Zinc-Catalyzed Oxidation of 5-*S*-Cysteinyldopa to 2,2′-Bi(2*H*-1,4-benzothiazine): Tracking the Biosynthetic Pathway of Trichochromes, the Characteristic Pigments of Red Hair

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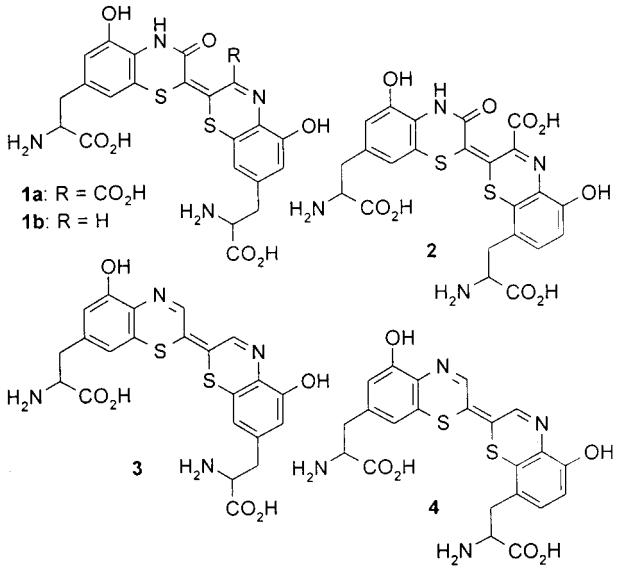
Trichochromes, the peculiar pigments of red human hair, featuring the $\Delta^{2,2'}$-bi(2*H*-1,4-benzothiazine) skeleton, are known to arise from cysteinyldopas, mainly the 5-*S*-isomer (**5**). However, the mode of formation and the direct precursors have remained largely undefined. To fill this gap, we investigated the oxidation of **5** in air or with chemical and enzymatic agents under biomimetic conditions. In the presence of zinc ions, which occur in epidermal tissues at significant concentrations, the reaction course is diverted toward the formation of a labile 3-carboxy-2*H*-1,4-benzothiazine intermediate (**11**), which was identified by direct NMR analysis. Structural formulation was supported by characterization of the analogous compound **13** isolated from oxidation of the model 5-methyl-3-*S*-cysteinylcatechol (**12**) after methylation. In the further stages of the oxidation, diastereomeric 2,2′-bi(2*H*-1,4-benzothiazine) **15** and **14** were obtained from **5** and **12**, respectively, the reaction proceeding at a higher rate and to a greater extent in the presence of acids. The dimers were shown to readily convert to each other in the presence of acids. In the case of the methylated dimers **14**, a 2,2′-bi(4*H*-1,4-benzothiazine) intermediate (**16**) was isolated and characterized. In acidic media, trichochrome C (**1a**), the most abundant in red human hair, was smoothly formed from aerial oxidation of **15**, and under similar conditions, trichochrome-related products (**17** and **18**) were obtained from **14** prior to or after methylation. The presence of **1a** and precursors **5** and **15** was investigated by HPLC analysis of red hair samples following mild proteolytic digestion. On the basis of these data, a likely biosynthetic route to trichochrome pigments of red human hair is depicted.

Introduction

The distinctive pigments of carrot-red human hair have attracted continuous research interest following demonstration of their ease of photodegradation with the production of highly reactive, mutagenic species.[1] Such photodynamic properties pointed to a major contributing role of these pigments in the abnormal susceptibility of red-haired, fair-skinned, Celtic-type Caucasians to actinic damage and skin cancer.[2]

The biochemical uniqueness of these pigments was first recognized as early as 1878 by Sorby who extracted from red human hair a pigment with a pH-dependent chromophore, later termed trichosiderin, as it was believed to contain iron.[3] Elucidation of the structure and biogenesis followed from the extensive studies carried out in Naples on similar pigments isolated from red feathers of domestic fowls.[4] Alkali extraction of the tissues afforded, besides polymeric red-brown pigments, pheomelanins,[5] a group of low-molecular weight pigments featuring the peculiar chromophoric properties that were formulated as the $\Delta^{2,2'}$-bibenzothiazines **1**−**4** and



named trichochromes.[4,6] The common biogenetic origin from dopa-cysteine conjugates, cysteinyldopas, was well

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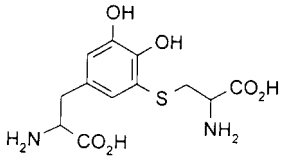
apparent from the structures, although the mode of formation was not defined.[4]

In vitro oxidation of isomeric cysteinyldopas, particularly the major biosynthetic precursor 5-*S*-cysteinyldopa (**5**), under a variety of biomimetic conditions, including



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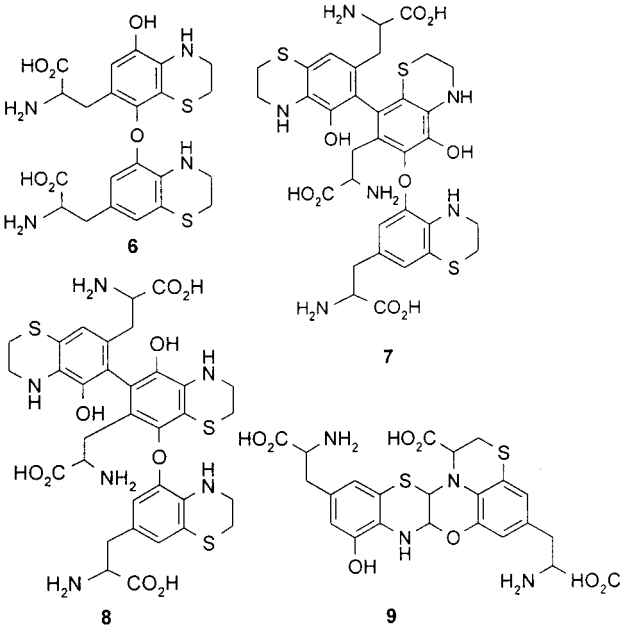
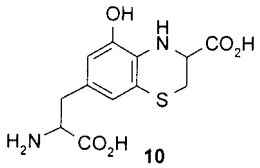
enzymatic systems, chemical agents, and aerial oxidation, led to polymeric materials resembling natural pheomelanins.[7] Oligomers of the type **6**−**8**, all characterized by C−C and C−O bonds between the benzothiazine units, were isolated in the early stages of the peroxidase/H_2O_2 oxidation of **5** after reductive treatment.[8] An alternate reaction path occurs in the tyrosinase-catalyzed oxidation involving an unusual cycloaddition process leading to oligomer intermediates such as **9**.[9] However, in neither



6 **7** **8** **9**

case have transient benzothiazine intermediates been shown to undergo symmetric coupling at the 2-position to give trichochrome-like structures. Moreover, in contrast to trichochromes B (**2**) and C (**1a**), which are more abundant in natural sources,[4] the benzothiazine units of dimers **6** and **9** are prevalently noncarboxylated at the 3-position. This is consistent with the observed tendency of the intermediate dihydrobenzothiazine *o*-quinonimine, derived from oxidative cyclization of **5**, to rearrange with decarboxylation at physiological pHs and temperatures.[10]

On the other hand, the formation of the 3-carboxy-3,4-dihydro-1,4-benzothiazine **10** was shown to prevail upon



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oxidation of **5** in air or at low oxidant:substrate molar ratios.[10b] No evidence, however, was ever presented for a biomimetic route to 3-carboxylated 1,4-benzothiazine intermediates, which might provide a clue to the formation of trichochromes.

