Melanogenesis: a search for pheomelanin and also, what is lurking behind those dark colors?

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Abstract: We investigated the synthesis of melanin-like materials from DOPA, dopamine, norepinephrine and epinephrine in the presence of L-cysteine. We observed that L-cysteine delayed the formation of pigment from these catecholamines and that the presence of L-cysteine yielded darker-colored reaction mixtures. No reddish pigment was observed that would indicate the synthesis of pheomelanin-like material. The reactions were performed in the presence of Na2CO3 and through the addition of CaCl2 at the end of the reaction; the black, eumelanin-like material was co-precipitated with CaCO3. The remaining supernatant solutions were observed to be light-yellow to rusty-orange in color depending on the catecholamine used in the reaction. Size exclusion chromatography (SEC) analyses indicated that the removal of the black pigment left behind an oligomeric material that exhibited a strong absorbance band around 280nm. Our experimental and analytical observations prompt us to raise a number of points of discussion or hypotheses. 1) The presence of L-cysteine during the air-mediated oxidation of catecholamines leads to darker-colored pigments; not reddish or lighter-colored pigments that would visually resemble pheomelanin-like pigments, 2) SEC analyses suggested that the black pigment generated during the air-mediated oxidation of catecholamines is not necessarily the main reaction product, 3) The pre-formed, dark-colored pigments obtained through the air-mediated oxidative melanogenesis process can readily be deposited on insoluble mineral surfaces using an in situ co-precipitation procedure, 4) The air-mediated oxidation of catecholamines leads to a binary product that contains an insoluble, melanin-like substance and a soluble, oligo- or polymeric substance containing unoxidized precursor units, 5) The melanogenesis process leads to a binary product involving a non-covalently bonded combination of dark-colored pigment and a lighter-colored or colorless substance; the latter being understudied or ignored in the in vitro or in vivo studies of the melanogenesis process, 6) The kinetics of the melanogenesis process may determine the balance between insoluble and soluble components of the binary product generated; the slower the reaction the more dark-colored, insoluble pigment generated, 7) One should consider the possibility of intermolecularly, N-to-C, bonded units of catecholamines when evaluating the structure of melanins, polydopamines, etc. and 8) There is a need for a systematic study of the effect of amino acids (beyond just L-cysteine) and amines in general on the melanogenesis process.

Keywords: eumelanin, pheomelanin, catecholamines, polydopamine, L-cysteine

1. Introduction

Melanins (MNs) constitute a ubiquitous class of dark-colored pigments which can be found in all kingdoms of life. It is a heterogeneous class of pigments as a wide variety of molecules can serve as precursor for these pigments and many reviews regarding their biosynthesis, chemistry, classification and functions have been written.1-4 Despite many decades of intense research and computer modelling, there is no consensus on the precise chemical structure of these biomolecules or a precise explanation for their dark color, exhibited by their broad-range, monotonic
absorbance profile over the entire ultraviolet and visible region of the electromagnetic spectrum. Based upon degradation reactions, various model structures have been proposed and it has been suggested that MNs should be described as “heterogeneous polymers derived by the oxidation of phenols and subsequent polymerization of intermediate phenols and their resulting quinones”. The main MN pigments found in mammalian cells are eumelanin and pheomelanin. Eumelanin is built from L-DOPA, an oxidation product of L-tyrosine, and has a dark brown to black color. Pheomelanin is built from a combination of L-DOPA and L-cysteine and is typically reddish in color. These types of MNs are found in melanocytes, stored in specialized organelles, the melanosomes, and the formation of melanosomes is a complex, multi-stage process. The biosynthesis and enzymology of MNs in melanocytes have been extensively studied and reviewed. In this context the enzyme tyrosinase has received a lot of attention. Tyrosinase is responsible for the first steps of the MN biosynthesis: the hydroxylation of L-tyrosine to L-DOPA and subsequent oxidation to L-dopaquinone. Dopaquinone can undergo an intramolecular substitution reaction ultimately leading to 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) as the building blocks for eumelanin. Alternatively, dopaquinone can undergo an intermolecular reaction with L-cysteine, leading to cysteinyl-DOPA molecules which then convert into benzothiazines as precursors for pheomelanin (see Scheme 1).

Apart from tyrosinase, the pre-melanosomal protein (PMEL; also known as PMEL17, SILV or gp100) has been discussed as an important contributor in the biosynthesis and deposition of MNs. PMEL is an amyloid protein that forms a fibrous matrix inside early stage melanosomes upon which the pigment is deposited. Thus, PMEL captures what would otherwise have been “diffusible” pigments. Regulation of pH levels inside melanosomes is
another important factor determining MN synthesis as the enzymatic or non-enzymatic formation of MNs from DOPA does not proceed in acidic environments.12

Eumelans can readily be synthesized in the laboratory using enzymatic (when starting from tyrosine) or non-enzymatic (when starting from DOPA or other catechols) reaction conditions. Thus, a vast quantity of literature does exist on the synthesis, characterization and applications of eumelans derived from L-DOPA, dopamine or other catecholic precursors.4, 13-15 and references within. Much less literature exists on the synthesis, characterization and potential applications of the pheomelans. The different steps proposed for the in vivo synthesis of pheomelanin are outlined in the review paper by Simon and Peles and are shown in Scheme 1.5 Thus, synthetic 5-S-cysteynydopa is often used as a precursor for the in vitro synthesis of pheomelanins.16-19 The endproducts obtained in some of these studies have been described as brown precipitate or as orange-brown to brown oil.16, 17 Ito established a protocol for the in vitro synthesis of pheomelanin involving the tyrosinase-mediated oxidation of DOPA in the presence of cysteine.20 Reaction mixtures were described as initially pale yellow, followed by the appearance of a reddish color after some lag time. The isolated endproduct of the reaction was described as being dark brown in color.

