0
C-10′	118.10		6.11, 6.83,	7.2, 3.8, 3.8
	118.74		6.11, 6.83, 3.75	7.2, 3.8, 3.8
αCH′	53.91 (d)[f]	4.21	2.92	
	54.05 (d)[f]	4.10	2.92	
bCH_2'	35.73 (t)	2.92	6.83	
COOH,	171.45			
COOH′	171.49			
	171.88			

[a] Assignments based on $^{13}C-^1H$ shift correlation experiments optimized for $J = 140$, 10, and 6 Hz. Operating temperature 298 K. [b] Multiplicities determined from DEPT spectra. [c] Mean value for the two conformers, unless otherwise stated. [d] Determined by *J*-resolved experiments. Estimated error on *J* values ±0.5 Hz. [e,f] These resonances may be interchanged.



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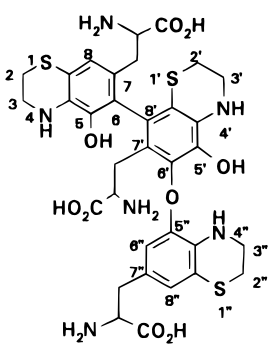

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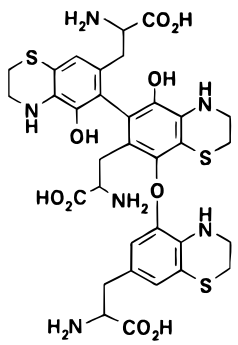

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Scrutiny of the aliphatic region aided by COSY experiments allowed identification of the signals of the alanyl side chains and the $-NHCH_2CH_2S$ systems expected for the trimeric structures. On the other hand, the aromatic resonances consisted of one 1H singlet (δ 6.90 for C, δ 6.63 for D) and two 1H doublets (δ 6.06, 6.88 for C, δ 5.99, 6.52 for D) with meta coupling constants ($J = 1.6$ Hz). The unsubstituted aromatic positions were identified as two C-8 (δ 122.64, 123.46 for C and δ 121.97, 123.14 for D) and one C-6 (δ 110.48 for C, δ 110.02 for D) based on analysis of the carbon spectra and the one-bond $^{13}C-^{1}H$ correlations. These data can be accommodated by the isomeric structures **18** and **19**, both having two terminal dihydro-2*H*-1,4-benzothiazine units linked through the 6-position or the C-5 oxygen to the 6- and 8-position of a central dihydro-1,4-benzothiazine ring. Careful analysis of the carbon chemical shifts in comparison with those of dimer **17** led eventually to assign structure **18** and **19** to the compounds eluted under HPLC peaks D and C, respectively.

It is worth noting in this connection that both the proton and carbon spectra of trimers **18** and **19** display single sets of sharp signals. However, in view of the restriction to internal rotation observed in the case of dimer **17**, it seems unlikely that this should be taken as evidence for a rapid spanning of the whole conformational space; rather, the possibility may be envisaged that for each trimer a preferred conformation exists, while the population of the other rotational isomers is negligible. Alternatively, it can be surmised that combination of a hindered diaryl ether subunit with an *o*,*o*′-disubstituted biphenyl may have resulted in a significant increase of the rotational barriers, making the isomerization processes sufficiently slow to allow isolation of individual conformers. The likelihood of this latter hypothesis is difficult to assess on theoretical grounds owing to the interference of the several conceivable isomerization pathways of the system. Attempts to accelerate the postulated equilibration processes by raising the temperature up to 363 K did not provide clear-cut results owing to supervening degradation reactions.

Consideration of structures **15**−**19**, coupled with current knowledge of peroxidase promoted oxidations,[17] would suggest a reaction pathway involving initial one-electron transfer of the benzothiazine intermediates **8a**,**b** to the peroxidase−hydrogen peroxide complex to yield an aryloxy radical species. The latter may then undergo self-coupling through the oxygen atom and the positions *ortho* and *para* to the hydroxyl group, in close analogy with the oxidative polymerization of simple phenols.[18] This represents an unprecedented facet of the oxidation chemistry of 3-unsubstituted 1,4-benzothiazines which is typically characterized by oxidative coupling at 2-position to give dimers or dehydrodimers depending on the reaction conditions.[12,19] The differences in the regiochemical features of the polymerization of **2a** with respect to that of the 3-*S*-cysteinyl catechol **2b** may be accounted for in terms of the steric hindrance offered by the alanyl side chain which favors coupling of the benzothiazine units through the oxygen bridge over that involving the nuclear positions, as reported in the case of other hindered phenols.[20]