On the basis of previous studies showing the ability of metal ions to affect in vitro melanogenesis,[11,3b] we have investigated in the present work the course of the oxidation of **5** in the presence of metal ions that occur at significant concentrations in epidermal tissues.[12] In the presence of zinc ions, the oxidation course was diverted since it proceeded through the intermediacy of a 1,4-benzothiazine-3-carboxylic acid. This labile intermediate was readily converted to a 2,2′-bibenzothiazine and then to trichochrome C upon standing in air under acidic conditions. On this basis, a possible origin of trichochrome-type pigments in red hair was formulated and assessed by direct analysis of red human hair samples.

Results and Discussion

Zinc-Catalyzed Oxidation of 5 and a Related Thioalkylcatechol. Addition of zinc ions to a buffered solution of **5** (1 mM) up to a 1:1 molar ratio resulted in a progressive batho- and hyperchromic shift of the original chromophore to 310 nm suggesting the formation of a complex.[11b] Aerial oxidation of the **5**−zinc complex was marked by the smooth generation of a new intense chromophore at 390 nm. HPLC inspection of the reaction mixture showed the disappearance of the starting catechol with concomitant formation of a species more retained under reverse-phase elutographic conditions (t_R = 7 min, eluant A) which could not be identified as any of the known intermediates of the oxidation of **5**. Halving of the concentration of **5** is observed in about 4 h, while in the absence of zinc ions, the consumption of the starting catechol is much slower, not exceeding 10% after 20 h, and leads to chromatographically undefined species. An even more rapid development of the 390 nm chromophore from the **5**−zinc complex was obtained using chemical oxidants such as persulfate, ferricyanide, or the peroxidase/H_2O_2 system. The 390 nm species proved to be rather unstable, and all attempts of isolation met with failure. Upon reduction with sodium borohydride, this species was rapidly converted to a colorless product identified as the dihydrobenzothiazine acid **10**. Acidification of the oxidation mixture caused the rapid disapperance of the t_R = 7 min peak with a shift of the chromophore to around 340 nm. The same

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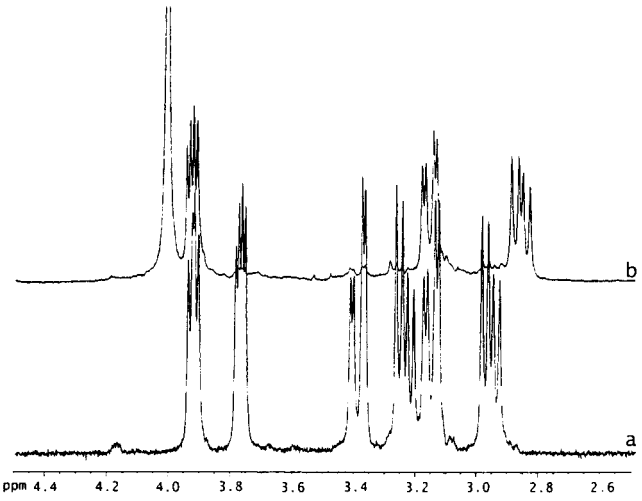
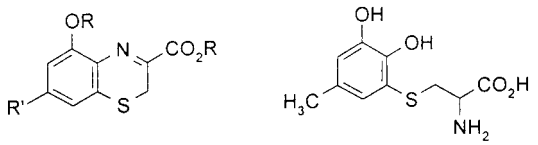


Figure 1. ^{1}H NMR spectrum of a mixture of **5** and zinc sulfate in a 1:1 molar ratio in 0.05 M phosphate buffer (pH 7.4) prior to (plot a) and 6 min after (plot b) the addition of 2 molar equiv of sodium persulfate.

shift was produced by the addition of EDTA suggesting that the reaction products also formed complexes with zinc ions.

To gain information on the nature of the labile intermediate, the oxidation of a 1:1 **5**−zinc complex was carried out in phosphate buffer in D_2O at neutral pH. The reaction was monitored by ^{1}H NMR at 400 MHz. Upon addition of persulfate as the oxidant, the resonance of the complex progressively diminished and was replaced by a new set of signals attributable to a single species (Figure 1). In the sp^3 region, the resonances of the alanyl chain persisted while those of the original cysteinyl residue were replaced by a 2H singlet at δ 4.01 typical of H-2 protons of 2*H*-1,4-benzothiazine systems,[13,14] which showed a direct CH bond correlation with a signal at δ 23.2. Coupled with consideration of the reactivity, spectral analysis led to the assignment of the 3-carboxy-1,4-benzothiazine structure **11** as the intermediate.

Support for this proposal was provided by oxidation of the model thioalkylcatechol **12**. Ethyl acetate extraction of the oxidation mixture of **12** followed by treatment with diazomethane led to a major product that, after careful purification, was characterized as the 3-methoxycarbonyl-5-methoxy-7-methyl-2*H*-1,4-benzothiazine (**13**), analogous to **11** obtained from **5**, on the basis of the EI-MS and 1D/2D NMR analyses.



11: R = H, R' = $CH_2CH(NH_2)CO_2H$

13: R = R' = CH_3

12

Formation of 2,2′-Bibenzothiazines. Acidification of the oxidation mixture of the **5**−zinc complex when the development of the 390 nm chromophore reached the maximum resulted in a rapid decay of the peak corresponding to the benzothiazine acid **11** and generation of two main components (t_R = 9 and 11 min, eluant B). These were formed at a lower rate at neutral pH, significant yields (around 5%) being obtained only after 120 h of reaction time. The products were rapidly degraded to a complex mixture in alkaline media but proved to be stable under acidic conditions on the time scale of a few hours. The UV spectra of the HPLC fractions corresponding to the two products were virtually identical with an intense chromophore at 355 nm at pH 4.0 shifting to 398 nm upon addition of zinc ions and to 305 nm upon reduction with $NaBH_4$. Raising the pH of the mixture to **5** led to the separation of a red-brown precipitate, which was shown to consist mainly of the more retained product, while that at lower t_R was isolated by preparative HPLC. Unfortunately, the products exhibited a pronounced tendency to be converted, even under carefully controlled conditions, to a ca. 1:1 mixture, and all attempts to obtain them in pure form met with failure. NMR analysis of the products as a mixture was hampered by their high degree of insolubility and rapid decomposition under conditions such as in DMSO or concentrated DCl where an appreciable solubilization occurred. MALDI mass spectral analysis showed clusters of peaks in the 500−590 Da region, the more prominent ones being at 546 and 501 Da, corresponding to dimers of **11** decarboxylated at one or both the benzothiazine units, and an intense fragmentation peak at 297 Da.

