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# Evidence for covalent lipoyl adduction with dopaquinone following tyrosinase-catalyzed oxidation

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**Abstract**

Previous studies have examined the conjugation of sulfhydryl compounds such as L-cysteine and glutathione with DOPA-quinone following the oxidation of tyrosine and DOPA by tyrosinase. These covalent reactions play a key role in the regulation and metabolism of pigment cells. We report on the first direct evidence for the formation of lipoyl adducts in reactions of thiol groups with DOPA-quinone in dihydrolipoic acid (6,8-dimercaptooctanoic acid [DHLA]). Incubating DHLA with DOPA-quinone followed by tyrosinase-catalyzed oxidation resulted in the three products predicted by HPLC-UV and LC-ESI$^-$-MS analyses for DHLA DOPA conjugates. In the current study, we identified 5-*S*-lipoyl-DOPA among the principal products isolated by HPLC and characterized by FAB$^-$-MS, ESI$^-$-MS/MS, and $^1$H NMR, 2D-COSY studies. Collectively, these results suggest that DHLA undergoes sulfhydryl conjugation with DOPA-quinone, pointing to the involvement of thiol-reactive metabolites.


*Keywords:* Dihydrolipoic acid; DOPA; Quinone; Tyrosinase; Thiol; Melanin

Primarily synthesized within melanocytes, melanin contributes to the pigmentation of the skin, hair, brain, and eyes [1,2]. Melanin synthesis in mammals is regulated by several specific enzymes that participate in pigment-producing reactions. Tyrosinase plays a critical role in catalyzing the initial and rate-limiting reactions in melanogenesis, which involve tyrosine hydroxylation to 3,4-dihydroxyphenylalanine (DOPA), DOPA oxidation to DOPA-quinone, and 5,6-dihydroxyindole oxidation to indole-5,6-quinone [3–5]. Other relevant proteins including tyrosinase-related protein 1 (oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomer into melanin) and 2 (rearrangement of DOPAchrome to DHICA) function at points downstream in the cascade of pigment-producing reactions [6,7], although these reactions proceed under active regulatory control [8].

Melanins occur in animals as one of two basic types: eumelanin, derived from DOPAchrome metabolites, and pheomelanin, derived from cysteinyl DOPA metabolites [8]. The chemical and biological characteristics indicate that both melanins are heterogeneous biopolymers with subunits consisting of intermediate products derived from DOPA-quinone. Sulfhydryl compound(s) such as cysteine and/or glutathione (GSH) combine with DOPA-quinone in a reaction that proceeds faster than the series of spontaneous reactions involving cyclization, decarboxylation, and polymerization, which is known as the Raper–Mason pathway [9–12]. The ratio of those compounds within pigment cells has been implicated as a key determinant for an alternative pathway resulting in sulfur-containing pigments: in the presence of thiol groups, these compounds react rapidly with DOPA-quinone. Essential to the production of the cysteinyl DOPAs required for pheomelanin, these covalent reactions with thiols play a key role in regulating the proportions of the resulting products.

α-Lipoic acid (LA) including dihydrolipoic acid (DHLA), its reduced form, is a well-known prosthetic

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group in the α-keto acid dehydrogenase complexes of the mitochondria, playing a key role in metabolism [13,14]. Experimental studies and clinical trials clearly indicate that LA also has strong potential as an endogenous thiol antioxidant capable of altering the redox status of cells and interacting with thiols and other antioxidants [15,16]. Interestingly, in a neurochemical study, Spencer et al. [17] have shown that DHLA, not LA, inhibits the conjugation of nonenzymatically oxidized catecholamines, *o*-quinone of DOPA and dopamine (DA), with cysteine and GSH in vitro. The authors raise the possibility that this inhibition may result in the formation of an adduct between DHLA and the catecholamine. However, the chemical reactivity of sulfhydryl groups in LA and DHLA with DOPA-quinone is poorly understood due to the lack of direct evidence. In the course of this study, using HPLC with UV detection and LC/ESI-MS in a Q1-MS scan, we found that DHLA, not LA, reacted effectively with DOPA in the presence of tyrosinase to form lipoyl adducts. The present study characterizes 5-*S*-lipoyl-DOPA, one of the main adducts, as a novel thiol conjugation product.