The lack of carboxyl functionalities at the 3-positions of the isolated oligomers is in keeping with the results of our previous deuterium labeling experiments showing that, at neutral pHs, rearrangement of the *o*-quinonimines **7a**,**b** proceeds mainly with decarboxylation, with prevailing formation of the 1,4-benzothiazines **8a**,**b** with respect to the 3-carboxy analogues **9a**,**b**.[10]

Another point for consideration concerns the role of peroxidase in pheomelanin biosynthesis with respect to tyrosinase commonly regarded as the main melanogenetic enzyme.[21] In previous studies, evidence was reported that **2a** is a poor substrate of tyrosinase unless in the presence of a suitable redox cycler such as dopa,[11b,13a] but is readily converted to pheomelanins by the peroxidase/H_2O_2 couple.[10] Support of the actual involvement of peroxidase in melanogenesis comes from biochemical studies by Shibata *et al.* showing that some subcellular compartments of pigment cells contain high levels of peroxidase, while tyrosinase activity is low.[22] In the light of this, differences in the reaction paths of oxidation of **2a** as catalyzed by the two enzymic systems may prove relevant both to the biosynthetic process and to the structure of the final pigment. Thus, while in the tyrosinase-catalyzed oxidation the sole species detected prior to pigment formation are dimeric cycloaddition products,[11b] resulting from reaction of the *o*-quinonimine **7a** and the benzothiazine **8a** slowly produced during the oxidation, the course of the peroxidase/H_2O_2 process is mainly governed by the reactivity of **8a** toward the enzymic system, no other intermediate apparently accumulating in the mixture.

In conclusion, characterization of oxidative polymerization of **2a** under biologically relevant conditions at the stages following formation of the ultimate monomer intermediate, namely the benzothiazine **8a**, provides a new lead to the pigment structure. It should be emphasized in this connection that the general structure of pheomelanins as 1,4-benzothiazine polymers which emerges from this study is essentially different from that previously proposed based on analysis of degradation products (see formula **1**).[6,7] The structural isomerism evidenced in the oligomer species formed in the early stages of pheomelanogenesis, which differ in the type of linkage and the positions of coupling of the monomer units, would point to a high complexity of the final pigment, well in line with the observed properties of native pheomelanins. Of course, other factors, such as concurrent polymerization and copolymerization of the 1,4-benzothiazinylalanine isomers derived from cysteinyldopas **2a** and **3**−**5**, may account for the high degree of heterogeneity of the natural pigments.

Experimental Section

General. MALDI mass spectra were taken on a Reflex time-of-flight mass spectrometer, operating in positive linear mode; ions, formed by a pulsed UV laser beam (nitrogen laser,

(17) For a review, see: Yamazaki, I. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1977; Vol. 3, p 183.

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λ = 337 nm), were accelerated at 15 keV; matrix: 2,5-dihydroxybenzoic acid. For EIMS measurements, samples were ionized with a 70 eV beam, with the source at 230 °C. ^{1}H (^{13}C) NMR spectra were recorded at 400.1 (100.6) or 270.1 (67.9) MHz at an operating temperature of 298 K, unless otherwise stated, using TMS or dioxane as internal standards. Chemical shifts are reported in parts per million (ppm, δ). COSY and heteronuclear *J*-resolved experiments were run using a standard Bruker pulse program. 2D carbon−proton shift correlation experiments[23] were performed at 100.6 MHz using a Bruker XHCORR microprogram with a D_3 delay corresponding to *J* values of 140, 10, and 6 Hz. Experiments were recorded with 128 × 2048 matrix sizes. For line shape analysis, the equation of Gutowsky and Holm[24] was used, on the assumption of unperturbed line widths. The relevant parameters were determined by nonlinear least-squares fitting to the measured line shapes. HPLC analyses were run under conditions previously described.[25] Ion exchange chromatography was carried out using a Dowex 50W-X2 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. 5-*S*-Cysteinyldopa (**2a**)[26] and 3-*S*-cysteinyl-5-methylcatechol (**2b**)[27] were prepared as previously described.

