ELSEVIER

Biochimica et Biophysica Acta 1291 (1996) 75–82

BBA Biochimica et Biophysica Acta

5-*S*-Cysteinyldopa, a diffusible product of melanocyte activity, is an efficient inhibitor of hydroxylation/oxidation reactions induced by the Fenton system

Alessandra Napolitano [a], Sofia Memoli [a], Anthony J. Nappi [b], Marco d'Ischia [a], Giuseppe Prota [a,*]

[a] *Department of Organic and Biological Chemistry, University of Naples 'Federico II', Via Mezzocannone 16, I-80134 Naples, Italy*
[b] *Department of Biology, Loyola University of Chicago, Chicago IL, 60626, USA*

Received 29 February 1996; revised 24 April 1996; accepted 25 April 1996

Abstract

Interest in 5-*S*-cysteinyldopa (5-S-CD), a major excretion product of normal and malignant melanocytes, has traditionally concentrated on its significance as a biosynthetic precursor of pheomelanins, the characteristic pigments of red hair, and as a specific biochemical marker for monitoring melanoma progression. The present study shows that 5-S-CD is a potent inhibitor of hydroxylation/oxidation reactions mediated by hydrogen peroxide and the Fe^{2+}/EDTA complex under both aerobic and anaerobic conditions. The inhibitory effect of 5-S-CD, as determined by the deoxyribose and salicylic acid assays in phosphate buffer (pH 7.4), is much stronger than that of dopa, acetylsalicylic acid and mannitol, increases with increasing ligand-to-metal ratio, and is inversely proportional to the concentration of EDTA present in the Fenton system. Spectrophotometric evidence and competition experiments indicate that 5-S-CD forms a chelate complex with ferric ions (λ_{max} = 500 nm at pH 7.4), which may account for both an altered production of hydroxyl radicals by the Fenton reagent and a site-specific localization of oxidative damage on the chelate complex itself.

Keywords: 5-*S*-cysteinyldopa; Melanocyte; Pheomelanin; Hydroxylation

1. Introduction

Production and excretion of 5-*S*-cysteinyldopa (5-S-CD) is currently recognized as a central mechanism for the control of the levels of melanin pigments formed within functionally active melanocytes [1,2]. Depending on the concentrations of cysteine and related sulfydryl compounds in the melanogenic compartments, a certain proportion of the dopaquinone produced by tyrosinase-catalyzed oxidation of tyrosine is trapped through nucleophilic addition of the SH group of the amino acid. In this way, the normal course of eumelanin biosynthesis via 5,6-dihydroxyindole(s) is prevented and, depending on the redox potential of the local environment, the resultant 5-S-CD is either oxidized to reddish brown pigments, termed pheomelanins [3], or is excreted from melanocytes into surrounding tissues, thereby passing into circulation (see Scheme 1).

In healthy individuals, the serum levels of 5-S-CD may vary in the range 4-16 nmol/l, depending on the basal metabolic activity of the melanocytes, whereas in the urine they are usually higher with a wider fluctuation [4,5]. The excretion of 5-S-CD normally does not correlate with the amount and type of pigmentation, since it is found in the urine of both heavily-pigmented and fair-complexioned individuals, and there is no substantial difference between people of different races. Notably, however, under contingent physiological or pathological conditions where melanocyte activity is increased, e.g., in response to UV-irradiation, inflammation, or in patients with melanoma, the excretion levels of 5-S-CD rise dramatically. Thus,

Abbreviations: 5-S-CD, 5-*S*-cysteinyldopa; 3-S-CMC, 3-*S*-cysteinyl-5-methylcatechol; 5-S-CAD, 5-*S*-cysteaminyldopa; TBA, thiobarbituric acid; DBA, 2,3-dihydroxybenzoic acid; GA, 2,5-dihydroxybenzoic acid (gentisic acid).

* Corresponding author. Fax: +39 81 5521217; e-mail: prota@ds.unina.it.

PII S0304-4165(96)00047-5

after prolonged exposure to sunlight or during PUVA therapy, serum concentration increases markedly [6,7].

Realistic estimates indicate that in certain epidermal compartments the local concentration of 5-S-CD may reach abnormally high values [8]. Since 5-S-CD is not decarboxylated in the kidney or other organs, is not conjugated with glucuronic and sulphuric acids, and has a high renal plasma clearance, it represents a useful and reliable biochemical marker of melanocyte activity. This has warranted considerable interest in the use of this metabolite for monitoring melanoma progression, detecting metastases and for evaluating various therapeutic procedures [9].

This focus on a practical application of 5-S-CD, and the underlying perception of this major excretory product of epidermal melanocytes as a detoxification product of the melanin pathway, have somewhat hindered the development of a broader perspective on its possible functional role. In pursuing our programme on the functional significance of colourless products of melanocyte activity, we have now found that 5-S-CD is endowed with remarkable inhibitory properties toward hydroxylation and oxidation reactions induced by iron-mediated decomposition of hydrogen peroxide, in Fenton-type processes.