Related to the various studies involving eumelanin or pheomelanin, the chemistry and physic-chemical properties, particularly the adhesive properties, of polydopamine has received a lot of attention in many reviews and individual reports alike.15, 21, 22 This includes a compilation of papers on polydopamine published in a special Forum in ACS Applied Materials and Interfaces*. Upon evaluation of many such reports, one does note the lack of consistency in the experimental synthesis conditions and the physical properties (color, solubility) of the final product obtained.21, 23-27 Although polydopamine receives a lot of attention, it is clear that one should not limit themselves to the use of dopamine as the catecholic precursor for developing adhesive materials.28, 29

In recent years we have presented various studies on the air- or reactive oxygen species-mediated oxidation of various catecholic compounds into dark- or light-colored pigments.31-38 This report presents our first attempt to generate pheomelanin-like pigments by including L-cysteine into the reaction mixtures. As before, we evaluated our reactions through UV-Vis spectroscopic measurements and size exclusion chromatography (SEC) analyses. The air-mediated oxidation of four catecholamines, DOPA, dopamine, norepinephrine and epinephrine (see Figure 1), was performed using Na2CO3 as the base component to promote the oxidation reaction. The use of Na2CO3 allowed us to explore a co-precipitation method through the addition of CaCl2 at the end of the reaction. The resulting precipitation of CaCO3 served as a matrix for the adhesion and co-precipitation of the black pigment generated in the reactions. SEC analyses of the remaining, lighter-colored, supernatants of these co-precipitation procedures revealed that the black pigment was not necessarily the main reaction product generated. The supernatants contained reaction products that had UV-Vis absorbance features similar to those of the precursor catecholic compounds employed, hinting to the fact that they consisted of oligomeric species containing units of unoxidized precursor compounds. Details of these findings and their possible implications are presented.

* https://pubs.acs.org/toc/aamick/10/9#10YearsofPolydopamineCurrentStatusandFutureDirectionsEditorial
2. Materials and Methods

2.1 Materials and solutions

Dopamine.HCl and L-cysteine.HCl were obtained from Alfa Aesar (Tewksbury, MA, USA). Epinephrine.HCl, norepinephrine.HCl and DOPA were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals used were of analytical grade. Catecholamine stock solutions and dilutions were prepared in 100mM Na-acetate buffer (pH=6.5). L-cysteine stock solutions and dilutions were prepared in 50mM Na₂CO₃ solution.

2.2. UV-Vis spectroscopy

UV-Vis spectroscopic measurements were made in wells of a 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT). For measurements involving absorbance readings below 350nm, UV-transparent microplates were used.

2.3. Fluorescence spectroscopy

Fluorescence measurements were made in wells of an opaque 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT) with excitation filter set at 360nm, emission set at 460nm and sensitivity factor set at 75.

2.4 Size exclusion chromatography (SEC)

SEC analyses were performed on a Breeze 2 HPLC system equipped with two 1500 series HPLC pumps and a model 2998 Photodiode array detector from Waters, Co (Milford, MA). Analyses were performed using an Ultrahydrogel 500 column (300 X 7.8 mm) obtained from Waters, Co (Milford, MA) in isocratic fashion using a mixture of 25mM Na-acetate:methanol:acetic acid (90:10:0.05% v/v) as solvent. The pH of this solvent was measured to be 5.3.

2.5 FT-IR spectroscopy

FT-IR spectroscopic scans were made using the NicoletiS10 instrument equipped with the SmartiTR Basic accessory from ThermoScientific (Waltham, MA). Scans were taken with a resolution of 4 cm⁻¹ between 650 and 4,000 cm⁻¹ at room temperature using a KBr beam splitter and DTGS KBr detector. Each spectrum represents the accumulation of 24 scans.
2.6 Dialysis and freeze drying

Select samples were dialyzed using Spectrum Spectra/Por RC dialysis membranes with molecular-weight-cut-off (MWCO) of 3.5kDa obtained from Fisher Scientific (Suwanee, GA). Select dialyzed materials were frozen overnight and dried using a Labconco FreeZone Plus 4.5L benchtop freeze-dry system obtained from Fisher Scientific (Suwanee, GA).

2.7 Small scale reactions

Stock solutions and dilutions of the catecholamines were prepared in acetate buffer such that their concentrations ranged between 2 and 20mM. Stock solutions and dilutions of L-cysteine were prepared in 50mM Na$_2$CO$_3$ solution such that the L-cysteine concentration varied between 1 and 50mM. In wells of a 96-well microplate, 100μL of each catecholamine dilution and stock solution was mixed with 100μL of 50mM Na$_2$CO$_3$ or 100μL of all L-cysteine dilutions and stock solution. The absorbance at 450, 550 and 650nm was measured every 5 minutes for up to three hours with the plate incubated at 37°C.

2.8 Intermediate scale reactions

For all four catecholamines, stock solutions and dilutions of the catecholamine were prepared in acetate buffer such that their concentrations ranged between 4 and 20mM. Stock solutions and dilutions of L-cysteine were prepared in 50mM Na$_2$CO$_3$ solution such that the L-cysteine concentration varied between 4 and 30mM. In wells of a 24-well plate, 1,000μL of each catecholamine dilution and stock solution was mixed with 1,000μL of 50mM Na$_2$CO$_3$ or 1,000μL of all the L-cysteine dilutions and stock solution. The plate was incubated at 37°C for three days. After three days of reaction photographs were taken of the plates before further processing of any of the reaction mixtures. From each well, a 50μL aliquot was mixed with 150μL water and a spectrum in the visible region was recorded. In addition, from select reaction mixtures, 500μL samples were mixed with either 50μL water or 50μL 1M CaCl$_2$. These latter mixtures were left overnight at room temperature to let precipitations form and settle. Aliquots from the mixtures with water (termed “crude” mixtures) or from the supernatants after precipitation with CaCl$_2$ (termed “supernatants”) were diluted with SEC solvent, centrifuged and analyzed.

2.9 Large scale reactions

For each of the four catecholamines two reactions were set up: one without and one with L-cysteine. A fixed amount of catecholamine was dissolved in 50mL acetate buffer and mixed with 50mL Na$_2$CO$_3$ or 50mL Na$_2$CO$_3$ containing a fixed amount of L-cysteine such that the concentrations were 9mM for both the catecholamine and L-cysteine. The mixtures were kept at 37°C for multiple days till no catecholamine precursor was observed in the SEC analyses of aliquots from these reaction mixtures. The reaction mixtures were divided over 10mL fractions and 1mL of 1M CaCl$_2$ was added to each. The mixtures were centrifuged after overnight standing to let the precipitates settle. The precipitates were pooled and washed repeatedly with water and lyophilized. The supernatants were pooled and dialyzed against repeated changes of water (typically about 3L) and lyophilized. Some 50mL aliquots of the solution outside the dialysis bag after the first dialysis step (typically overnight) were collected and lyophilized. Table 1 presents an overview of the nomenclature used to describe the various fractions thus obtained.
### Table 1: Overview of the fractions from the large scale reactions that were analyzed and are discussed in this report.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude reaction mixture</td>
<td>Untreated reaction mixture</td>
</tr>
<tr>
<td>Precipitate</td>
<td>Washed and dried insoluble material following co-precipitation with CaCO₃</td>
</tr>
<tr>
<td>Crude supernatant</td>
<td>Untreated supernatant from the crude reaction mixture following co-precipitation with CaCO₃</td>
</tr>
<tr>
<td>Dialyzed supernatant</td>
<td>Material from the crude supernatant retained by dialysis bag with MWCO 3.5kDa.</td>
</tr>
<tr>
<td>Dialysate from supernatant</td>
<td>Material from the crude supernatant not retained by dialysis bag with MWCO 3.5kDa.</td>
</tr>
</tbody>
</table>