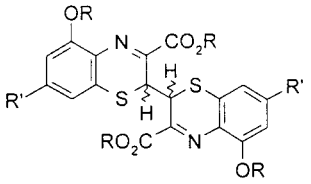
To gain deeper insight into the oxidation products of the 3-carboxy-1,4-benzothiazine system, the reaction was carried out using **12**, which, lacking the alanyl side chain, should lead to products more amenable to structural characterization. The reaction course proved to be very similar to that observed in the case of **5**, and two products (t_R = 14 and 17 min, eluant D), featuring UV absorption at 352 nm, were obtained by acidification of the oxidation mixture at the maximum formation of the 1,4-benzothiazine-3-carboxylic acid intermediate. These were isolated in pure form after methylation with diazomethane of the ethyl acetate-extractable fraction and PTLC purification. The MALDI mass spectrum of each compound showed a pseudomolecular ion peak at *m*/*z* 501 consistent with dimers of **13**. The EI mass spectrum showed, in addition to a weak molecular ion peak, a base peak at *m*/*z* 251 attributable to a fission of the dimer. The ^{1}H NMR spectra of the compounds were similar and provided evidence for a symmetric structure exhibiting as the most salient features two 1H × 2 broad singlets at δ 6.67/6.60 and 6.65/6.58 and a 1H × 2 singlet at δ 4.26/4.27. ^{1}H−^{13}C HETCOR and HMBC connectivity experiments allowed assignment of the signals at δ 6.65/6.58 (110.93/110.90) and 6.67/6.60 (120.66/120.25) to the 6- and 8- positions of the benzothiazine ring, respectively.[8] The H-2 proton signal of both compounds showed one bond correlation with a carbon resonance at δ 30.46/30.42 and cross-peaks in the HMBC experiments with carbon resonances at δ 123.88/123.10 (C-9), δ 144.17/143.86 (C-5), and notably δ 164.88/165.00 due to the carbomethoxy group. The signal at δ 156.95/157.01 was ascribed to the C-3 carbon on the basis of HMBC cross-peaks with the H-6 proton and the −OCH_3 protons at the 5-position.

Taken together, these data led to the formulation of the products obtained from **12** as the diasteromeric

(13) Prota, G.; Ponsiglione, E. *Tetrahedron Lett.* **1972**, *14*, 1327−1330.

(14) Sica, D.; Santacroce, C.; Prota, G. *J. Heterocycl. Chem.* **1970**, 1143−1147.

dimers corresponding to the general structure **14**. On the basis of the close analogy of the reaction course and the spectral properties, the compounds obtained from **5** can be tentatively assigned the analogous structure **15**.

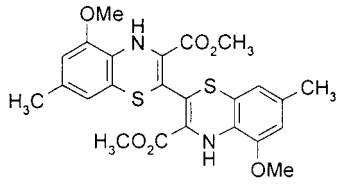


14: R = R' = CH_3
15: R = H, R' = $CH_2CH(NH_2)CO_2H$

In an attempt to assign the relative stereochemistry of the 2-positions in structures **14**, we ran the ^{1}H NMR spectrum of each compound in the presence of a chiral shift reagent. Addition of variable amounts of the reagent to a solution of either diastereomer, even at a 1:10 molar ratio, caused the rapid decay of the signals in the sp^2 region, which were replaced by two 1H signals at δ 6.34 and 6.43 and a broad D_2O/DCl-exchangeable signal at δ 6.84. On the other hand, the resonance at δ 4.2 for the H-2 proton disappeared and those due to the OCH_3 and CH_3 groups were shifted upfield. These changes were accompanied by a bathochromic shift of the absorption of the solution from 352 nm to 400 and 489 (sh) nm. HPLC analysis of the solution confirmed the disappearance of the peaks corresponding to the methylated dimers **14** with formation of a major component more retained under the elution conditions adopted ($t_R = 35'$ min, eluant E). EI mass spectral analysis of the isolated product showed a weak molecular ion peak at 500 Da. Analysis of the carbon resonances provided evidence for a symmetrical benzothiazine dimer lacking the C-2 and C-3 resonances of dimers **14**. The δ 6.83 proton signal did not show one bond correlation, whereas in the HMBC experiments, cross-peaks with carbons at δ 119.31 (C-8), δ 146.72 (C-5), δ 162.37 ($COOCH_3$), and notably with a low-field resonance at δ 115.48 were very apparent. Assignment of the carbon resonances of the benzene moiety on the basis of the connectivities of the H-6 and H-8 protons marked a profound modification in the nature of the nitrogen atom. Taken together, these observations led to the formulation of the $t_R = 35'$ min species as the 2,2′-bi(4*H*-1,4-benzothiazine) **16**. Following treatment with aqueous HCl, each of the dimers **14** in chloroform solution was converted to **16**. After the solution stood in the presence of acids, a mixture was eventually obtained comprising, in addition to **16** as the major component, the two diastereomers in a 1:1 ratio. The observed interchange can be straightforwardly interpreted in terms of an equilibrium between the two 2*H*-1,4-benzothiazine diastereomers through a single 4*H*-1,4-benzothiazine species, promoted by the shift reagent used and the presence of acids.[15]

Oxidation of 2,2′-Bibenzothiazines. To assess whether the 2,2′-bibenzothiazine dimers were converted to trichochrome-related structures and to determine the conditions required for such a conversion, a variety of oxidation conditions were explored in a series of further experiments. Upon exposure to chemical oxidants such as potassium ferricyanide, ammonium persulfate, sodium periodate, peroxidase/H_2O_2, and the Fenton system, the diastereomeric dimers from **5** gave rise to poorly defined patterns of products but showed a significant stability when kept in air at neutral pH over 2−3 days. A more defined reaction path was observed in aqueous solutions at acidic pH at room temperature in the presence of air under which conditions the dimers were converted extensively to a new, more retained species after 72 h (HPLC evidence). Notably, the kinetics and the extent of conversion were higher for the $t_R = 9'$ min (eluant B) dimer than for the more retained one. The UV features of the new compound and the shift of the chromophore in alkaline and acid media were strongly suggestive of trichochrome C.[17] The nature of the product, isolated by preparative HPLC, was substantiated by MALDI-MS analysis that showed a pseudomolecular ion peak at 561 Da and an intense peak at 517 Da due to a decarboxylated species generated under the conditions of the analysis, consistent with the reported ease with which trichochrome C undergoes decarboxylation.[4,17] The decarboxylated species was also obtained in high yields by heating tricochrome C in mineral acids and was identified as **1b** by the typical pH-dependent chromophore.[4,17]

Prolonged aerial oxidation of either of the diastereomeric dimers from the model catechol **12** prior to methylation in a 1:1 methanol/2 M HCl solution gave substantial amounts of a red compound featuring a pH-dependent chromophore virtually identical to that of tricochrome C. The product was also obtained by oxidation of the dimers with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in acetonitrile, persulfate in aqueous acetic acid, or periodate at pH 6 and was eventually isolated after extensive methylation with diazomethane. Also in this case, one of the diastereomers was preferentially converted to the product. Analysis of the features of the ^{1}H NMR and ^{13}C NMR spectra in comparison with those of the starting dimers suggested an asymmetric dehydro 2,2′-dimer consisting of a 3-methoxycarbonyl-1,4-benzothiazine and a 3-methoxy-1,4-benzothiazine unit. Assignment of the trichochrome-related structure **17** to the



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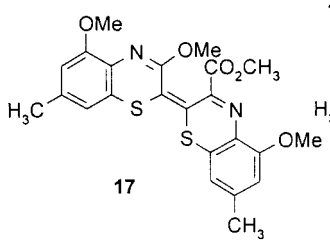

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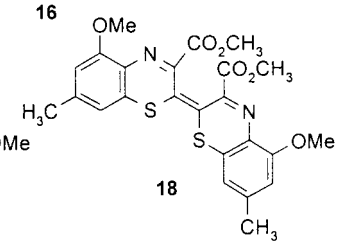

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compound was supported by the ^{1}H−^{13}C HETCOR and HMBC spectra and primarily by the EI-MS spectrum showing an intense molecular ion peak at *m*/*z* 470 and a series of fragmentation peaks, including, notably, those

(15) Hickmott, P. W. In *The Chemistry of Enamines. Part 1*; Patai, S., Rappoport, Z., Eds.; Wiley & Sons: New York, 1994; pp 731−735.