## Materials and methods

*Materials.* L-DOPA, DHLA, and tyrosinase from mushroom (EC 1.14.18.1) were purchased from Sigma Chemical Corp. (St. Louis, MO). LA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of the highest grade commercially available.

*Spectroscopy.* $^1$H NMR spectra were recorded on a JEOL (Tokyo, Japan) JNM-AL 400 NMR spectrometer system. Fast atom bombardment-mass spectrometry (FAB-MS) was performed with a JEOL JMS-SX102 mass spectrometer. UV–visible spectra were recorded on a JASCO Corp. (Tokyo, Japan) Model MD-2010 plus multi-UV detector.

*DOPAchrome formation and effects of LA and DHLA.* The reaction mixtures consisted of DOPA (2.5 mM) and tyrosinase (2.5 U) in a 10 mM phosphate buffer (pH 6.5). Mixtures were incubated at 37 °C and evaluated for DOPAchrome formation by a Perkin-Elmer Japan Corp., Ltd. (Kanagawa, Japan) 1420 ARVO series multilabel counter. The effects of LA and DHLA were investigated by adding DHLA or LA (500 μM) to the mixtures before tyrosinase. The DOPAchrome for standard curves was prepared as previously described [18,19]. The amount of DOPAchrome transformed was estimated at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$). For analytical experiments, the reaction mixtures were further analyzed by HPLC method II, as described below.

*HPLC.* HPLC was performed with a JASCO Corp. gradient system equipped with dual Model PU-2089 plus pumps (10 mL pump heads), a Rheodyne (Cotati, CA) Model 7725i equipped with a 5.0 mL sample loop, and a JASCO Corp. Model MD-2010 plus multi-UV detector. Two mobile phase solvents were employed: Solvent A, prepared by adding concentrated formic acid (0.1%) to deionized water; and Solvent B, prepared by adding formic acid (0.1%) to HPLC-grade acetonitrile. In Method I for isolation, HPLC incorporated a reverse-phase column (Develosil $C_{18}$, 5μm, 20 × 250 mm, Nomura Chemical Corp., Ltd., Aichi, Japan) and the following mobile gradient: 0–60 min, linear gradient from 100% solvent A to 30% solvent B; 60–70 min, linear gradient to 100% solvent B: 70–80 min, 100% solvent B. The flow rate was constant at 7.0 mL min$^{-1}$. For the analytical method, HPLC method II incorporated a reverse-phase column (Develosil $C_{18}$, 5 μm, 4.6 × 250 mm, Nomura Chemical Corp., Ltd.) and the following mobile gradient: 0–30 min, linear gradient from 100% solvent A to 75% solvent B; 30–40 min, linear gradient to 100% solvent B. The flow rate was constant at 1.0 mL min$^{-1}$.

*LC/ESI-MS.* Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) analyses were performed on an Applied Biosystems (Forster, CA) API 2000 triple-quadrupole mass spectrometer equipped with an ESI source operating in negative ion mode, maintaining the heated capillary of the ESI source at 450 °C. Nitrogen was used as the sheath, nebulization, and auxiliary gas and maintained at 40, 60, and 15 arbitrary units, respectively. The spray voltage was 4.5 kV.