Whenever possible, FT-IR spectra of all dried materials were recorded. Whenever possible, dilution series of any of the dried materials were prepared in water and UV-Vis and fluorescence spectroscopic measurements were made.

### 3. Results

#### 3.1 Small scale experiments

In these experiments we monitored the increase in absorbance in the visible region as a function of reaction time. Typical results obtained are illustrated in Figure 2, panels A and B. They present the increase in absorbance at 450nm of dopamine in the presence of L-cysteine with varying concentrations for both reagents.

![Figure 2](image-url)

**Panel A**

**Panel B**

**Figure 2**: Increase in absorbance at 450nm as a function of reaction time for the auto-oxidation of dopamine in the presence of L-cysteine with fixed concentration of dopamine and varying concentrations of L-cysteine (Panel A) or with varying concentrations of dopamine and fixed concentration of L-cysteine (Panel B).

A similar pattern of results was observed for absorbance readings at 550 or 650nm (results not shown). The results presented in Figure 2, panel A, indicate that the presence of L-cysteine delayed the increase in absorbance at 450nm. These measurements correlated with visual observations that the emergence of color in the reaction mixtures was delayed in the presence of L-cysteine. In the absence of L-cysteine, the auto-oxidation of dopamine instantly resulted in the formation of a yellow color that quickly darkened. The results presented in Figure 2 indicate that this delay in color formation may be dependent on the concentration of L-cysteine and independent of the concentration...
of dopamine although more detailed studies may be needed to confirm this in a statistical manner. The pattern of results observed for dopamine was observed for the other catecholamines tested (results not shown).

### 3.2 Intermediate scale experiments

As outlined in the Materials and Methods section, reactions between varying concentrations of all four catecholamines and varying concentrations of L-cysteine were set up for three days at 37°C. Figure 3, panels A through D, illustrates photographs taken of the 24-well plates at the end of these experiments.

![Panel A](image1)
![Panel B](image2)
![Panel C](image3)
![Panel D](image4)

**Figure 3:** Photographs of reaction mixtures containing DOPA (panel A), dopamine (panel B), norepinephrine (panel C) or epinephrine (panel D) at, from top to bottom, increasing concentrations between 2 and 11mM and containing L-cysteine at, from left to right, increasing concentrations between 0 and 16mM. The plates were incubated at 37°C for three days upon which the photographs were taken.

The following visual observations could readily be made and were consistent for all four catecholamines. In the absence of any L-cysteine (left columns), the color of the mixtures darkened with increasing concentration of catecholamine present. The presence of L-cysteine consistently resulted in even darker reaction mixtures; an effect
that was particularly true for the case of epinephrine (panel D in Figure 3). Figure 4, panels A through D, illustrate the spectra (visible region) of four-fold diluted reaction mixtures containing 2mM catecholamine (top rows in Figure 3) and between 0 to 16mM L-cysteine.

**Figure 4**: Spectra in the visible region of four-fold diluted mixtures from the top rows shown in Figure 3, panels A through D, of the reaction between DOPA (panel A), dopamine (panel B), norepinephrine (panel C) or epinephrine (panel D) at 2mM and varying concentrations of L-cysteine after three days at 37ºC.

The results presented in Figure 4 illustrate our visual observations that the presence of L-cysteine appeared to “darken” the reaction mixtures. For all four catecholamines tested, the absorbance profiles in the absence of L-cysteine were consistently weaker compared to the profiles in the presence of any of the L-cysteine concentrations tested. This was particularly true for the case of epinephrine (see Figure 4, panel D).

As described in the Materials and Methods section, from select reaction mixtures presented in Figure 3, SEC analyses of “crude” reaction mixtures and “supernatants” of the reaction mixtures were performed. In all instances, the addition of CaCl₂ to the crude reaction mixtures resulted in the formation of a very dark precipitate and a supernatant solution that was yellow or rusty-orange in color (photographs of such observations will be presented in Section 3.3). Figures 5 through 8 illustrate 3D profiles of the SEC analyses obtained from select reaction mixtures shown in Figure 3. For each selected reaction mixture, the SEC profiles for the “crude” and “supernatant” are
presented and for each compound tested, SEC profiles for the reaction mixtures without L-cysteine and in the presence of a select concentration of L-cysteine are shown. The inserts shown in all these 3D plots illustrate the UV-Vis spectra of the sample at its peak retention time.

Figure 5, panels A through D, show results obtained for DOPA tested at 7mM (third row from top in Figure 3, panel A) without (first column from left in Figure 3, panel A) or with L-cysteine tested at 4mM (third column from left in Figure 3, panel A). Independent of the presence of L-cysteine, these “crude” reaction mixtures were black in color (with no signs of precipitations), while the “supernatant” samples were yellow in color.
Panel B

Panel C
Panel D

**Figure 5:** SEC profiles (between 12 and 14 min retention time) of tenfold diluted crude mixture (panels A and C) and supernatant (panels B and D) from reaction mixtures containing DOPA (7mM) in the absence (panels A and B) or presence (panels C and D) of L-cysteine (4mM). The inserts illustrate the UV-Vis spectrum of the peak with a retention time of 12.7 min.