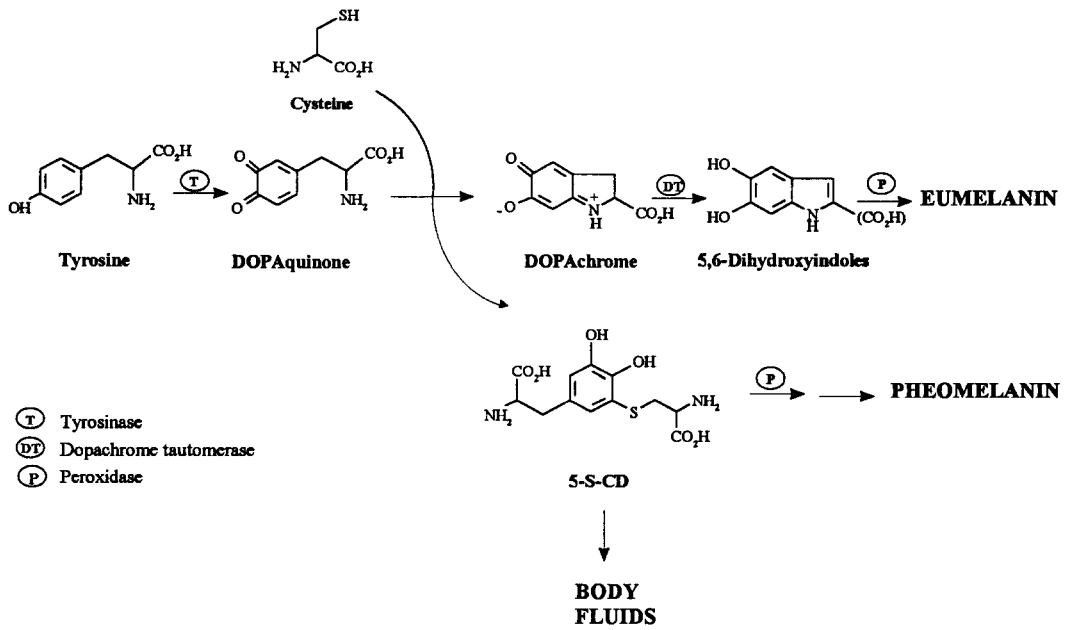
2. Materials and methods

2.1. Materials

Salicylic acid, 2,3-dihydroxybenzoate (DBA), 2,5-dihydroxybenzoate (gentisic acid, GA), catechol, L-dopa, acetylsalicylic acid, mannitol and hydrogen peroxide (30% in water) were purchased from Sigma Chemical Co. (St. Louis, MO). EDTA disodium salt, deoxyribose, 4-methylcatechol, L-cysteine, L-histidine and 2-aminoethanethiol were from Aldrich Chemie (Steinheim, Germany). Chelex 100 (200–400 mesh) was from Bio-Rad.

5-S-CD [10] and 3-*S*-cysteinyl-5-methylcatechol (3-S-CMC) [11] were synthesized as previously reported. 5-*S*-Cysteaminyldopa (5-S-CAD) was prepared by a standard procedure [10] modified as follows. Cerium ammonium nitrate (2.74 g) in 25 ml of 2 M sulphuric acid was added to a solution of 0.49 g of L-dopa in 12.5 ml of 2 M sulphuric acid. The resultant red solution, containing dopaquinone, was added to 1.12 g of 2-aminoethanethiol in 12.5 ml of 2 M sulphuric acid. The reaction mixture was then purified by ion-exchange chromatography over a Dowex 50W-X8 (H^+ form, 100–200 mesh) column (30 × 2 cm), eluted with water (280 ml), 0.5 M HCl (1 l) and 3 M HCl (1.4 l). Fractions from 3 M HCl were re-chromatographed on a Dowex 50W-X8 column (50 × 2 cm), equilibrated with 0.5 M HCl. Elution was performed with a 0.5 M to 4 M HCl gradient and monitored by UV spectrophotometry. Fractions from 3 M HCl, showing absorption maxima at 251 nm and 290 nm at an about 1.4 ratio, were collected and evaporated to afford 5-*S*-cysteaminyldopa hydrochloride as a grey solid (510 mg, 59% yield). UV λ max (0.1 M HCl) 290, 252 (sh) nm (log ε 3.23, 3.35); ^{1}H-NMR (0.1 M DCl) δ(ppm): 6.96 (1H, d, J = 1.8 Hz), 6.86 (1H, d, J = 1.8 Hz), 4.26 (1H, t, J = 6.6 Hz), 3.22 (2H, t, J = 8.4 Hz), 3.15 (2H, m), 3.11 (2H, t, J = 8.4 Hz).

TBA was from Aldrich and was crystallized from hot water before use. All other chemicals were of the highest purity available and were used without further purification. All solvents were HPLC grade. Standard aqueous solutions



Scheme 1. Schematic outline of melanogenesis in epidermal melanocytes showing the formation of 5-S-CD by nucleophilic attack of cysteine to dopaquinone.

of $FeCl_3$, $(NH_4)_2Fe(SO_4)_2$ and hydrogen peroxide were prepared fresh before use. $FeCl_3$ and H_2O_2 concentrations were determined from their absorbances at 343 nm (ϵ = 2987 cm^{-1} M^{-1} in HCl 6M) and 240 nm (ϵ = 43.6 cm^{-1} M^{-1} in water) [12], respectively. Glass-distilled, deionized water was used for preparation of all solutions. All buffers were freed from metal ions by passage through a column of Chelex 100 resin.