(16) Goldstein, S.; Meyerstein, D.; Czapski, G. *Free Radical Biol. Med.* **1993**, *15*, 435−445.

(17) Nicolaus, R. A.; Prota, G.; Santacroce, C.; Scherillo, G.; Sica, D. *Gazz. Chim. Ital.* **1969**, *99*, 323−350.

at *m/z* 250 and 222 corresponding to the two benzothiazine moieties. In this connection, it is interesting to note that formation of the 3-methoxy-2*H*-1,4-benzothiazine system apparently resulting from methylation of the tautomer of the benzothiazin-3-one unit, as in trichochrome C, is closely reminiscent of that obtained from 5-hydroxy-7-methyl-8-(2-hydroxy-4-methylphenoxy)-3,4-dihydro-2*H*-1,4-benzothiazine-3-one upon treatment with diazomethane.[18] Treatment of either methylated dimer **14** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in dioxane at 109 °C led to the formation of two red species, which were purified by PTLC fractionation. One of these products ($R_f = 0.5$) was also obtained by aerial oxidation of the 3-methoxycarbonyl-1,4-benzothiazine **13** in acidic methanol. The product exhibited structural features remarkably similar to those of the starting dimers except for the lack of the H-2 proton and carbon resonances, which were apparently replaced by a quaternary sp^2 carbon at δ 128.52. An intriguing feature of the proton and carbon spectra, taken at 298 K, was the presence of another closely related series of signals at a 0.5:1 ratio with respect to the main series suggesting a minor isomeric component. The ratio of the areas of the proton signals changed when the sample was allowed to remain in solution, reaching a 0.3:1 value, which was not altered when the spectrum was run at higher temperatures of up to 330 K. A possible interpretation would envisage *cis*−*trans* isomerism in line with the results of X-ray analysis of $\Delta^{2,2'}$-bibenzothiazine systems bearing large substituents at the 3-position. The latter were shown to exist largely in the *cis* configuration (red) with respect to the *trans* one (yellow), each of the isomers being readily converted to a ca. 0.5:1 *trans*:*cis* equilibrium mixture at relatively low temperatures.[19] However, neither HPLC nor TLC analysis of the $R_f = 0.5$ fraction under different conditions allowed us to identify a minor component. $NaBH_4$ reduction readily afforded a single species (TLC evidence) absorbing at 304 nm, closely resembling that obtained from dimers **14**. On the basis of these data, the compound was formulated as the $\Delta^{2,2'}$-bi(3-carboxy-5-hydroxy-7-methyl-1,4-benzothiazine) **18**. The other component of the oxidation mixture ($R_f = 0.9$) was identified as the bi(4*H*-1,4-benzothiazine) **16** obtained from dimers **14** by acid treatment.

Identification of 1,4-Benzothiazine Dimers and Trichochromes in Red Hair. Further experiments were directed at assessing whether any of the benzothiazine intermediates obtained in vitro by zinc-catalyzed oxidation of **5** may represent a precursor of trichochrome pigments in hair. To this aim, a recently developed procedure[20] for pigment isolation from human hair was adopted that involves enzymatic digestion of the keratin matrix to allow the release of the pigment at neutral pH. The soluble pigment fraction obtained from red human hair samples after one digestion treatment, run under an argon atmosphere to avoid oxidation of labile intermediates, was analyzed by HPLC. Although the product pattern was complex, the absence of trichochrome C (**1a**) was evident (Figure 2, profile a). By contrast, **1a** was readily obtained from the hair pigmented fraction following treatment in mineral acids as described[17] or after prolonged exposure (15 days) to air at neutral pH (Figure 2, profile b). The nature of the species was confirmed by treatment of the eluted peak with hot acids, which rapidly afforded **1b** (UV and HPLC evidence).

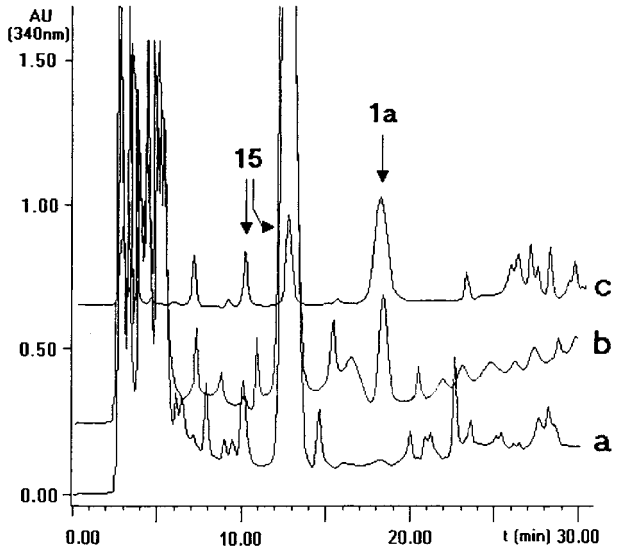

Figure 2. HPLC elution profiles of the pigmented fraction from red hair after enzymatic hydrolysis as obtained (profile a)or after 15 days of exposure to air(profile b). Prolonged oxidation in acids of the fraction eluting between 9 and 15 min is shown in profile c. UV detection was recorded at 340 nm, eluant B (see Experimental Section for details).

Close inspection of the elutographic profile of the soluble pigment fraction obtained with UV detection at 340 nm showed the presence of two peaks at the retention times of dimers **15**, with the more retained one obscured by another more abundant component of the mixture. Given the complexity of the chromatographic profile, neither spiking with authentic dimers nor direct LC-MS analysis provided definitive evidence for the nature of the compounds. We then resolved to collect the fraction eluting in the corresponding time interval and to subject it to prolonged aerial oxidation in acids, after which the formation of **1a** was observed (UV and HPLC evidence, Figure 2, profile c). In looking for the direct precursor of dimers **15**, we also fractionated an aliquot of the enzymatic hydrolysate by the procedure currently used for analysis of **5** in body fluids.[21] A small but significant amount of **5** (50 ± 5 and 39 ± 2 ng/g of hair) was observed in two different hair samples by comparison with external calibration curves.

Trichochrome Route. The oxidation chemistry of **5** and the related catechol **12** under the biomimetic conditions explored in this study provides the basis with which to track a path to the 2,2′-bibenzothiazine pigments of red hair as outlined in Scheme 1.