Despite the dramatic visual differences between the “crude” samples and the “supernatant” samples (black vs. yellow color), the SEC analyses reveal very little qualitative differences between both; independent of the presence of L-cysteine. All SEC profiles are dominated by a single peak with a UV-Vis spectrum that shows a declining absorbance with increasing wavelength, but containing a very distinct absorbance band around 280nm. However, the spectrum obtained for the main peak in the crude reaction mixture containing DOPA and L-cysteine (insert of panel C) does show a second, much weaker, absorbance band around 330nm. Overall the SEC profiles of the supernatants do differ from the crude mixtures in that a reduced absorbance over the entire UV range of the spectrum can be observed. This can be seen in the 3D plots in the form of the strong absorbance band at 280nm superimposed on a more “tilted” surface in the case of the crude mixtures and a more “flat” surface in the case of the supernatant samples. Thus, the precipitation with CaCl$_2$ resulted in the removal of a black pigment from the crude reaction mixtures, but appeared not to affect the compound(s) with the strong absorbance band around 280nm.

Figure 6, panels A through D, show results obtained for dopamine tested at 7mM (third row from top in Figure 3, panel B) without (first column from left in Figure 3, panel B) or with L-cysteine tested at 4mM (third column from left in Figure 3, panel B). Independent of the presence of L-cysteine, these “crude” reaction mixtures were black in color (with no signs of precipitations), while the “supernatant” samples were yellow in color.
Panel A

Panel B
Figure 6: SEC profiles (between 12 and 14 min retention time) of tenfold diluted crude mixture (panels A and C) and supernatant (panels B and D) from reaction mixtures containing dopamine (7mM) in the absence (panels A and B) or presence (panels C and D) of L-cysteine (4mM). The inserts illustrate the UV-Vis spectrum of the peak with a retention time of 13.2 min.

In general, our observations for the mixtures involving dopamine are very similar to what was observed for DOPA. Despite the dramatic visual differences between the “crude” samples and the “supernatant” samples (black...
vs. yellow colored), the SEC analyses reveal very little qualitative differences between both; independent of the presence of L-cysteine. All SEC profiles are dominated by a single peak with a UV-Vis spectrum that shows a declining absorbance with increasing wavelength, but containing a very distinct absorbance band around 280nm.

For all reaction mixtures, independent of the presence of L-cysteine, the precipitation with CaCl₂ did lead to a reduced absorbance over the UV range of the spectrum. This is clear from comparing the overall SEC 3D profiles; panel A vs. panel B and panel C vs. D. In both cases, the profiles of the crude reaction mixtures (panels A and C) show the main peak on top of a “slanted surface”, while the profiles of the supernatant samples (panels B and D) show the main peak on top of a much “flatter surface”. Similar observations can be made from comparing the UV-Vis spectra of the inserts shown in the panels of Figure 6.

Figure 7, panels A through D, show results obtained for norepinephrine tested at 7mM (third row from top in Figure 3, panel C) without (first column from left in Figure 3, panel C) or with L-cysteine tested at 4mM (third column from left in Figure 3, panel C). Independent of the presence of L-cysteine, the “crude” reaction mixtures were black in color (with no signs of precipitations), while the “supernatant” samples were rusty-orange in color.
Figure 7: SEC profiles (between 12 and 14 min retention time) of tenfold diluted crude mixture (panels A and C) and supernatant (panels B and D) from reaction mixtures containing norepinephrine (7mM) in the absence (panels A and B) or presence (panels C and D) of L-cysteine (4mM). The inserts illustrate the UV-Vis spectrum of the peak with a retention time of 13.2 min.

As for the cases of DOPA and dopamine, the “crude” and “supernatant” samples were visually dramatically different. The SEC profiles obtained for both sets of crude and supernatant samples obtained from the reaction mixtures, were dominated by a single a peak with a retention time of about 13.2 minutes. This peak exhibited a distinct and sharp absorbance maximum around 280nm. However, for the reaction mixture containing L-cysteine (Figure 7, panels C and D), the spectrum exhibited a distinct shoulder around 320nm visible in the SEC analyses for both the crude and supernatant samples. As for the reaction with DOPA and dopamine, the supernatants of the reaction mixtures with norepinephrine, with or without L-cysteine, exhibited a reduced absorbance over the entire UV range of the spectrum. This is clear from comparing the overall SEC 3D profiles shown; i.e., comparing panel A vs. panel B and comparing panel C vs. D. In both cases, the profiles of the crude samples (panels A and C) show the main peak on top of a “slanted surface”, while the profiles of the supernatant samples (panels B and D) show the main peak on top of a much “flatter surface”. Similar observations can be made from comparing the UV-Vis spectra of the inserts shown in the panels of Figure 7.

Figure 8, panels A through D, show results obtained for epinephrine tested at 7mM (third row from top in Figure 3, panel D) without (first column from left in Figure 3, panel D) or with L-cysteine tested at 4mM (third column from left in Figure 3, panel D). The reaction mixture in the absence of L-cysteine was orange-rusty in color and following precipitation with CaCl₂ a minimal amount of dark precipitate was formed with the supernatant having a rusty-orange color. In the presence of L-cysteine, the reaction mixture was much darker in color. Precipitation with CaCl₂ yielded much more dark precipitate and the supernatant was rusty-orange colored.
Figure 8: SEC profiles (between 12 and 14 min retention time) of tenfold diluted crude mixture (panels A and C) and supernatant (panels B and D) from reaction mixtures containing epinephrine (7mM) in the absence (panels A and B) or presence (panels C and D) of L-cysteine (4mM). The inserts illustrate the UV-Vis spectrum of the peak with a retention time of 13.2 min.

The SEC profiles for the reactions with epinephrine are drastically different from those obtained for the reaction mixtures involving the other catecholamines. As for the other catecholamines, all epinephrine appeared to have
reacted away after three days of reaction at 37°C. The UV-Vis spectrum of the resulting materials exhibited distinct absorbance maxima around 255, 280 and 315 nm. The inserts shown in the plots above correspond to the UV-Vis spectra of the peak with retention time of about 13.2 min. However, the 3D plots clearly show the mixtures contain multiple different peaks with closely matching retention times. As observed for the other catecholamines, the coprecipitation with CaCO₃ leads to a reduced absorbance in the UV range (particularly noticeable in the very low UV range) while the other aspects of the UV-Vis spectra appear not to be affected by the co-precipitation process.

It is worth noting that the UV-Vis spectra of the main peak in the SEC profiles of the samples involving reactions with epinephrine show a very small, but noticeable maximum around 711 nm. In their study of the properties of eumelanin, using experimental and computational approaches, Chen et al. evaluated the broad band optical absorptions of small and large scale systems built from oligomeric and polymeric species of oxidized dopamine. In their study they predicted, and observed, a specific absorption band around 724 nm that was attributed to a tetrameric, porphyrin-like structure. Alternatively, Micillo presented UV-Vis spectra obtained during the oxidation of DHI dimer quinones (typical intermediates obtained in the synthesis of eumelanin from DOPA; see Scheme 1). They observed a very faint absorbance band around 725 nm. Thus, we could speculate that the observed absorption band around 711 nm suggests the presence of an oligomeric species of oxidized epinephrine.