2.2. Methods

UV spectra were recorded on a Perkin Elmer Lambda 7 spectrophotometer having the cell compartment controlled at 25 ± 0.1°C with circulating water. ^{1}H-NMR (270 MHz) spectra were recorded on a Bruker AC 270 spectrometer. HPLC analyses were performed with a Gilson instrument equipped with model 305 and 306 pumps, using a Spherisorb S5 ODS2 (25 × 4.6 mm) column. Detection was carried out with a 119 UV/VIS spectrophotometer set at 295 or 280 nm, and a LC-4B amperometric detector equipped with a Bioanalytical Systems detector cell; the detector potential was set at +650 mV vs an Ag/AgCl reference electrode. Instrument sensitivity was varied from 100 to 5 nA. Different elution conditions were employed as follows: 0.05 M triethylammonium phosphate, pH 2.0-acetonitrile, 85:15 v/v (eluant A); 0.03 M sodium citrate and sodium acetate, pH 4.7 (eluant B); 0.05 M sodium citrate, pH 4.0-methanol 85:15 v/v (eluant C). The flow rate was 1.0 ml/min. Quantitation was carried out by comparing integrated peak areas with external calibration curves. Reactions were carried out in thermostatted tubes controlled at 25 ± 0.1 and 37 ± 0.2°C.

2.2.1. Deoxyribose assay

The experimental procedure used was essentially as described by Zhao et al. [13] with minor modifications. The concentrations of the additives present in the reaction mixtures are reported in the figure legends. $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$ and EDTA were premixed just before addition to the reaction mixture and were added to start the reaction. In some experiments, EDTA was omitted and 5-S-CD or dopa were added at a final concentration of 0.15 mM. After incubation at 37°C for 1 h, 1.5 ml of 2.8% (w/v) cold trichloroacetic acid was added to the reaction mixture and 1.0 ml of the resultant solution was added to 1.0 ml of TBA (1% w/v in 50 mM NaOH). After heating at 100°C for 20 min, the mixture was allowed to cool and the absorbance at 532 nm was measured against appropriate blanks [14]. None of the compounds tested gave interference with the TBA assay.

2.2.2. Salicylate hydroxylation

Salicylate hydroxylation was carried out at 25°C, under both aerobic and anaerobic conditions. In a typical experiment, to a stirred solution of 3 mM salicylic acid in 0.1 M phosphate buffer (pH 7.4), the following additives were added in the order stated: 5-S-CD, dopa or mannitol in 0.1 M HCl up to the desired concentration, H_2O_2 (0.1 mM), Fe(II)-EDTA complex, obtained by premixing $(NH_4)_2Fe(SO_4)_2$ and EDTA, at a final concentration of 0.1 and 0.5 mM, respectively. Anaerobic experiments were performed in rubber-capped glass cuvettes, fluxed with argon or oxygen-free nitrogen for at least 20 min prior to addition of the Fe(II)-EDTA complex. After 30 min incubation, 300-μl aliquots of the reaction mixtures were withdrawn via syringe, treated with an excess of solid sodium borohydride (ca. 10 mM final concentration) and, after 30 s, acidified to pH 1 with 20 μl of 2 M HCl prior to HPLC analysis. Solutions containing mannitol or 5-S-CD were analysed using eluant A, while eluant B was employed for analysis of mixtures containing dopa. Eluant A was found to give a better resolution compared to those recommended by other authors [15,16], permitting a good analysis of scavenger oxidation products without overlap with detector products.

2.2.3. Catechol-iron complexes

Formation of Fe(III) chelates of 5-S-CD, dopa, 5-S-CMC or 5-S-CAD was studied spectrophotometrically at different metal to ligand ratios. In a typical experiment, ligand was added, at the stated concentration, to a solution of 0.15 mM $FeCl_3$ in 0.1 M Tris buffer (pH 7.4), which had been fluxed with argon for at least 20 min. The UV spectrum was recorded after 15 min equilibration. In another set of experiments, deareated solutions of 0.15 mM $FeCl_3$ and 0.75 mM ligand were examined in 0.1 M phosphate buffer at different pH values. In competition experiments, varying amounts of EDTA were added to a solution of 0.1 mM $FeCl_3$ and 0.2 mM 5-S-CD in 0.1 M phosphate buffer (pH 7.4) under argon atmosphere

2.2.4. Reaction of 5-S-CD with the Fenton system

A solution of 1.0 mM 5-S-CD in 0.1 M phosphate buffer (pH 7.4) was treated sequentially with 0.25 mM H_2O_2, 0.2 mM $(NH_4)_2Fe(SO_4)_2$ and 0.2 mM EDTA and taken under vigorous stirring at 25°C, under nitrogen atmosphere. Aliquots of the reaction mixture were periodically withdrawn and injected for HPLC analysis after reduction with sodium borohydride and acidification as above, using eluant C as the mobile phase. Products were identified by comparison of their chromatographic and spectral properties with those of authentic samples [17,18].

2.2.5. Data presentation

The results are the mean of at least three separate experiments with a typical coefficient of variation of less than 5%.

3. Results

In the present study we chose as the Fenton reagent a complex consisting of 0.14 mM EDTA and 0.13 mM Fe^{2+}

(molar ratio 1.1:1) in 0.1 M phosphate buffer (pH 7.4), with hydrogen peroxide at 0.85 mM concentration, according to a reported procedure [13]. Although use of phosphate may complicate the interpretation of iron-binding reactions [19], this was preferred to organic buffers, such as Hepes, Mes or Tris, because of the known ability of the latter to scavenge hydroxyl radicals [13,20].