The presence of zinc ions is critical for the formation of the 3-carboxylated benzothiazines. Indeed, these metal ions are apparently capable of affecting the course of a key step of the oxidation of cysteinyldopas involved in the biosynthesis of pheomelanins and trichochromes, i.e.,

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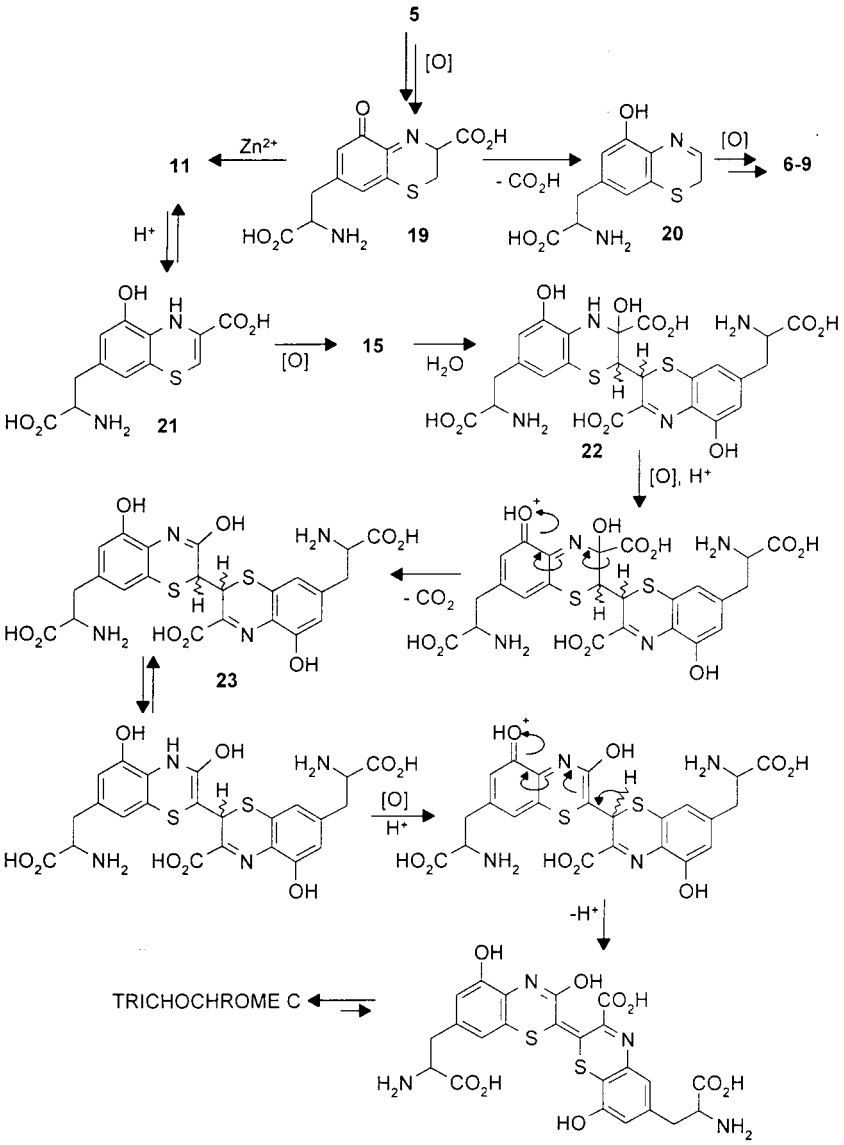
Scheme 1



the rearrangement of *o*-quinonimine intermediates of the type **19** (Scheme 1). Notably, zinc is among the most abundant trace element present in skin and hair.[22] The highest levels are associated with deeply pigmented hair such as red hair, and reach values of about 680 μg/mg of dry weight tissue in melanosomes.[23] On the basis of these data, it could be argued that the levels of zinc ions may represent a regulatory factor in the first stages of the biosynthesis of red hair pigments by determining the ratio of noncarboxylated vs carboxylated benzothiazine intermediates **20** and **11**. This closely parallels the eumelanogenic pathway in which the intervention of metal ions, particularly zinc, diverts the rearrangement of the aminochrome dopachrome toward the formation of 5,6-dihydroxyindole-2-carboxylic acid in place of 5,6-dihydroxyindole prevailing in the absence of metal or enzymatic catalysis.[3b,11b] Of interest in this connection is also the reactivity of thioalkylkcatechol compounds related to **5** and **12**, arising from the addition of cysteine to catecholamine neurotrasmitters. Oxidation of these compounds in aqueous buffer at physiological pH is reported to proceed without decarboxylation to give 3-carboxy-3,4-dihydro-1,4-benzothiazines as the main reaction products.[24]

The role of zinc ions extends further to the stabilization of the benzothiazine acid **11** as shown by the rapid decomposition of the latter upon addition of chelating agents. At variance with the 3-unsubstituted benzothiazine **20** for which the mode of dimerization is markedly affected by the oxidation conditions leading to different oligomers such as **6**−**9**,[8,9] the 3-carboxybenzothiazine **11** gives exclusively 2,2′-linked dimers **15**, which are not susceptible to further polymerization.

Another intriguing issue of the reaction pathway presented in Scheme 1 is the dimerization of **11**. Literature data for the oxidative coupling of 1,4-benzothiazine systems are rather scanty, and no conclusive mechanism

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for the reaction has been offered.[14,19,25] In principle, the formation of **15** from **11**, and similarly of **14** from **12**, under the conditions adopted in the present study may be envisaged as the result of either ionic or radical oxidation processes. In the ionic-type mechanism, tautomerization of **11** to the corresponding 4*H*-benzothiazine **21** is followed by addition of the nucleophilic enamine β-position to the 2-position of an oxidized enamine-*o*-quinonimine counterpart. The alternate radical oxidation pathway is likely to proceed by the symmetric coupling of a benzothiazinyl radical generated by one-electron oxidation of the readily oxidizable 4*H*-benzothiazine tautomer.[26] In such a radical, the spin density at the 2-position would be expectedly high, in line with the results of ESR measurements on a persistent neutral radical from 2,3-dimethoxycarbonyl-4*H*-benzothiazine.[27] Although no direct experimental evidence was obtained to discriminate between the two processes, the formation of the 2,2′-dimers from the *O*-methylated benzothiazine **13**, to which oxidation to the corresponding *o*-quinonimine benzothiazine is precluded, would argue in favor of a radical coupling. Moreover, it could be reasoned that the catalysis to the dimerization offered by strong acids should expectedly enhance the electrophilicity of the oxidized benzothiazine but strongly reduce the nucleophilicity of the enamine. The proposed mechanism would be in accord also with the reported tendency of 3-substituted thiazines and benzothiazines not bearing a hydroxyl group at the 5-position to dimerize at the 2-position.[18,28]

As for the formation of **1a**, a likely origin of the 3-oxo benzothiazine unit would involve addition of the elements of water to the electrophilic site of the 2*H*-benzothiazine unit of **15** followed by decarboxylation. The process is expectedly catalyzed by acids and requires the intermediacy of the oxidized form of the initial adduct **22**. This reaction path is closely reminiscent of the formation of the 3,5-dihydroxy-3,4-dihydro-1,4-benzothiazine from benzothiazine **20** evidenced in a previous study by direct NMR analysis.[29] Tautomerization of the resulting 3-hydroxy-2,2′-bibenzothiazine **23** followed by further oxidation would favor hydrogen abstraction with formation of the double bond in the 2,2′-dimer leading eventually to trichochrome C. In line with the proposed mechanistic route is the requirement of a mild and prolonged oxidation such as that effected by molecular oxygen for the formation of trichochrome C.