In general, the limited SEC analyses that were performed on the mixtures shown in Figure 3, panels A through D, indicated that in the presence of the catecholamine at its highest concentrations and L-cysteine at its highest concentration, unreacted catecholamine could be detected in their SEC profiles after three days of reaction at 37°C. This suggested that the presence of L-cysteine at its higher concentrations delayed the consumption of the precursor compound. Apart from the analyses discussed above, visual observations confirmed the findings of the small scale experiments that the presence of L-cysteine, particularly at its highest concentrations, delayed the formation of color significantly; up to more than 10 hours. After three days of reaction at 37°C, the reaction mixture involving dopamine at its highest concentration and L-cysteine at its highest concentration (Figure 3, panel B; lower right corner), exhibited black-colored precipitate and the walls of the well were coated with black pigment.

Overall our observations and SEC analyses indicated that the air-mediated oxidation of the four catecholamines, independent of the presence of L-cysteine, leads to the generation of at least two different materials. One has a very dark, black color and readily co-precipitates with CaCO₃. The other has no or a much lighter color and does not co-precipitate with CaCO₃. These materials exhibited UV-Vis spectra with a strong absorbance band around 280 nm. In the case of epinephrine, there are clear indications that more than two compounds were generated; some with UV-Vis spectra exhibiting a distinct absorbance band around 315 nm. The presence of L-cysteine appeared not to affect these basic findings. However, the presence of L-cysteine, depending on the concentration present, resulted in: a) darker colored reaction mixtures, b) delayed formation of any colored material and c) slower consumption of the starting catecholamine.
3.3 Large scale experiments

All reaction mixtures of the large scale experiments, with the exception of the mixture containing epinephrine in the absence of L-cysteine, were black in appearance at the end of the reaction time. Upon addition of CaCl₂ and overnight settling, all mixtures exhibited black precipitations, but the supernatant solutions were yellow in color in the cases of L-DOPA and dopamine or rusty-orange in the cases of norepinephrine and epinephrine. The photographs shown in Figures 9 through 12 illustrate some of these observations.

![Panel A](image1.png)  ![Panel B](image2.png)

**Figure 9:** Pooled crude supernatants collected from the reaction mixtures containing DOPA (panel A) and DOPA with L-cysteine (panel B) as described in the Materials and Methods section.
Figure 10: Panel A Comparison of the crude reaction mixture involving dopamine without L-cysteine before (left in Panel A) and after (right in Panel A) co-precipitation with CaCO₃. Panel B Comparison of the pooled crude supernatants from the reaction mixtures involving dopamine (panel B) and dopamine with L-cysteine (panel C) as described in the Materials and Methods section.

Figure 11: Pooled crude supernatants collected from the reaction mixtures containing norepinephrine (panel A) and norepinephrine with L-cysteine (panel B) as described in the Materials and Methods section.
Figure 12: Comparison of the crude reaction mixtures involving epinephrine without L-cysteine (panel A) and with L-cysteine (panel B) before (left in Panels A and B) and after (right in Panels A and B) co-precipitation with CaCO$_3$ as discussed in the Materials and Methods section.

The photographs shown in Figures 9 through 12 illustrate and confirm the visual observations made from the intermediate scale experiments: the dark reaction mixtures obtained through the air-mediated oxidation of the catecholamines, independent from the presence of L-cysteine, can be fractionated into a black precipitate and a lighter-colored supernatant following a co-precipitation process with CaCO$_3$. It is worth noting that for the reaction involving epinephrine without L-cysteine (Figure 12, panel A) very little black precipitate was obtained.

The following sets of figures compare the FT-IR spectra of the washed and dried precipitates from the reaction mixtures involving all four catecholamines with or without L-cysteine. Included in these figures is a spectrum of precipitated and washed CaCO$_3$ prepared in our laboratory using the same conditions as for the setup of the large scale reactions involving the catecholamines.
Panel A

Panel B
Figure 13: Comparison of the FT-IR spectra of the washed and dried precipitates for reactions involving DOPA (panel A), dopamine (panel B), norepinephrine (panel C) or epinephrine (panel D) with or without L-cysteine as discussed in the Materials and Methods section.
The FT-IR spectrum of CaCO$_3$ exhibits a sharp, weak peak at wavenumber 874 cm$^{-1}$ and sharp, strong peaks at wavenumbers 1,408 and 1,440 cm$^{-1}$. These features are visible in the FT-IR spectra of all the precipitates obtained from the reaction mixtures involving all four catecholamines, with or without L-cysteine. This indicates that the black pigments co-precipitated with CaCO$_3$. The absorbance features at 1,408 and 1,440 cm$^{-1}$ attributed to CaCO$_3$ somewhat interfere with the typical absorbance bands around wavenumbers 1,350 and 1,560 cm$^{-1}$ one can observe in spectra of MN-like pigments. The FT-IR spectra obtained for all the precipitates exhibited absorbance bands, flanking the absorbance regions associated with CaCO$_3$, that are within the range of the typical absorbance bands associated with MN materials. In addition, all precipitates exhibited a strong and broad absorbance band in the 3,200 to 3,400 cm$^{-1}$ range that was not observed in the sample of CaCO$_3$. In general, for all four catecholamines, the FT-IR spectra of the precipitates exhibited features that hint to the presence of melanin-like materials mixed with CaCO$_3$. It is important to note that no qualitative differences could be observed in the FT-IR spectra for the precipitates obtained from any of the reactions in the absence or presence of L-cysteine.

As outlined in the Materials and Methods section, the supernatants from the crude reaction mixtures were dialyzed against water using a dialysis membrane with MWCO of 3.5kDa. It was observed that during this dialysis process the lighter colored supernatants inside the dialysis bag would lose some of that color. This made us suspect that some of the supernatant material was diffusing through the dialysis membrane and 50mL aliquots were collected and dried as outlined in the Materials and Methods section. The suspicion that supernatant material was diffusing out of the dialysis membrane was confirmed from SEC analyses (see below) and the fact that following freeze drying of the dialyzed supernatant mixture, very little material was often recovered (2-3mg in the cases of DOPA and dopamine; about 10mg in the cases of norepinephrine and epinephrine). Figure 14 presents a comparison of the SEC profiles obtained for the crude reaction mixture, crude supernatant, dialyzed supernatant and dialysate from supernatant (see Table 1 in Materials and Methods section) involving the reaction of DOPA in the absence of L-cysteine.