Oxidation of 2b by Peroxidase/H_2O_2. To a solution of **2b** (1.0 g, 4.12 mmol) and horseradish peroxidase (4400 pyrogallol units) in 0.1 M phosphate buffer, pH 7.0 (1.5 L) was added 1.5% H_2O_2 (8.4 mL) under vigorous stirring. After 20 s, the reaction mixture was reduced by addition of $NaBH_4$ (0.5 g), the pH of the solution rising to ca. 8. The mixture was extracted with AcOEt (4 × 300 mL), and the combined organic layers were washed with brine, dried over anhyd Na_2SO_4, and taken to dryness to give a brown oil (600 mg). The aqueous layer was acidified to pH 3.0 with 2 M HCl and extracted with AcOEt to give, after removal of the solvent, a residue (350 mg). On HPLC (mobile phase: 30−100% acetonitrile gradient in 0.05 M citrate buffer pH 4.0, in 20 min; flow rate: 1 mL/min) and TLC (eluants: $CHCl_3$/Et_2O 6:4 and $CHCl_3$/MeOH/AcOH 8:2:1 for the neutral and acidic extracts, respectively) analysis, the acidic fractions were found to contain a predominant product, identified as **10b** by comparison of the chromatographic and spectral properties with those of an authentic sample,[11a] whereas the neutral phase consisted, in addition to significant amounts of polymeric material, of three major components. Preparative HPLC fractionation (mobile phase: 0.05 M citrate buffer, pH 4.0/CH_3CN 55:45; flow rate: 8 mL/min) followed by AcOEt extraction of the eluates corresponding to the bands at t_R 7, 15, and 28 min afforded **14b** (200 mg, 27% yield), **15** (50 mg, 7% yield), and **16** (80 mg, 11% yield), respectively.

5-Hydroxy-7-methyl-3,4-dihydro-2*H*-1,4-benzothiazine (14b): mp 160−162 °C (white needles from ethanol);[14] UV λ_{max} (EtOH) 307 (logϵ 3.46); ^{1}H NMR (acetone-d_6) δ 2.06 (bs, 3H), 3.00 (m, 2H) 3.57 (m, 2H), 4.60 (bs, 1H), 6.24 (dq, *J* = 1.4, 0.7 Hz, 1H), 6.32 (d, *J* = 1.4 Hz, 1H), 8.20 (bs, 1H); ^{13}C NMR (acetone-d_6) δ 20.54 (q), 26.96 (t), 42.55 (t), 112.28 (d), 116.32 (s), 119.40 (d), 126.36 (s), 129.82 (s), 144.50 (s); EIMS (*m*/*z*, relative intensity) 181 (M^+, 100), 166 (70); exact mass calcd for $C_9H_{11}NOS$ 181.0561, found 181.0566.

5,5′-Dihydroxy-7,7′-dimethyl-3,3′,4,4′-tetrahydro-6,6′-bi-2*H*-1,4-benzothiazine (15): glassy oil >95% pure by HPLC and TLC analysis; UV λ_{max} (EtOH) 316 , 295 (sh) , 233 nm, λ_{max} (EtOH + HCl) 295, 287 (s), 261 nm; ^{1}H NMR (acetone-d_6) δ 1.76 (d, 3H × 2, *J* = 0.7 Hz), 3.06 (m, 2H × 2) 3.62 (m, 2H × 2), 6.36 (q, 1H × 2, *J* = 0.7 Hz); 7.01 (br s, 1H × 2); ^{13}C NMR (acetone-d_6) δ 19.13 (q), 26.91 (t), 42.40 (t), 115.67 (s), 118.16 (s), 120.23 (d), 126.17 (s), 130.21 (s), 142.44 (s); EIMS (*m*/*z*, relative intensity) 360 (M^+, 54), 358 (24), 181 (100), 180 (91); exact mass calcd for $C_{18}H_{20}N_2O_2S_2$ 360.0966, found 360.0968.

5,5′-Dihydroxy-7,7′-dimethyl-3,3′,4,4′-tetrahydro-6,8′-bi-2*H*-1,4-benzothiazine (16): glassy oil >95% pure by HPLC and TLC analysis; UV λ_{max} (EtOH) 310 , 238 nm, λ_{max} (EtOH + HCl) 295, 287 (shoulder, sh), 256 nm; ^{1}H NMR (acetone-d_6) δ 1.67, 1.68 (br s, 6H), 2.83 (m, 2H), 3.00 (m, 2H), 3.39 (m, 2H), 3.48 (m, 2H), 4.88 (br s, 2H), 6.29 (q, 1H, *J* = 0.5 Hz), 6.38 (s, 1H), 7.29 (br s, 1H), 9.15 (br s, 1H); ^{13}C NMR (acetone-d_6) δ 18.98, 19.17 (q), 26.92, 27.18 (t), 41.80, 42.57 (t), 112.86 (d), 115.02 (s), 118.48 (s), 119.76 (d), 122.03 (s), 124.46 (s), 125.70 (s), 126.13 (s), 129.83 (s), 130.68 (s), 141.65 (s), 143.92 (s); EIMS (*m*/*z*, relative intensity) 360 (M^+, 100), 358 (28), 181 (21), 180 (31); exact mass calcd for $C_{18}H_{20}N_2O_2S_2$ 360.0966, found 360.0962.