Samples (10 μL) were analyzed by online LC/ESI-MS to acquire full scan ESI spectra. Online LC separations were performed with an Agilent Technologies, Inc. (Palo Alto, CA) model 1100 HPLC series incorporating a binary gradient pump, a vacuum degasser, auto sampler, and diode array detector (DAD), proceeding as described above for analytical HPLC conditions. LC/ESI-MS was used in Q1 negative ion scan mode. The column used was a Develosil $C_{18}$, 5 μm, 2.0 × 250 mm (Nomura Chemical Corp., Ltd.) set to a flow rate of 150 μL min$^{-1}$. To prevent contamination during mass spectrometry, a switching valve was used, then introduced elution between 18 and 25 min, corresponding to the peak elution period of DHLA conjugate products into the spectrometer.

Direct injection of the HPLC-purified adduct was performed by introducing 10 μL of the sample dissolved in 50% acetonitrile in 0.1% formic acid into the mass spectrometer to obtain daughter ion spectra for the adduct. The MS/MS collision energy was 34 V.

*Preparation and purification of the lipoic acid–DOPA conjugate.* DHLA (5 mM) and DOPA (2.5 mM) were dissolved in 1 L of a 10 mM phosphate buffer (pH 6.5). The pH was readjusted to 6.5 and tyrosinase (25 U) was added. The progress of the crude reaction mixture was monitored by HPLC method II. The reaction time was 2 h at 37 °C. When HPLC analysis indicated lipoyl-DOPA formation, the enzyme reaction was terminated by adding 4 M perchloric acid until achieving a pH of 1.0. The reaction vial was then placed in an ice-water bath, and perchlorate was precipitated by adding 5 M potassium hydroxide until achieving a pH of 6.5. After separation by centrifugation, the supernatant was freeze-dried. The crude mixture was dissolved in ethanol and purified by HPLC method I. The solution eluted under the chromatographic peak corresponding to the product was collected and immediately frozen at −80 °C (dry ice bath) and the resulting eluents freeze-dried to produce a dry, solid product. Spectroscopic data as evidence for the proposed product structure are presented below. Assignment of proton resonances observed in the $^1$H NMR spectra of the

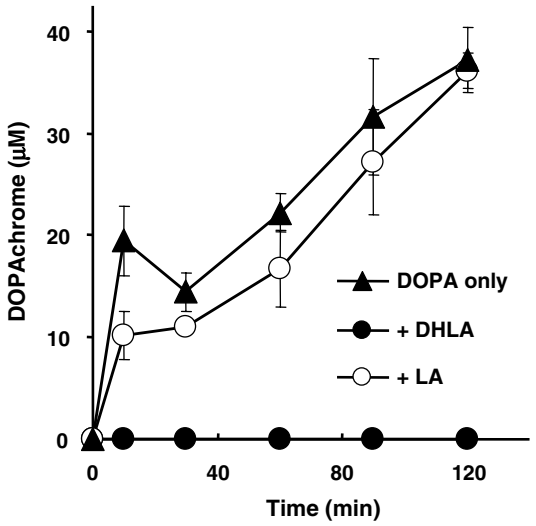



Fig. 1. DOPAchrome formation in the presence of tyrosinase and effects of DHLA and LA. The reaction mixtures consisted of DOPA (2.5 mM) and tyrosinase (2.5 U) in 10 mM phosphate buffer (pH 6.5). Mixtures were incubated at 37 °C and measured for DOPAchrome formation using a 1420 ARVO series multilabel counter. The effects of LA and DHLA were examined by adding DHLA or LA (500 μM) to the mixtures before tyrosinase. Data points refer to the concentrations (μM) of DOPAchrome plotted, along with the SD from the mean.

product was based on comparisons with the spectra of DOPA and DHLA and confirmed by two-dimensional correlated spectroscopy (2D-COSY) experiments.