Figure 14: SEC profiles of the various fractions obtained for the large scale reaction involving DOPA in the absence of L-cysteine as outlined in the Materials and Methods section. The right axis applies to the “dialysate from supernatant” sample.
The SEC profiles shown in Figure 14 indicate that, as observed during the evaluations of the intermediate scale reactions, the co-precipitation with CaCO₃ does not lead to significantly different SEC profiles. Despite the dramatic differences in visual appearance, black solution for the crude reaction mixture vs. yellow solution for the crude supernatant mixture (see Figure 9, panel A), very little qualitative or quantitative differences can be observed in the SEC profiles of both. This indicated that the black pigment generated in the reaction may not be the main reaction product. Following dialysis against water (at least three changes of water in two days), the dialyzed supernatant solution appeared to have lost significant amounts of material as observed from the SEC analysis shown in Figure 14. It is worth noting that the aliquot analyzed from the dialyzed supernatant solution was diluted in the same way as for the aliquot analyzed from the crude supernatant or crude reaction mixture. The SEC profile of the dialysate exhibited the same qualitative aspects as for the SEC analyses of all the other fractions, despite the significant dilution factor involved: 100mL reaction mixture inside the dialysis membrane surrounded by 3L of water and twofold dilution with SEC solvent of the aliquot prior to analysis (see Figure 14). This latter observation confirmed that during the dialysis process some reaction product(s) diffused through the dialysis membrane. As this was an unexpected outcome of our experiments, we have not yet established any procedures to purify and characterize this/these reaction product(s). The observations made for DOPA and shown in Figure 14 were observed for the reactions of the other catecholamines and were independent of the presence of L-cysteine.

Figure 15, panels A through D, present the FT-IR spectra of the dried dialyzed supernatants from the reaction mixtures of all four catecholamines, with or without L-cysteine.
Panel B

Panel C
The FT-IR spectra of the dialyzed supernatants exhibited all the typical absorbance features attributed to melanin-like materials.\textsuperscript{41, 42} We suspect that this material represents left-over pigment that was not precipitated in the CaCO\textsubscript{3} co-precipitation process but was retained by the dialysis membrane with MWCO of 3.5kDa. In addition, very little qualitative differences can be observed between the materials prepared in the absence or presence of L-cysteine.

Figure 16, panels A through D, illustrate the UV-Vis spectra of the dried dialyzed supernatant materials, redissolved in water at a concentration of 0.15 mg/mL, for the reaction mixtures involving all four catecholamines with or without L-cysteine.
Figure 16: UV-Vis spectra of the dried dialyzed supernatant fractions obtained for the large scale reactions involving DOPA (panel A), dopamine (panel B), norepinephrine (panel C) and epinephrine (panel D), with or without L-cysteine, as outlined in the Materials and Methods section.

In the UV-Vis spectra presented in Figure 16, qualitative differences can be observed for reaction mixtures with or without cysteine. This was particularly true for the case of DOPA (panel A). In the absence of any L-cysteine, the dialyzed supernatant material showed a very strong and narrow absorbance band around 280nm. Such a feature was not present in the sample from the reaction mixture containing DOPA and L-cysteine. The spectrum of the material obtained from the reaction between norepinephrine and L-cysteine (panel C) shows a distinct absorbance band at 320nm, which is not present in the spectrum of the material obtained from the reaction involving norepinephrine without L-cysteine. No strong absorbance bands around 320nm were present in the spectra of the materials obtained from epinephrine, with or without L-cysteine, despite such features can be observed in the UV-Vis spectra of the main peaks in the SEC profiles of the crude reaction mixtures (see Figure 8).

Figure 17, panels A through D, illustrate the UV-Vis spectra obtained from the dried dialysates, redissolved in water at a concentration of 2mg/mL, of all the reaction mixtures as outlined in the Materials and Methods section.
Figure 17: UV-Vis spectra of the dried dialysates of the supernatant fractions obtained for the large scale reactions involving DOPA (panel A), dopamine (panel B), norepinephrine (panel C) and epinephrine (panel D), with or without L-cysteine, as outlined in the Materials and Methods section.

Before making any comparisons, one should be aware that the spectra shown in Figure 17 were obtained from dried dialysate fractions that are presumed to contain the buffer salts used in the reaction mixtures (Na acetate) and CaCl$_2$ that was added for the coprecipitation process. However, it was presumed that these salts would have little significant impacts in the UV-Vis spectra for wavelengths above 230nm. In the UV-Vis spectra shown in Figure 17 qualitative differences between the reaction mixtures with or without L-cysteine can be observed. The spectra exhibit fairly similar patterns as the corresponding spectra shown in Figure 16. An exception to these comparisons involves the spectra of the samples obtained from the reaction mixtures containing epinephrine (compare panels D in Figures 16 and 17). The spectra shown in Figure 17, panel D, contain a distinct absorbance band around 330nm that cannot be observed in the corresponding spectra of the dialyzed supernatant fractions as shown in Figure 16, panel D.
Figure 18 presents the fluorescence emission measured as a function of concentration for the dialysate and dialyzed supernatant fractions obtained from all reaction mixtures as outlined in the Materials and Methods section. The dialysate materials are not purified and are presumed to contain the buffer salts used in the reaction mixtures (Na acetate) and CaCl$_2$ that was added for the coprecipitation process. However, it was presumed that these salts would have little significant impacts in the fluorescence emission properties. Thus, in the case of the dialysate materials, only qualitative evaluations should be made.

**Figure 18:** Fluorescence emission as a function of concentration of the dried and redissolved dialyzed supernatants (panels A and B) and dialysates (panels C and D) obtained from the reaction mixtures containing the catecholamines without (panels A and C) or with (panels B and D) L-cysteine as outlined in the Materials and Methods section.

In general the fluorescence emission appeared to be independent of the concentration for the dialyzed supernatant materials, while the fluorescence emission increased as a function of the concentration for the dialysates materials.
4. Discussion

Based upon our findings and observations we would like to raise a number of points of (future) research and discussion and some hypotheses.