In preliminary experiments, not shown, we compared the effect of 5-S-CD on the above system and other relevant reagents, particularly one based on the EDTA-Fe^{3+} chelate [21,22]. No significant difference was observed for what concerns general trends and relative effects.

Fig. 1 shows the effect of varying concentrations of 5-S-CD, dopa and three hydroxyl radical scavengers, i.e., acetylsalicylic acid, mannitol and histidine, on the oxidation of deoxyribose with the Fe^{2+}/EDTA system [13,20], as determined by the TBA assay [14].

At comparable concentrations, 5-S-CD appears to be by far more active than dopa and, notably, acetylsalicylic acid and mannitol, at inhibiting the formation of TBA reactive substances. The effect is especially observable at 5-S-CD concentrations higher than 2 mM, with Fe^{2+} at 0.13 mM, and, more in general, at high scavenger-to-metal ratios, as apparent from separate experiments carried out at lower concentrations of closer physiological relevance. At low concentrations, the behavior of 5-S-CD, like that of dopa, deviates from linearity, suggesting a direct interaction with the hydroxyl radical generating system [13], e.g., by iron chelation. This prevents calculation of a rate constant for the reaction of 5-S-CD with hydroxyl radicals.

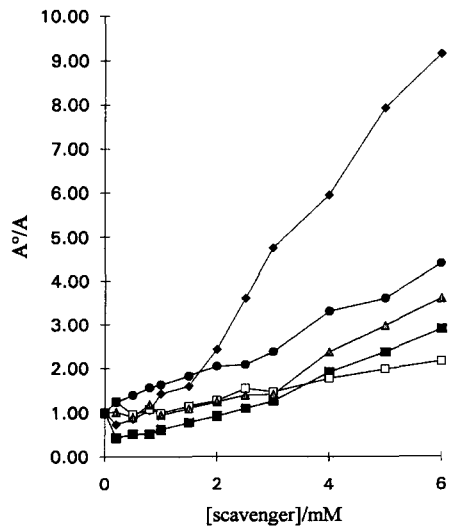


Fig. 1. Effect of 5-S-CD and other hydroxyl radical scavengers on deoxyribose degradation induced by the Fenton reagent. A^0/A indicates the relative extent of deoxyribose degradation in the absence (A^0) and in the presence (A) of scavenger, as determined by the TBA assay. Deoxyribose was 3 mM, H_2O_2 was 0.85 mM, Fe^{2+} was 0.13 mM, EDTA was 0.14 mM. The reaction was carried out in 0.1 M phosphate buffer at pH 7.4 at 37°C for 1 h. Absorbance at 532 nm in the absence of scavenger was 0.35 ± 0.02 (mean value for 4 experiments). [♦], 5-S-CD; [●], dopa; [▲], acetylsalicylic acid; [■], histidine; [□], mannitol.

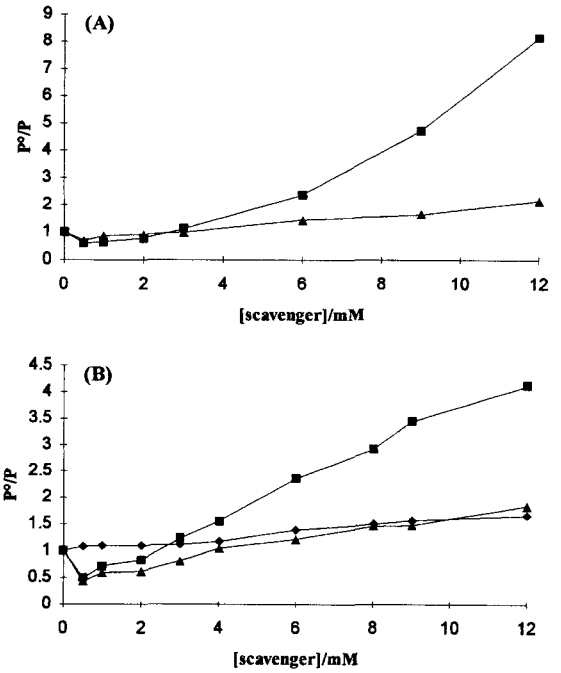


Fig. 2. Effect of 5-S-CD and other hydroxyl radical scavengers on salicylic acid hydroxylation by the Fenton reagent under aerobic (A) and anaerobic (B) conditions. P^0/P denotes the sum of DBA, GA and catechol in the absence (P^0) and in the presence (P) of scavenger. Salicylic acid was 3.0 mM, H_2O_2 was 0.1 mM, Fe^{2+} was 0.1 mM, EDTA was 0.5 mM. The reaction was carried out in 0.1 M phosphate buffer at pH 7.4 at 25°C for 30 min. [■], 5-S-CD; [▲], dopa; [♦], mannitol.

In subsequent experiments we investigated the effect of 5-S-CD on Fenton reactions using as detector molecule sodium salicylate [23,24]. Specific quantitation of hydroxylation products, i.e., DBA, GA and catechol [16] by HPLC with electrochemical detection, using an improved eluting system, allowed selective determination of hydroxylation processes, permitting a more in-depth analysis of the mechanism of the inhibitory effect of 5-S-CD in the Fenton process.

As shown in Fig. 2, at high concentrations 5-S-CD exerted a consistently efficient inhibitory effect, compared to dopa and mannitol, on the generation of hydroxylation products of salicylic acid, whereas a slight pro-oxidant effect was apparent at concentrations lower than 2 mM.