Oxidation of 2a by Peroxidase/H_2O_2. A solution of **2a** (2.0 g, 6.32 mmol) in 0.1 M phosphate buffer, pH 7.0 (2 L), containing horseradish peroxidase (15 000 pyrogallol units), was treated with 1.5% H_2O_2 (20.4 mL) under vigorous stirring. After 10 min, $NaBH_4$ (0.75 g) was added and, after an additional 3 min, the mixture, acidified to pH 2 with 3 M HCl, was applied on a Dowex 50W-X2 (H^+ form, 200−400 mesh) column (60 × 2 cm). After washing with water (1.0 L), the elution was performed with a 0.5 to 4 M HCl gradient and monitored by HPLC analysis (mobile phase: 0.05 M citrate buffer, pH 4.0/MeOH 8:2, eluant A, or 0.05 M Et_3N/CH_3CN 90:10, adjusted to pH 3 with concentrated phosphoric acid, eluant B; flow rate: 1 mL/min) and UV/vis spectrophotometry. Fractions from 2 M HCl (500 mg), consisting mainly of the product responsible for HPLC peak E, were purified by preparative HPLC (eluant: 0.05 M Et_3N/CH_3CN 93:7, adjusted to pH 3 with concentrated formic acid; flow rate: 8 mL/min) to afford, after extensive drying under reduced pressure, dimer **17** (160 mg, 10% yield) as a white amorphous powder, homogeneous to HPLC (eluant A and B). Preparative HPLC purification (eluant: 0.05 M citrate buffer, pH 4.0/methanol, 85:15; flow rate 8 mL/min) of fractions from 3 M HCl, containing the products eluted under HPLC peaks C and D, gave two major bands at t_R 25 and 30 min in the above eluant which, after desalting by ion exchange chromatography (Dowex 50W-X2, H^+ form, 20 × 1 cm column, eluant HCl gradient), yielded trimers **18** (t_R 30′, 40 mg, 2% yield) and **19** (t_R 25′, 20 mg, 1% yield), respectively, as glassy oils homogeneous to HPLC (eluant A and B).

7-(2-Amino-2-carboxyethyl)-8-[[7-(2-amino-2-carboxyethyl)-3,4-dihydro-2*H*-1,4-benzothiazinyl]-5-oxy]-5-hydroxy-3,4-dihydro-2*H*-1,4-benzothiazine (17): UV λ_{max} (0.1 M HCl) 295, 288 (sh) nm (log ϵ 3.75, 3.72); ^{1}H NMR (1 M DCl, 298 K) δ 2.89−2.95 (m, 7.6 H), 3.13 (m, 3.8 H), 3.29 (m, 3.8 H), 3.64 (m, 3.8 H), 3.75 (m, 3.8 H), 4.10 (m, 2.8 H), 4.21 (t, 1H, *J* = 6.8Hz), 6.11 (d, 1.9 H, *J* = 1.7 Hz), 6.73 (s, 1H), 6.78 (s, 0.9 H), 6.82 (d, 0.9 H, *J* = 1.7 Hz), 6.83 (d, 1 H, *J* = 1.7 Hz); ^{1}H NMR (1M DCl, 343 K), δ: 2.88 (m, 4H), 3.04 (m, 2H), 3.12 (m, 2H), 3.54 (m, 2H), 3.60 (m, 2H), 3.99 (t, 1H, *J* = 6.5 Hz), 4.04 (m, 1H), 5.96 (d, 1H, *J* = 1.6 Hz), 6.65 (s, 1H), 6.66 (d, 1H, *J* = 1.6 Hz); ^{13}C NMR (1 M, 298 K) δ 23.14 (t), 24.01 (t), 24.13 (t), 31.23 (t), 31.46 (t), 35.73 (t), 42.24 (t), 43.10 (t), 53.91 (d), 54.05 (d), 54.30 (d), 110.65 (d), 110.75 (d), 114.23 (d), 114.45 (d), 117.60 (s), 117.77 (s), 118.10 (s), 118.74 (s), 123.68 (d), 125.18 (s) 129.74 (s), 130.11 (s), 130.22 (s), 130.58 (s), 135.45 (s), 135.94 (s), 140.07 (s), 140.24 (s), 149.60 (s), 149.68 (s), 150.09 (s), 150.48 (s) 171.45 (s), 171.49 (s), 171.88 (s); ^{13}C NMR (1 M DCl, 343 K) δ 23.46 (t), 24.65 (t), 31.44 (t), 35.77 (t), 42.57 (t), 43.22 (t), 53.98 (d), 54.44 (d), 110.84 (d), 114.57 (d), 119.52 (s) , 119.62 (s), 123.58 (d), 123.84 (s), 127.89 (s), 128.45 (s), 133.40 (s), 140.92 (s), 148.50 (s), 149.30 (s), 171.28 (s), 171.56 (s); MALDI MS *m*/*z* 507 (M + H)$^+$, 529 (M + Na)$^+$, 545 (M + K)$^+$.