1. As is well established, when catecholamines are exposed to air in an alkaline environment, a dark-colored solution/precipitate is readily obtained. In the hope of obtaining pheomelanin-like, lighter-colored materials, we mixed the catecholamines with varying concentrations of L-cysteine. However, much to our surprise, the addition of L-cysteine resulted in darker-colored solutions, particularly in the case of epinephrine, as can be observed from the photographs shown in Figure 3. For all compounds tested, the presence of L-cysteine delayed the appearance of a dark color in the reaction mixtures in a concentration-dependent manner (see Figure 2). In addition, SEC analyses performed on the intermediate scale and large scale experiments indicated that reaction mixtures containing L-cysteine needed more time to completely react away the starting precursor. Thus, all our observations combined indicate that the presence of L-cysteine slows down the air-mediated oxidation of catecholamines. **The presence of L-cysteine during the air-mediated oxidation of catecholamines leads to darker-colored pigments; not reddish or lighter-colored pigments that would visually resemble pheomelanin-like pigments.**

2. We chose Na$_2$CO$_3$ as the base component to initiate the air-mediated oxidation of the catecholamines. This allowed, upon addition of CaCl$_2$, for a co-precipitation process whereby the black pigment generated in our reactions was bound to CaCO$_3$ and removed from the reaction mixture following centrifugation. In many instances, the supernatant resulting after the centrifugation of the precipitates had a light yellow to orange-rusty color (see Figures 9 through 12). This prompted us to compare the SEC profiles obtained for the crude reaction mixtures with the SEC profiles obtained for the supernatants resulting from this co-precipitation process (see Figures 5 through 8). Despite the dramatic visual differences, black-colored crude reaction mixture vs. light-colored supernatant solution, very little quantitative and qualitative differences could be observed between the SEC profiles of the crude reaction mixture and the supernatant solutions. **SEC analyses suggested that the black pigment generated during the air-mediated oxidation of catecholamines is not necessarily the main reaction product.**

3. The co-precipitation process we employed in our studies is a reflection of the capacity of MN-like materials to adhere to virtually any surface available. However, it is in stark contrast with discussions that MN-coatings need to be deposited in situ; that pre-formed MN materials will not adhere to available surfaces.\cite{3, 44} Preliminary experiments conducted on the side of this report indicated that the black pigments generated from the catecholamines can co-precipitate with, and adhere to, a wide variety of water-insoluble minerals (carbonates, hydroxides, phosphates, etc. or Ca$^{2+}$, Cu$^{2+}$, Fe$^{2+/3+}$, Zn$^{2+}$, etc. based minerals). In addition, this co-precipitation process is not limited to nitrogen-containing precursors as initial experiments indicate that, e.g., pyrogallol-based MN-like materials, co-precipitate with CaCO$_3$.\footnote{Personal communication} However, there may be differences in the efficiency with which a particular mineral co-precipitates a particular pigment and this aspect is currently under investigation. **The pre-
formed, dark-colored pigments obtained through the air-mediated oxidative melanogenesis process can readily be deposited on insoluble mineral surfaces using an in situ co-precipitation procedure.

4. In the cases of DOPA and dopamine (see Figures 5 and 6), the SEC profiles of the crude reaction mixtures and the supernatants appear to be dominated by a single peak with absorbance over a broad range, but exhibiting a very sharp and strong absorbance band around 280nm. This UV-Vis pattern is very similar to that described for polydopamine or melanin-like materials obtained from DOPA or DHI containing unoxidized precursor units.23, 26, 40 A similar UV-Vis pattern was observed for the case of norepinephrine (see Figure 7), but the peak exhibited a shoulder around 320nm in addition to the absorbance band around 280nm. The case of epinephrine appeared to be far more complex (see Figure 8). The SEC profiles exhibited the presence of more than one peak with varying UV-Vis absorbance profiles. The combined observations made from the intermediate and large scale experiments, particularly the comparison of the SEC, FT-IR and UV-Vis analyses of the various fractions (see Figures 14 through 17), leads us to propose a hypothesis that the air-mediated oxidation of catecholamines yields a solution or dispersion of at least two distinct oligo- or polymeric materials that are non-covalently bonded to each other. One material would constitute an insoluble, black pigment similar to the “classic” eumelanin materials. The other material would constitute a soluble, much lighter, if not colorless, material that exhibits a UV-Vis spectrum dominated by sharp absorbance bands akin to unoxidized precursor units. The differences in UV-Vis spectra of soluble dopamine-eumelanin and dopamine-eumelanin deposited on the surface of quartz slides, particularly the loss of the sharp absorbance band at 280nm for the deposited material, presented by Ball et al. could support our above-mentioned hypothesis.26, 42 The air-mediated oxidation of catecholamines leads to a binary product that contains an insoluble, melanin-like substance and a soluble, oligo- or polymeric substance containing unoxidized precursor units.

5. In an earlier report we discussed the presence of strong absorbance bands around 280nm in DOPA-based MN-like materials and attributed this to the possible presence of unreacted precursor or intermediates, physically embedded into the MN-material.34 In that report we also discussed that dialysis against salt improved the removal of these “impurities”. We now believe this to be wrong and that the DOPA-based materials generated then were a mixture of at least two oligo- or polymeric materials that could be separated through the process of dialysis, particularly in the presence of salt. Putting these earlier observations into the context of the hypothesis discussed in point 4, and based upon our current observations, we speculate that ionic interactions or cation-π interactions46, 47 or π-π interactions48 hold the two distinct materials together. In the co-precipitation process, the addition of excess Ca2+ (or any other cation) could lead to the disruption of any ionic or π-based interactions47, releasing the insoluble, black pigment from its counterpart and adhering instantly to the in situ formed insoluble CaCO3 (or any other mineral). The notion that the oxidation of catecholamines leads to a binary product may explain the ongoing discussions (and inconsistencies) in trying to elucidate the precise chemical structure of polydopamine or eumelanin and the origins of the broadband absorbance profiles of these materials.13, 21, 22, 24, 25, 39, 49, 50 All too often melanogenesis research is focused on the, visually dramatic, colored pigments that are formed; ignoring the lighter-colored or colorless substances that may be generated and may be more dominant. Whether our hypothesis regarding the in vitro melanogenesis process yielding a binary material has any implications for the in vivo melanogenesis
process remains to be seen. We would like to suggest that when studying the *in vivo* melanogenesis process one should be aware that the dark eumelanin pigment, as captured and immobilized by the pre-melanosome protein PMEL<sup>4,11</sup>, may not be the only (not even the dominant) product generated. One should be aware of the possibility that a soluble, lighter-colored material may have been generated with its own cell-biological properties. **The melanogenesis process leads to a binary product involving a non-covalently bonded combination of dark-colored pigment and a lighter-colored or colorless substance; the latter being understudied or ignored in the *in vitro* or *in vivo* studies of the melanogenesis process.**