The yield and distribution of hydroxylation products was found to vary significantly depending on whether the reaction was carried out in air or in an oxygen-free atmosphere, as previously reported [23]. In all cases examined, under both aerobic and anaerobic conditions, the inhibitory effect of 5-S-CD was associated to a substantial decomposition of the amino acid, as determined by HPLC. Additional experiments were run in argon or nitrogen-saturated atmosphere, unless otherwise stated, to focus on those processes that are specifically dependent on the redox

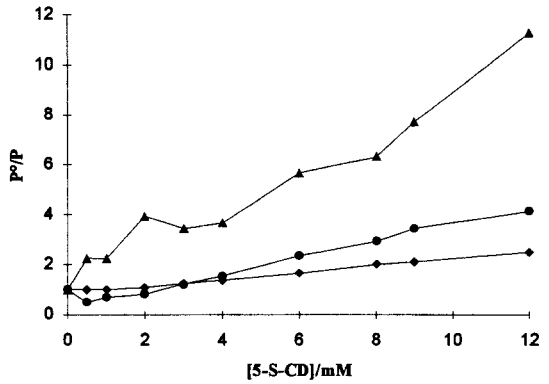


Fig. 3. Effect of 5-S-CD on salicylic acid hydroxylation by the Fenton reagent as a function of EDTA concentration. P^0/P denotes the sum of DBA, GA and catechol in the absence (P^0) and in the presence (P) of scavenger. The reaction was carried out in 0.1 M phosphate buffer at pH 7.4 at 25°C for 30 min, under anaerobic conditions. Salicylic acid was 3 mM, H_2O_2 was 0.1 mM, Fe^{2+} was 0.1 mM. EDTA was absent [▲]; 0.5 mM [●]; 10 mM [◆].

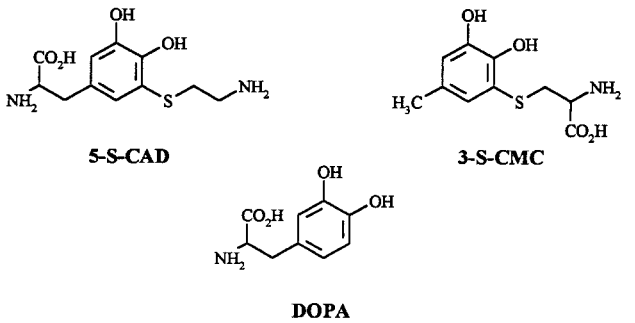
interaction of hydrogen peroxide and ferrous ions, without concomitant participation of molecular oxygen and species thereof.

Fig. 3 shows the effect of different concentrations of EDTA on the inhibition of salicylate hydroxylation by 5-S-CD.

The inhibitory effect of 5-S-CD on the Fenton-mediated reaction was inversely proportional to the concentration of EDTA present in the medium, being maximum in the absence of the chelator and almost negligible with 10 mM EDTA. This is well consistent with the ability of EDTA to chelate iron and to act at the same time as an interceptor of hydroxyl radicals [13,19].

Some insight into the nature of the 5-S-CD-iron complexes was gained by spectrophotometric investigation of the interaction of 5-S-CD with both ferrous and ferric ions. Mixing 5-S-CD and ferrous ions rigorously in a oxygen-free atmosphere at various molar ratios caused no significant change in the absorption maximum of 5-S-CD. By contrast, interaction with ferric ions led to the development of a red-purple chromophore, suggesting formation of a chelate complex akin to those formed by other catechol compounds, including dopa [25].

Formation of ferric chelates of 5-S-CD was examined at various ligand-to-metal ratios and different pH values using for comparative purposes two structural analogues of 5-S-CD, namely 5-*S*-cysteaminyldopa (5-S-CAD), lacking the carboxyl group on the cysteine chain, and 3-*S*-cysteinyl-5-methycatechol (3-S-CMC), in which the alanyl side chain on the aromatic ring is missing. Dopa was included as a reference catechol system.



The data, reported in Table 1, show little or no differences in the absorption spectra of 5-S-CD compared to those of the other alkylthiocatechols, but a marked ipsochromic shift with respect to the spectrum of the dopa complex.

A significant bathochromic shift of the absorption maximum occurred at lower pH in all cases examined. The maximum absorbance for the 5-S-CD/Fe^{3+} complex is apparently attained at a ligand-to-metal ratio of about five, which, of course, does not reflect the actual composition of the complex but rather the lowest ligand-to-metal ratio ensuring full complexation.

In control experiments, the stability of the 5-S-CD-Fe^{3+} complex (at various ligand-to-metal ratios) was examined by HPLC under both anaerobic and aerobic conditions. No detectable decay of 5-S-CD was observed in the former

Table 1
Absorption maxima of Fe^{3+} chelates of 5-S-CD, 3-S-CMC, 5-S-CAD and dopa as a function of pH