The exact sequence of the steps leading to trichochrome-like structures, i.e., dehydrogenation of the 2-position and oxygenation at the 3-position of the 2,2′-dimers, cannot be assessed on the basis of the available data. On the other hand, the formation of **18** from dimers **14**, to which the decarboxylation route is obviously precluded, and, more generally, the formation of 2,2′-dehydrodimers from 3-substituted 1,4-benzothiazine systems under a variety of oxidizing conditions suggest that hydrogen abstraction is a general feature of 2,2′-bibenzothiazines.[19,30] By contrast, the formation of the 3-oxo functionality in trichochrome-like structures is ascribed to the presence of the 5-hydroxy group allowing the generation of an *o*-quinonimine dihydrobenzothiazine and may thus represent the peculiarity of the trichochrome route with respect to the oxidation paths of 2*H*-1,4-benzothiazine systems.

Conclusions

The oxidation reactivity exhibited by the labile 2*H*-1,4-benzothiazine-3-carboxylic acid **11** under the biologically relevant conditions explored in the present study contributes detail to the picture of the chemistry of the 1,4-benzothiazine system highlighting the profound effects of substitution at the 3-position. This is also of interest in relation to the implication of (dihydro) 1,4-benzothiazine-3-carboxylic acid derivatives as putative nigral endotoxins in Parkinson's disease[24,31] and in the mechanism of the renal-specific toxicity of 2-bromo-3-(glutathion-*S*-yl)hydroquinone.[32] In addition, the results of this study have provided new elements to track a route to the 2,2′-bibenzothiazine pigments of red human hair. Although evidence for the occurrence of trichochrome pigments was not obtained by direct HPLC analysis of red hair samples, the presence of the biosynthetic precursor **5** and of the intermediate 2,2′-dimers **15** was clearly demonstrated. The potential of zinc ions as new regulatory factors in the biosynthesis of red hair pigments determining the nature of the benzothiazine intermediates and their subsequent oxidation pathways is outlined.

Experimental Section

General. MALDI mass spectra were taken on a Reflex time-of-flight mass spectrometer operating in positive linear mode.[33] For electron impact (EI-MS) and high-resolution (HR-MS) mass spectra, samples were ionized with a 70 eV beam with the source at 230 °C. ^{1}H and ^{13}C spectra were recorded at 400.1 (100.6) and 270.1 (67.9) MHz, respectively. ^{1}H−^{1}H COSY, ^{1}H−^{13}C HETCOR, and ^{1}H−^{13}C HMBC NMR experiments were carried out at 400 MHz. Analytical and preparative HPLCs were performed with an instrument equipped with a variable-wavelength UV detector and an electrochemical detector with dual, fully porous graphite electrodes. A 4.6 × 250 mm octadecylsilane-coated column (5 μm particle size, rate of 1 mL/min) and a 22 × 250 mm column (10 μm particle size, rate of 18 mL/min) were used for analytical and preparative runs, respectively. The following elution conditions were used: 85:15 v/v 0.05 M sodium citrate (pH 4.0)/methanol (eluant A), 10−60% methanol gradient in 0.05 M sodium citrate (pH 4.0) in 25 min (eluant B), 60:40 v/v methanol/H_2O (eluant C), 30−60% acetonitrile gradient in 0.05 M formic acid in 25 min (eluant D), 60:40 acetonitrile/H_2O (eluant E), and 0.1 M phosphoric acid/0.1 M methanesulfonic acid/0.1 mM EDTA (pH 3.1) (eluant F). Analytical and preparative TLC analyses were performed on F254 0.25 and 0.5 mm silica gel plates using 80:20 v/v chloroform/ethyl ether as the eluant unless otherwise stated. 5-*S*-Cysteinyldopa(3-[(*R*)-2-amino-2-carboxyethylthio]-5-[(*S*)-2-amino-2-carboxyethyl]-1,2-

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dihydroxybenzene) (**5**),[34] 3-[(*R*)-2-amino-2-carboxyethylthio]-1,2-dihydroxy-5-methylbenzene (**12**),[35] and 7-(2-amino-2-carboxyethyl)-3-carboxy-5-hydroxy-3,4-dihydro-2*H*-1,4-benzothiazine (**10**)[36] were prepared as previously reported. Zinc sulfate heptahydrate, ammonium persulfate, sodium periodate, potassium ferricyanide, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, praseodymiumtris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorate], threo-1,4-mercapto-2,3-butanediol, horseradish peroxidase (210 units/mg), and proteinase K (14 units/mg) were used as they were obtained from commercial sources. Red human hairs were collected from healthy volunteers.

Zinc-Catalyzed Oxidation of 5. To a solution of **5** (10 mg, 0.032 mmol) in 0.05 M phosphate buffer (pH 7.4, 32 mL) was added zinc sulfate in variable amounts up to a 1 mM concentration under vigorous stirring at room temperature. Formation of the zinc−catechol complex was monitored spectrophotometrically following the development of the 310 nm chromophore. Ammonium persulfate was then added up to a 1.9 mM concentration. Aliquots of the reaction mixture were periodically withdrawn and analyzed by HPLC (eluant A, λ = 280 nm). In some experiments, the reaction mixture was reduced with sodium borohydride after 20 min of reaction time and acidified to pH 2. The major reaction product obtained under the latter conditions was identified as **10**, and a comparison of the chromatographic properties and UV absorptions with those of an authentic sample was performed.[36] The oxidation of **5** by peroxidase (1.5 pyrogallol units/mL)/H_2O_2 (0.8 mM), by potassium ferricyanide (21 mg, 0.064 mmol), or in air was run under similar conditions.

7-(2-Amino-2-carboxyethyl)-3-carboxy-5-hydroxy-2*H*-1,4-benzothiazine (11). For identification of the title compound, oxidation of **5** (3.0 mg, 9.5 μmol) was repeated in a NMR cuvette in deuterated 0.05 M phosphate buffer (pH 7.4, 1 mL) containing zinc sulfate (3.0 mg, 10 μmol) and sodium persulfate (4.1 mg, 18 μmol), and spectra were recorded at 2 min intervals. ^{1}H NMR (D_2O) of **11**: δ 2.85 (1H, dd, J = 14.4, 8.8 Hz), 3.15 (1H, dd, J = 14.4, 4.4 Hz), 3.92 (1H, dd, J = 8.8, 4.4 Hz), 4.01 (2H, s), 6.42 (1H, bs), 6.43 (1H, bs).

3-Methoxycarbonyl-5-methoxy-7-methyl-2*H*-1,4-benzothiazine (13). To a solution of **12** (54 mg, 0.22 mmol) in 0.05 M phosphate buffer (pH 7.4, 110 mL) containing zinc sulfate (76 mg, 0.26 mmol) was added ammonium persulfate (95 mg, 0.42 mmol) under vigorous stirring. After 20 min, the pH was lowered to 3 with 4 M HCl and the mixture was extracted with AcOEt. The organic layers were dried over sodium sulfate, and the residue was dissolved in chloroform and treated with diazomethane. PTLC fractionation of the mixture thus obtained afforded pure **13** (R_f = 0.89, 15 mg, 27% yield) as a glassy oil, homogeneous to TLC analysis (eluant = 98:2 v/v chloroform/methanol). UV ($CHCl_3$) λ_{max}: 360 nm (log ϵ = 3.89). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H), 3.53 (s, 2H), 3.92 (s, 3H), 3.94 (s, 3H), 6.52 (d, 1H, J = 0.8 Hz), 6.74 (d, 1H, J = 0.8 Hz). ^{13}C NMR ($CDCl_3$): δ 22.24 (CH_3), 22.65 (CH_2), 54.00 (CH_3), 56.76 (CH_3), 110.30 (CH), 120.10 (CH), 121.94 (C), 127.40 (C), 141.47 (C), 145.03 (C), 156.81 (C), 165.09 (C). EI-MS *m*/*z* (relative intensity): 251 (M^+, 80), 236 (M − CH_3, 50), 192 (M − CO_2CH_3, 100). HREI-MS: exact mass calcd for $C_{12}H_{13}NO_3S$, 251.0616; found, 251.0618.