6. As discussed in point 1., the presence of L-cysteine slows the reaction and creates darker colored solutions. These current observations and a number of loose-end observations made over the years, suggests to us that the kinetics of the reaction could be an important factor determining the physical properties (color, solubility) of the pigments generated during the air-mediated oxidation of catecholamines. When the reaction proceeds slowly, as evaluated through the analysis of the disappearance of the precursor, the material generated will be black and can precipitate without the need for any co-precipitation process; often staining the sides of the reaction container. When the reaction proceeds faster, a diffusible material, brown to black in color, is obtained without any observable precipitations or staining of the reaction container. From these latter types of mixtures, a black pigment can be precipitated through a co-precipitation process as described in this report. Adding these observations to our earlier-stated hypothesis (see point 4.), we suspect that during the melanogenesis process, the kinetics of the reaction may be an important factor determining the balance or ratio between the soluble, light-colored material and the insoluble, black material that comprises the binary material generated from the catecholic precursor. Slow reactions will lead to black solutions or precipitates (akin to eumelanin), while faster reactions will lead to brown-colored solutions from which a black pigment (akin to eumelanin) can be co-precipitated; leaving behind a light-colored supernatant that contains a material akin to poly-DOPA, -dopamine, norepinephrine or –epinephrine. **The kinetics of the melanogenesis process may determine the balance between insoluble and soluble components of the binary product generated; the slower the reaction the more dark-colored, insoluble pigment generated.**

7. Our observations that the melanogenesis process may result in the generation of a binary complex may be a reflection of the different reaction pathways the catecholamines can undertake during their oxidation reactions as discussed by Della Vecchia et al. They discussed three different reaction pathways: a) intramolecular cyclization followed by polymerization (similar to what is outlined in Scheme 1), b) polymerization through intermolecular C-C bonds between individual catecholamine units followed by cyclization and polymerization and c) a hybrid version of these two other pathways. In this context it is unclear to these authors why the possibility of an intermolecular reaction involving the primary amines of the catecholamines is not considered as a possible reaction pathway, leading to C-N bonded catecholamine units. Such intermolecular substitution reaction could be considered a direct competition for the intramolecular substitution reaction typically discussed in the reaction pathways leading to MNs. This point of discussion is outlined in Scheme 2.
Scheme 2: Comparison between the intra- and intermolecular substitution reactions possible for the quinone form of dopamine.

Intermolecular nucleophilic substitution that prevent indole ring closure was proposed by Ambrico et al. for their studies of the polymerization between dopamine and 3-aminotyrosine or p-phenylenediamine.\(^2^3\) In addition, nitrogen-containing buffer components like Tris have been observed to be covalently incorporated into polydopamine materials\(^2^4\) and recommendations to avoid such buffer components have been made.\(^1^5\) In general, catechols are very reactive chemical species and could be involved in many different types of reactions\(^5^1\), including reactivity towards amino acids or amines in general.\(^5^2\) The reactivity between catechols and amines is exploited in the development of multifunctional coatings through a reaction between a catechol and diamine species.\(^2^8, 30, 5^3, 5^4\)

One should consider the possibility of intermolecularly, N-to-C, bonded units of catecholamines when evaluating the structure of melanins, polydopamines, etc.

8. Our observations regarding the effect of L-cysteine on the air-mediated oxidation of catecholamines was unexpected. Unexpected observations, i.e., deviating from published reports, were also made regarding the effect of the presence of amino acids on the oxidation of chlorogenic acid.\(^5^5\) In that particular study, it was observed that L-cysteine, L-serine and L-threonine created a green pigment that disappeared fairly quickly, while many other amino acids yielded a permanent green pigment (or red in the case of tryptophan). Similarly, the air-mediated oxidation of pyrogallol in the presence of L-cysteine yielded a greenish color in the initial minutes of the reaction, which disappeared within the hour.\(^1\) In addition, L-proline and L-hydroxyproline have been observed to create a temporary red-pinkish color during the oxidation process of catechol\(^5^6\) and this was confirmed in our laboratory.\(^8\) There is a need for a systematic study of the effect of amino acids (beyond just L-cysteine) and amines in general on the melanogenesis process.

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\(^1\) Personal observations.
\(^8\) Personal observations.
References

2. d'Ischia, M., Wakamatsu, K., Cicoira, F., Di Mauro, E., Garcia-Borron, J. C., Commo, S., Galván, I.,
   Ghanem, G., Kenzo, K., Meredith, P., Pezzella, A., Santato, C., Sarna, T., Simon, J. D., Zecca, L.,
   1452-1460.
   System* (Nordlund, J. J., Boissy, R. E., Hearing, V. J., King, R. A., Oetting, W. S., and Ortonne, J., Eds.),
   pp 261-281, Blackwell Publishing Ltd.
7. Maranduca, M. A., Branisteanu, D., Serban, D. N., Branisteanu, D. C., Stoleriu, G., Manolache, N.,
   4183-4187.
   (HsTYRP1) Solves an Old Problem and Poses a New One, *Angew Chem Int Ed Engl* 56, 14352-14354.
    *Protein Sci* 24, 1360-1369.
   Wakamatsu, K., Larsson, J., Ulfendahl, M., Kulle, K., Raap, G., Kerje, S., Hallbook, F., Marks, M. S.,
   and Andersson, L. (2011) Inactivation of Pmel alters melanosome shape but has only a subtle effect on
    pigmentation by regulating melanosome pH and size, *Proceedings of the National Academy of Sciences
    of the United States of America* 113, 5622-5627.
    human red hair pheomelanin: benzothiazolylthiazinodihydroisoquinolines as key building blocks, *J Nat
    Prod* 74, 675-682.
    2,2′-bi(2H-1,4-benzothiazine): tracking the biosynthetic pathway of trichochromes, the characteristic
    of Pheomelansins: A Reassessment†, *Photochemistry and Photobiology* 84, 593-599.