Ligand/Fe^{3+}	Buffer (0.1 M)	pH	λ_{max}(nm) (absorbance)			
			5-S-CD	5-S-CAD	3-S-CMC	Dopa
0.5	Tris	7.4	520 (0.10)	540 (0.08)	560 (0.07)	560 (0.09)
1	Tris	7.4	516 (0.24)	515 (0.16)	558 (0.18)	560 (0.17)
2	Tris	7.4	500 (0.38)	500 (0.26)	564 (0.34)	570 (0.31)
3	Tris	7.4	500 (0.54)	500 (0.35)	519 (0.41)	570 (0.44)
4	Tris	7.4	500 (0.64)	500 (0.51)	512 (0.51)	570 (0.44)
5	Tris	7.4	500 (0.73)	500 (0.57)	508 (0.62)	570 (0.45)
8	Tris	7.4	500 (0.75)	500 (0.73)	506 (0.68)	570 (0.44)
10	Tris	7.4	500 (0.76)	500 (0.83)	505 (0.72)	570 (0.46)
5	Phosphate	6.0	565 (0.33)	552 (0.18)	561 (0.14)	560 (0.02)
5	Phosphate	7.4	498 (0.67)	525 (0.56)	519 (0.52)	560 (0.07)
5	Phosphate	8.0	500 (0.66)	515 (0.61)	507 (0.69)	549 (0.17)

Fe^{3+} concentration was maintained at 0.15 mM and ligand varied as indicated.

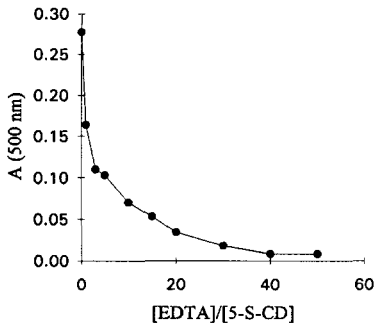


Fig. 4. Absorbance at 500 nm of the 5-S-CD-Fe^{3+} complex as a function of the concentration of added EDTA. 5-S-CD was 0.2 mM, Fe^{3+} was 0.1 mM, EDTA was varied in the range 0.2–10 mM. All experiments were run under an argon atmosphere, in 0.1 M phosphate buffer (pH 7.4).

case even after 16 h. This can be taken to indicate that, at least at the ligand-to-metal ratios examined and under anaerobic conditions, 5-S-CD does not undergo redox exchange with ferric ions. In air, no significant effect of ferric ions on autoxidation of 5-S-CD was observed (not shown). Also, no significant reaction was observed between 5-S-CD and hydrogen peroxide in the absence of added iron over a period of two or more hours.

Fig. 4 shows the effect of increasing concentrations of EDTA on the absorbance of the 5-S-CD-Fe^{3+} complex.

As the EDTA/5-S-CD ratio increases, the absorbance of the orange-red chromophore of the 5-S-CD-Fe^{3+} complex gradually diminishes, being virtually suppressed with a 40-fold excess of EDTA over 5-S-CD at 0.2 mM concentration.

In another series of experiments, the potential of the 5-S-CD-Fe^{2+} and the 5-S-CD-Fe^{3+} systems to promote Fenton-type processes was investigated by measuring their ability to mediate hydrogen peroxide-induced deoxyribose oxidation in comparison with that of dopa- and EDTA-iron complexes. The data shown in Fig. 5 indicate a somewhat higher formation of TBA reactive material with the dopa chelate, and no substantial difference between the 5-S-CD- and EDTA-containing systems assayed under similar conditions.

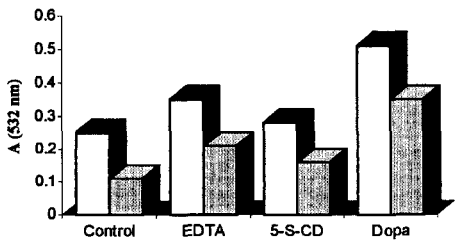


Fig. 5. Yield of degradation products of deoxyribose formed by reaction of hydrogen peroxide with Fe^{2+} (open bars) and Fe^{3+} (hatched bars) complexes of 5-S-CD, dopa and EDTA. Deoxyribose was 3.0 mM, H_2O_2 was 0.85 mM, Fe^{2+} or Fe^{3+} was 0.13 mM, ligand, when added, was 0.15 mM. All other conditions were as described in Fig. 1. Deoxyribose oxidation was determined by the TBA assay, measuring the absorbance at 532 nm.