2,2′-Bi[7-(2-amino-2-carboxyethyl)-3-carboxy-5-hydroxy-2*H*-1,4-benzothiazine] (15). The oxidation of **5** (200 mg, 0.63 mmol) was carried out as described above in 0.05 M phosphate buffer (pH 7.4, 316 mL) using ammonium persulfate (273 mg, 1.20 mmol). The reaction course was followed by HPLC (eluant B, λ = 280 and 340 nm). After 20 min of reaction time, the oxidation mixture was acidified to pH 0 with 6 M HCl, and after an additional 20 min interval, corresponding to the optimum formation of the t_R = 9 and 11 min products, the pH of the solution was raised to 5. The precipitate thus formed was collected by centrifugation and was found to consist mainly of the t_R = 11 min compound by HPLC analysis. The t_R = 9 min species was obtained from the mother liquors and purified by repeated preparative HPLC (eluants A and C, λ = 340 nm). Upon standing, both the t_R = 9 and 11 min compounds were converted to a ca. 1:1 mixture of either compound. A suspension of the mixture of the products in 0.05 M phosphate buffer was treated with sodium borohydride and then acidified to pH 3. The resulting colorless solution was analyzed spectrophotometrically. UV λ_{max}: 355 nm (0.1 M HCl), 305 nm ($NaBH_4$, pH 3). MALDI-MS *m*/*z*: 546, 501, 297.

2,2′-Bi[3-methoxycarbonyl-5-methoxy-7-methyl-2*H*-1,4-benzothiazine] (14). The oxidation of **12** (300 mg, 1.23 mmol) was carried out as described above for the isolation of **13**. After 20 min of reaction time, the oxidation mixture was acidified to pH 0, and after an additional 20 min, corresponding to the maximum formation of the t_R = 14 and 17 min species (HPLC evidence, eluant D, λ = 280 and 340 nm), the oxidation mixture was extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate, and the residue taken up in ethyl ether was treated with diazomethane overnight. The mixture thus obtained was fractionated by PTLC to give pure dimers **14** of R_f = 0.72 (25 mg, 8% yield) as a powder and of R_f = 0.29 (18 mg, 6% yield) as a glassy oil, both homogeneous to TLC (eluant = 98:2 chloroform/methanol) and HPLC (eluant E, λ = 340 nm). In other experiments, a solution of either dimer **14** (3 mg, 6 μmol) in $CDCl_3$ (1 mL) was added with praseodymiumtris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorate] in variable amounts up to a 1:1 molar ratio and proton spectra were recorded at 2 min intervals. Separate aliquots of the two compounds (3 mg in 500 μL of $CHCl_3$) were treated with 0.1 M HCl (20 μL) and analyzed by HPLC (eluant E, λ = 340 nm).

R_f = 0.72. UV ($CHCl_3$) λ_{max}: 379, 297 nm (log ϵ = 3.92, 3.86). ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H × 2), 3.85 (s, 3H × 2), 3.94 (s, 3H × 2), 4.26 (s, 1H × 2), 6.65 (bs, 1H × 2), 6.67 (bs, 1H × 2). ^{13}C NMR ($CDCl_3$): δ 22.92 (CH_3), 30.46 (CH), 54.04 (CH_3), 56.86 (CH_3), 110.93 (CH), 120.66 (CH), 123.88 (C), 129.85 (C), 142.21 (C), 144.17 (C), 156.95 (C), 164.88 (C). MALDI-MS: 501 (M + 1)$^+$. EI-MS *m*/*z* (relative intensity): 500 (M^+, 3), 441 (M − CO_2CH_3, 2), 381 (M − 2×CO_2CH_3, 5), 251 (M/2 + H, 100), 190 (M/2 − CO_2CH_3, 100). HREI-MS: exact mass calcd for $C_{24}H_{24}N_2O_6S_2$, 500.1076; found, 500.1079.

R_f = 0.29. UV ($CHCl_3$) λ_{max}: 377, 303 (log ϵ = 3.71, 3.69). ^{1}H NMR ($CDCl_3$): δ 2.32 (s, 3H × 2), 3.86 (s, 3H × 2), 3.97 (s, 3H × 2), 4.27 (s, 1H × 2), 6.58 (bs, 1H × 2), 6.60 (bs, 1H × 2). ^{13}C NMR ($CDCl_3$): δ 23.00 (CH_3), 30.42 (C), 54.05 (CH_3), 56.95 (CH_3), 110.90 (CH), 120.25 (CH), 123.10 (C), 129.85 (C), 141.92 (C), 143.86 (C), 157.01 (C), 165.00 (C). MALDI-MS: 501 (M + 1)$^+$. EI-MS *m*/*z* (relative intensity): 500 (M^+, 2), 485 (M − CH_3, 1), 441 (M − CO_2CH_3, 2), 381 (M − 2×CO_2CH_3, 3), 251 (M/2 + H^+, 100), 190 (M/2 − CO_2CH_3, 100). HREI-MS: exact mass calcd for $C_{24}H_{24}N_2O_6S_2$, 500.1076; found, 500.1073.

2,2′-Bi[3-methoxycarbonyl-5-methoxy-7-methyl-4*H*-1,4-benzothiazine] (16). A solution of either dimer **14** (20 mg, 0.04 mmol) in chloroform (5 mL) was treated with 0.5 M HCl (1 mL), and the biphasic system was taken under vigorous stirring for 1 h. The organic layer was separated and dried over sodium sulfate to give a red glassy oil. Upon TLC and HPLC analyses (eluant E, λ = 340 nm), the reaction mixture was shown to consist of compound **16**. UV ($CHCl_3$) λ_{max}: 400, 489 (sh) nm. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H × 2), 3.71 (s, 3H × 2), 3.82 (s, 3H × 2), 6.34 (bs, 1H × 2), 6.43 (bs, 1H × 2), 6.84 (bs, 1H × 2). ^{13}C NMR ($CDCl_3$): δ 21.82 (CH_3), 53.57 (CH_3), 56.57 (CH_3), 111.29 (CH), 115.48 (C), 118.74 (C), 119.31 (CH), 129.29 (C), 131.12 (C), 134.03 (C), 146.72 (C), 162.37 (C). EI-MS *m*/*z* (relative intensity): 500 (M^+, 8), 251 (M/2 + H, 4). HREI-MS: exact mass calcd for $C_{24}H_{24}N_2O_6S_2$, 500.1076; found, 500.1078.