6′-[[7-(2-Amino-2-carboxyethyl)-3,4-dihydro-2*H*-1,4-benzothiazinyl]-5-oxy]-7,7′-bis(2-amino-2-carboxyethyl)-5,5′-dihydroxy-3,3′,4,4′-tetrahydro-6,8′-bi-2*H*-1,4-benzothiazine (18): UV λ_{max} (0.1 M HCl) 295, 258 (sh) nm; ^{1}H NMR (1 M DCl) δ 2.24 and 2.51 (dd, 1H, *J* = 6.8, 13.6 Hz and dd, 1H, *J* = 10.3, 13.6 Hz), 2.62 and 2.80 (dd, 1H, *J* = 9.0, 14.1 Hz and dd, 1H, *J* = 7.5, 14.1 Hz), 2.85−3.00 (m, 2H), 3.05 (m,

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6H), 3.34 (dd, 1H, J = 6.8, 10.3 Hz), 3.42 (t, 1H, J = 8.5 Hz), 3.50 (m, 6H), 3.70 (t, 1H, J = 5.4 Hz), 5.99 (d, 1H, J = 1.6 Hz), 6.52 (d, 1H, J = 1.6 Hz), 6.63 (s, 1H); ^{13}C NMR (1 M DCl) δ 25.45 (t), 25.47 (t), 26.09 (t), 30.12 (t), 35.63 (t), 35.96 (t), 41.62 (t), 42.25 (t), 42.37 (t), 54.91(d), 55.29 (d), 55.94 (d), 110.02 (d), 114.90 (s), 119.15 (s), 119.66 (s), 119.90 (s), 121.88 (s), 121.97 (d), 123.14 (d), 124.15 (s), 126.34 (s), 127.94 (s), 128.41 (s), 130.72 (s), 132.72 (s), 141.11 (s), 143.37 (s), 146.01 (s), 146.71 (s), 173.91 (s), 174.16 (s), 174.41 (s); MALDI MS m/z 759 $(M + H)^+$, 781 $(M + Na)^+$, 797 $(M + K)^+$.

8′-[[7-(2-Amino-2-carboxyethyl)-3,4-dihydro-2*H*-1,4-benzothiazinyl]-5-oxy]-7,7′-bis(2-amino-2-carboxyethyl)-5,5′-dihydroxy-3,3′,4,4′-tetrahydro-6,6′-bi-2*H*-1,4-benzothiazine (19): UV λ_{max} (0.1 M HCl) 295, 258 (sh) nm; ^{1}H NMR (1 M DCl) δ 2.54 and 2.72 (dd, 1H, J = 4.7, 14.7 Hz and dd, 1H, J = 9.8, 14.7 Hz), 2.68 and 2.87 (dd, 1H, J = 14.4, 11.7 and m, 1H), 2.92 (m, 2H), 3.20−3.42 (m, 6H), 3.73 (m, 1H), 3.74 (m, 1H), 3.61−3.82 (m, 6H), 3.96 (t, 1H, J = 6.9 Hz), 6.06 (d, 1H, J = 1.6 Hz) 6.88 (d, 1H, J = 1.6 Hz), 6.90 (s, 1H); ^{13}C NMR (1 M DCl) δ 23.92 (t), 24.55 (t), 24.76 (t), 29.48 (t), 35.44 (t), 36.08 (t), 41.77 (t), 42.76 (t), 42.77 (t), 54.03 (d), 54.28 (d), 55.19 (d), 110.48 (d), 112.53 (s), 118.20 (s), 118.62 (s), 119.02 (s), 119.19 (s), 121.79 (s), 122.64 (d), 123.46 (d), 127.09 (s), 129.22 (s), 131.02 (s), 132.86 (s), 135.77 (s), 142.60 (s), 144.07 (s), 148.80 (s), 150.40 (s), 171.80 (s), 171.90 (s), 172.00 (s); MALDI MS m/z 759 $(M + H)^+$, 781 $(M + Na)^+$, 797 $(M + K)^+$.

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Supporting Information Available: ^{13}C spectra at 298 and 343 K, direct and long-range ^{13}C−^{1}H correlation spectra of compound **17**, and COSY and ^{13}C−^{1}H correlation spectra of compounds **18** and **19** (24 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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