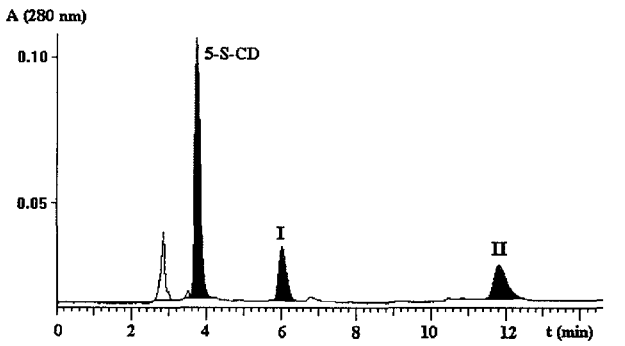


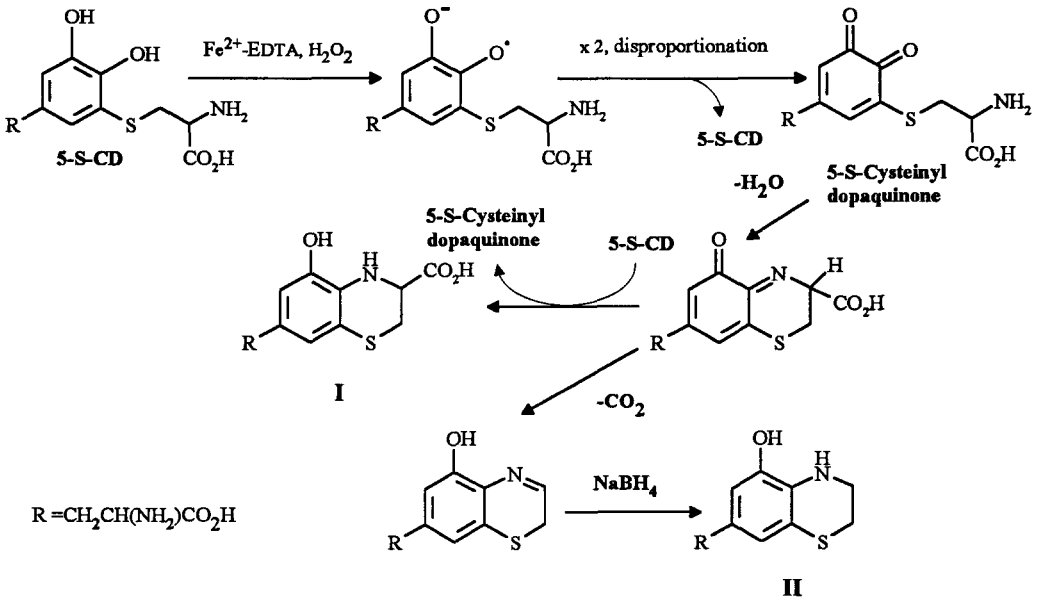
Fig. 6. HPLC elution profile of the products formed by reaction of 5-S-CD with the Fenton reagent. 5-S-CD was at 1.0 mM concentration, H_2O_2 was 0.25 mM, Fe^{2+} was 0.2 mM, EDTA was 0.2 mM. The reaction was carried out in 0.1 M phosphate buffer at pH 7.4 at 25°C and was stopped after 30 min by reduction with sodium borohydride.

The chemical fate of 5-S-CD following exposure to the Fenton reagent was also investigated. HPLC analysis of the reaction mixture after reduction with sodium borohydride (Fig. 6) showed that, under the reaction conditions, 5-S-CD undergoes oxidative cyclization, as evidenced by formation of the dihydrobenzothiazines I and II after reduction of the mixture with sodium borohydride [18].

4. Discussion

Traditional accounts of melanocyte function and interaction with neighbouring structures have focused largely on the pigmentary activity of these cells, and the complex sequence of events underlying the transfer and re-distribution of melanosomes to the surrounding keratinocytes [26]. It seems, however, that most of those studies did not clearly distinguish between the role of the pigments and that of related intermediates of the biosynthetic pathway, so that the potential contribution of the latter was largely overlooked. Following some papers (reviewed in Refs. [27] and [28]) that drew attention to this oversight, there has been in the last few years an upsurge of interest in the functional significance of colourless diffusible products of melanocyte metabolism. The results of the present study are clearly supportive of this trend, as they throw light for the first time on an important property of 5-S-CD other than its role as pigment precursor.

The inhibitory effect of 5-S-CD on Fenton-induced hydroxylation reactions is apparent at high catechol-to-metal ratios and is larger than predicted by simple competition for hydroxyl radical in free solution. This can be accounted for in terms of the ability of 5-S-CD to form a chelate complex with ferric ions, thereby interfering with production of hydroxyl radicals by the Fenton reagent. In particular, chelation may alter the redox potential of the Fe^{2+}/Fe^{3+} couple, may hinder attack of hydrogen peroxide to the metal ion, which is crucial for hydroxyl radical formation to occur [29], and/or may localize damaging



Scheme 2. Mechanism of oxidation of 5-S-CD by the Fenton reagent.

processes to the catechol-iron complex itself, so sparing the detector molecule and other possible targets. In any case, whatever the actual mechanism, 5-S-CD should not classify as a simple hydroxyl radical scavenger, since this definition applies stricto jure to those compounds that operate on the basis of a random competition for hydroxyl radicals in free solution [30].

The slight pro-oxidant effect seen at low 5-S-CD/metal ratios is similar to that observed in the case of dopa and other related substances, and is possibly due to the ability of the catechol to chelate ferric ions, thus altering the redox potential of the Fe^{2+}/Fe^{3+} couple without providing antioxidant protection.

A detailed investigation of the 5-S-CD-iron complex is beyond the scope of the present study. Available evidence, however, would suggest that an important coordination site for the metal is provided by the sulfur atom, on account of the marked ipsochromic shift of the Fe^{3+} complex of 5-S-CD compared to that of dopa. On the other hand, the alanyl side chain on the aromatic ring of 5-S-CD and the carboxy group on the cysteine residue seem to be involved only marginally.

From a biological perspective, the implications of our results are manifold. A key point to emerge is that 5-S-CD may serve a hitherto overlooked function as a strong competitor for site-specific hydroxyl radical induced damaging processes, acting specifically on those fractions of the iron pool which are not tightly bound to proteins. It has been shown that upon exposure to UV radiation levels of Fe^{2+} and Fe^{3+} in the skin increase, and that these changes are accompanied by the synthesis of additional pigment [1,28]. The increase in the eumelanin/pheomelanin content in the melanosomal compartment following exposure to UV radiation has been viewed differently by various investigators who proposed either a prooxidant or an antioxidant function to some of the melanin precursors. Increased levels of Fe^{2+}/Fe^{3+} can only be viewed as alarming, considering the potential for hydroxyl radical generation by Fenton chemistry.