3-Methoxycarbonyl-3′,5,5′-trimethoxy-7,7′-dimethyl-$\Delta^{2,2'}$bi(2*H*-1,4-benzothiazine) (17). The oxidation mixture of **12** (200 mg, 0.43 mmol) containing the t_R = 14 and 17 min species (HPLC evidence, eluant D, λ = 280 and 340 nm) was extracted as described above with ethyl acetate after 20 min of reaction time. After removal of the volatile components, the residue (50 mg) was taken up in 1:1 methanol/2 M HCl and

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allowed to stand in air under vigorous stirring at room temperature for 72 h. The mixture thus obtained was extracted with ethyl acetate, and the combined organic layers were dried over sodium sulfate. The residue taken up in ethyl ether was treated with diazomethane overnight and then fractionated by PTLC to give **17** (R_f = 0.60, 5 mg, 5% yield), as a glassy oil, homogeneous to TLC (98:2 chloroform/methanol). In other experiments the residue obtained as described above from the oxidation mixture of **12** (10 mg) was treated (i) in acetonitrile (2 mL) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (9.2 mg, 0.04 mmol), (ii) in 8:2 v/v acetic acid/water (5 mL) with ammonium persulfate (18.3 mg, 0.08 mmol), or (iii) in 9:1 v/v phosphate buffer (pH 6, 0.025 M)/acetonitrile (10 mL) with sodium periodate (17.1 mg, 0.08 mmol). The reaction mixture was taken to dryness in the case of i or ii or extracted with ethyl acetate in the case of iii. The residues taken up in ethyl ether were treated with diazomethane overnight. UV ($CHCl_3$) λ_{max}: 299, 373, 455 nm. ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H), 2.35 (s, 3H), 3.89 (s, 6H), 3.90 (s, 3H), 3.94 (s, 3H), 6.59 (s, 2H), 6.74 (s, 1H), 6.75 (s, 1H). ^{13}C NMR: δ 23.06 (CH_3), 24.11 (CH_3), 54.84 (CH_3), 55.38 (CH_3), 57.68 (CH_3), 58.00 (CH_3), 109.23 (CH), 110.39 (CH), 116.36 (CH), 117.28 (CH), 122.89 (C), 127.03 (C), 128.01 (C), 136.90 (C), 139.80 (C), 140.90 (C), 146.36 (C), 151.16 (C), 154.62 (C), 156.55 (C), 164.93 (C). EI-MS *m*/*z* (relative intensity): 470 (M^+, 100), 439 ($M - OCH_3$, 16), 411 ($M - CO_2CH_3$, 43), 250 (14), 235 (40), 222 (10), 191 (30). HREI-MS: exact mass calcd for $C_{23}H_{22}N_2O_5S_2$, 470.0970; found, 470.0972.

$\Delta^{2,2'}$-Bi(3-methoxycarbonyl-5-methoxy-7-methyl-2*H*-1,4-benzothiazine) (18). To a stirred solution of either dimer **14** (30 mg, 0.06 mmol) in anhydrous dioxane (5 mL) at 109 °C was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (12.4 mg, 0.05 mmol), and the reaction mixture was allowed to stand overnight. After removal of the volatile fraction, the mixture was fractionated by PTLC to give two products identified as **16** (R_f = 0.9, 10 mg, 33% yield) and **18** (R_f = 0.5, 17 mg, 57% yield) as red glassy oils. R_f = 0.5. UV ($CHCl_3$) λ_{max}: 310 (sh), 400, 497 (sh) nm. ^{1}H NMR ($CDCl_3$, 298 K): δ 2.33 (s, 0.9H × 2), 2.36 (s, 3H × 2), 3.86 (s, 0.9H × 2), 3.92 (s, 3H × 2), 3.93 (s, 0.9H × 2), 4.01 (s, 3H × 2), 6.57 (bs, 1H × 2), 6.58 (bs, 0.3H × 2), 6.59 (bs, 1H × 2), 6.75 (bs, 0.3H × 2). ^{13}C NMR ($CDCl_3$) (major/minor component): δ 22.81 (CH_3), 54.30/54.17 (CH_3), 56.90/56.99 (CH_3), 110.94/111.14 (CH), 118.25/118.53 (C), 121.84/119.71 (C), 127.54/127.85 (C), 128.52/129.24 (C), 142.64 (C), 144.06/143.50 (C), 157.29/157.61 (C), 164.72/165.01 (C). EI-MS *m*/*z* (relative intensity): 498 (M^+, 9), 439 ($M - CO_2CH_3$, 9), 424 ($M - CO_2CH_3 - CH_3$, 2), 409 ($M - CO_2CH_3 - 2\times CH_3$, 13). HREI-MS: exact mass calcd for $C_{24}H_{22}N_2O_6S_2$, 498.0919; found, 498.0922.

Oxidation of 15. To a solution of **15** (10 mg, 0.02 mmol) in 0.05 M phosphate buffer (pH 7.4, 20 mL) were added (i) ammonium persulfate (7.7 mg, 0.03 mmol), (ii) sodium periodate (7.2 mg, 0.03 mmol), (iii) peroxidase (10 pyrogallol units) and H_2O_2 (0.04 mmol), or (iv) the Fenton reagent (H_2O_2, 0.04 mmol, ammonium iron(II) sulfate/EDTA, 4 μmol). The reaction was monitored by periodical HPLC analysis (eluant B, λ = 280 and 340 nm).

Trichochrome 1a,b. For the generation of trichochrome **1a**, a solution of **15** (20 mg, 0.04 mmol) in 0.1 M HCl (35.5 mL) was kept under vigorous stirring at room temperature for 72 h. The reaction course was followed by periodical HPLC analysis (eluant B, λ = 280 and 340 nm). After 72 h of reaction time, the mixture was fractionated by preparative HPLC (eluants A and C, λ = 280 nm) to give small amounts of **1a** identified by comparison of the chromatographic and spectral properties of the reaction product with those of an authentic sample.[17] MALDI-MS: 561 $(M + 1)^+$, 517 ($M - CO_2$). Product **1b** was obtained by heating a 1 M HCl solution of **1a** at 80 °C as previously described.[17]

Identification of 5, 15, and 1a,b in Red Hair. Samples of red human hair (2 g) were washed with acetone, finely minced, and dissolved in 0.05 M phosphate buffer (pH 7.4, 66 mL) using a glass/glass potter. The resulting mixture was incubated with proteinase K (16 mg) and dithiothreitol (200 mg) in an argon atmosphere under vigorous stirring at 37 °C overnight. The soluble fraction of the pigment was analyzed by HPLC (eluant B, λ = 280 and 340 nm) after centrifugation to remove the protein residue. In some experiments, fractions eluting between 9 and 15 min were collected, acidified to pH 0 with 1 M HCl, and stirred vigorously for 3 h. After centrifugation, the supernatant was reduced to a small volume and analyzed by HPLC. Analysis of **5** in the supernatant (0.5 mL) obtained after one digestion treatment was carried out according to a procedure currently adopted for catecholamine analysis involving enrichment of the sample by selective adsorption on acid-treated alumina.[21] HPLC analysis was carried out with electrochemical detection with the first electrode set at −0.1 V, the second at +0.4 V, and the sensitivity at 200 nA. Eluant F was used as the mobile phase.

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Supporting Information Available: NMR spectra of compounds **13**, **14**, and **16**−**18**, including ^{1}H, ^{13}C, HETCOR, and HMBC. This material is available free of charge via the Internet at http://pubs.acs.org.

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