5-*S*-Lipoyl-DOPA $^1$H NMR (400 MHz, DMSO-$d_6$ containing formic acid-$d_2$ (1%)) spectrum of 5-*S*-lipoyl-DOPA was assigned as follows: signals for the catecholamine part $\delta$ 6.67 (d, 1H, $J = 1.7$ Hz), 6.61 (d, 1H, $J = 1.7$ Hz), 4.01 (dd, 1H, $J = 12.4$, 6.1 Hz), 3.03 (t, 1H, $J = 4.6$ Hz), 2.95 (m, 1H), and for the lipoic acid part $\delta$ 3.05 (s, 1H), 2.58 (m, 2H), 2.24 (m, 3H), 1.91 (m, 1H), 1.63–1.82 (m, 2H), 1.52 (m, 5H). FAB-MS (glycerol matrix) *m/z* calculated for $C_{17}H_{24}NO_6S_2$ $[M - H]^-$, 402.10; found, 402.11. The UV spectrum showed separable peaks with $\lambda_{max}$ at 292 and a shoulder at about 256 nm.

## Results and discussion

The sulfhydryl compound(s) cysteine and/or GSH undergo nucleophilic reactions with DOPA-quinone to form thiol conjugation product(s). Our study demonstrates that the thiol groups of DHLA react with DOPA-quinone to form lipoyl-DOPA adducts.

In preliminary experiments, we performed spectrophotometric monitoring at 475 nm to investigate the effects of DHLA on DOPAchrome formation. As shown in