The increased production of 5-S-CD by stimulated melanocytes can therefore be envisaged as part of the biochemical machinery which is set into motion to counteract threatening events consequent to oxidative stress. Under conditions of high catechol-to-metal ratios, which are likely to occur considering the exceedingly low biological levels of loosely bound iron, 5-S-CD would be superior to conventional antioxidants by virtue of its ability to strip iron away from the site of injury into body fluids for excretion. Moreover, within oxidative environments, conversion of the 5-S-CD-iron chelate would result in the formation of dihydrobenzothiazine derivatives, e.g., I, which in principle may be endowed with antioxidant and chelating properties similar to those of the parent catechol (Scheme 2). This would provide additional chemical arguments to rationalize the outstanding inhibitory properties of 5-S-CD toward Fenton-promoted oxidation processes.

Acknowledgements

This work has been supported in part by grants from MURST and CNR. S.M. thanks the University of Naples for a graduate scholarship at Loyola University. A.J.N. thanks NSF (IBN 950 4796) for support. We also thank Miss Silvana Corsani for technical assistance.

References

[1] Prota, G. (1992) Melanins and Melanogenesis, Academic Press, San Diego, CA.
[2] Prota, G. (1993) J. Invest. Dermatol 100 (suppl.), 156–161.
[3] Prota, G. (1995) Fortsch. Chem. Org. Naturst. (Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, Ch. eds.), vol. 64, pp. 94–148, Springer, Wien.
[4] Rorsman, H., Agrup, G., Hansson, C. and Rosengren, E. (1983) in Pigment Cell (MacKie, R.M., ed.), Vol. 6, pp. 93–115, Karger, Basel.
[5] Rorsman, H. and Pavel, S. (1990) in Cutaneous Melanoma Biology and Management (Cascinelli, N., Santimani, M. and Veronesi, U., eds.), pp. 79–87, Masson, Milan.
[6] Rorsman, H., Agrup, G., Falck, B., Rosengren, A.-M. and Rosengren, E. (1976) in Pigment Cell (Riley, V. ed.), vol. 2, pp. 284–289, Karger, Basel.
[7] Hansson, C., Rorsman, H., Rosengren, E. and Tegner, E. (1981) Acta Derm. Venereol. (Stockholm) 61, 251–255.
[8] Chedekel, M.R. and Zeise, L. (1988) Lipids 23, 587–591.
[9] Horikoshi, T., Ito, S., Wakamatsu, K., Onodera, H. and Eguchi, H. (1994) Cancer 73, 629–636.
[10] Chioccara, F. and Novellino, E. (1986) Synth. Commun. 16, 967–971.
[11] Prota, G., Scherillo, G. and Nicolaus, R.A. (1968) Gazz. Chim. Ital. 98, 495–510.
[12] Bielski, B.H.J. and Allen, A.O. (1977) J. Phys. Chem. 81, 1048–1050.
[13] Zhao, M.J., Jung, L., Tanielian, C. and Mechin, R. (1994) Free Rad. Res. 20, 345–363.
[14] Aruoma, O.I. (1994) Methods in Enzymol. 233, 57–66.
[15] Grootveld, M. and Halliwell B. (1986) Biochem. J. 237, 499–504.
[16] Floyd, R.A., Watson, J.J. and Wong, P.K. (1984) J. Biochem. Biophys. Methods 10, 221–235.
[17] Prota, G., Crescenzi, S., Misuraca, G. and Nicolaus, R.A. (1970) Experientia 26, 1058–1059.
[18] Napolitano, A., Costantini, C., Crescenzi, O. and Prota, G. (1994) Tetrahedron Lett. 35, 6365–6368.
[19] Gutteridge, J.M.C. (1987) Biochem. J. 243, 709–714.
[20] Halliwell, B., Gutteridge, J.M.C. and Aruoma, O.I. (1987) Anal. Biochem. 165, 215–219.
[21] Nappi, A.J., Vass, E., Prota, G. and Memoli, S. (1995) Pigment Cell Res. 8, 283–292.
[22] Motohashi, N. and Mori, I. (1986) J. Inorg. Biochem. 26, 205–212.
[23] Maskos, Z., Rush, J.D. and Koppenol, W.H. (1990) Free Rad. Biol. Med. 8, 153–162.
[24] Maskos, Z., Rush, J.D. and Koppenol, W.H. (1992) Arch. Biochim. Biophys. 296, 521–529.
[25] Sotomatsu, A., Nakano, M. and Hirai, S. (1990) Arch. Biochem. Biophys. 283, 334–341.
[26] Pathak, M.A., Jimbow, K. and Fitzpatrick, T.B. (1981) In: Pigment Cell (Seiji, M. ed.) pp. 655–670, University of Tokyo Press, Tokyo.
[27] Prota, G. and Misuraca, G. (1996) Excerpta Medica, in press.
[28] Jimbow, K. (1995) Keio J. Med. 44, 9–18.
[29] Goldstein, S., Meyerstein, D. and Czapski G. (1993) Free Rad. Biol. Med. 15, 435–445.
[30] Gelvan, D., Moreno, V., Gassmann, W., Hegenauer, J. and Saltman, P. (1992) Biochim. Biophys. Acta 1116, 183–191.