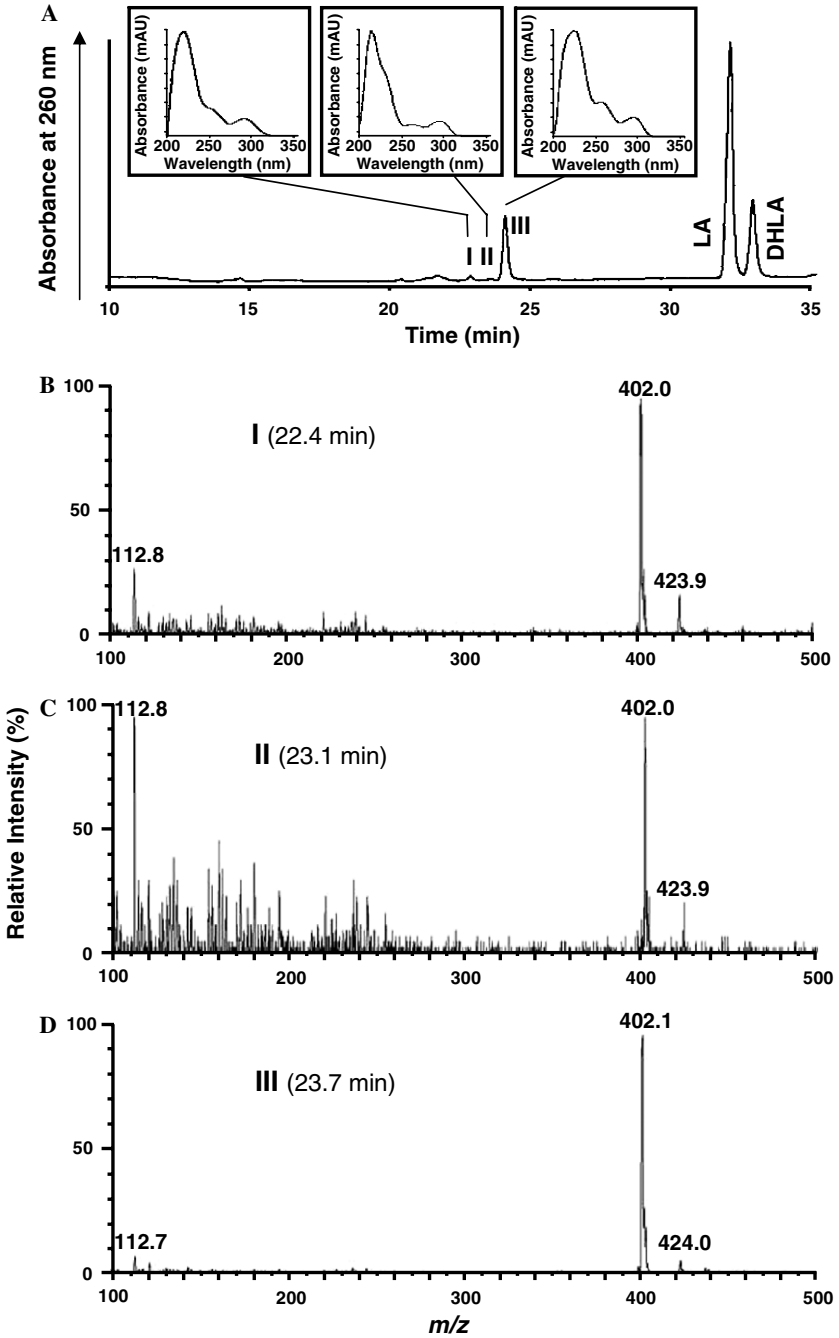



Fig. 2. HPLC profile for reaction mixture of DHLA incubated with DOPA following tyrosinase catalyzed oxidation (A). UV spectra for peaks I, II, and III (insets A). LC-ESI$^-$-MS profile for peaks I (B), II (C), and III (D) in Q1-MS scan from *m/z* 100 to 500 under the same chromatographic conditions.

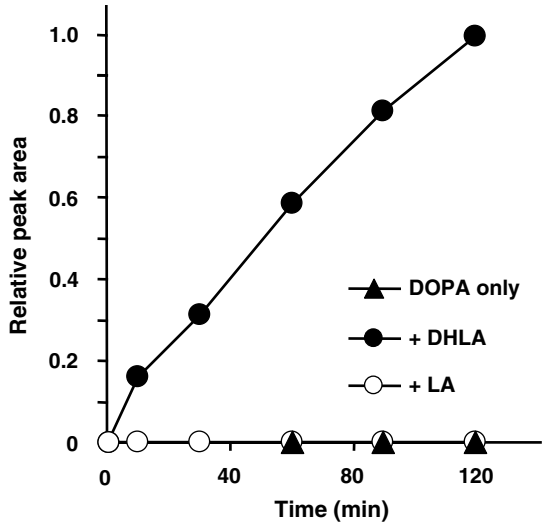



Fig. 3. Relative yields of peak III in the reaction mixture with DHLA or LA in the presence of tyrosinase. Relative products yields were estimated from peak areas measured by HPLC method II.

Fig. 1, DHLA, but not LA, significantly inhibited the formation of DOPAchrome. Interestingly, similar concentrations of cysteine also exerted effects, which proved to be relatively minor during a longer incubation period compared to the effects for the period resulting in permanent effects for DHLA (results not shown).

To examine the reactions of DHLA with DOPA in the present of tyrosinase, the reaction mixture was monitored by HPLC with UV detection. As shown in Fig. 2A, enzymatic reactions of DOPA with DHLA resulted in three unknown peaks that were detected as separable peaks with $\lambda_{max}$ at 292 and a shoulder at about 256 nm on a HPLC–photodiode array system. Analysis of the reaction mixtures by LC/ESI$^-$-MS in a Q1-MS scan from $m/z$ 100 to 500 under the same chromatographic conditions showed three ion peaks, peak I, II, and III, with $m/z = 402$ corresponding to the deprotonated molecular ion $[M–H]^-$ expected for the DHLA DOPA conjugate (Figs. 2B–D). These peaks were not detected when incubating DHLA or DOPA alone followed by tyrosinase-catalyzed oxidation and barely detected such peaks in the absence of tyrosinase (results not shown). Incubation of DOPA with DHLA followed by the tyrosinase-catalyzed oxidation gave product ratios peak I/peak II/peak III 4:1:80 based on HPLC peak areas measuring 260 nm.

The yields of peak III increased throughout the course of the reaction with DHLA, in contrast to undetectable

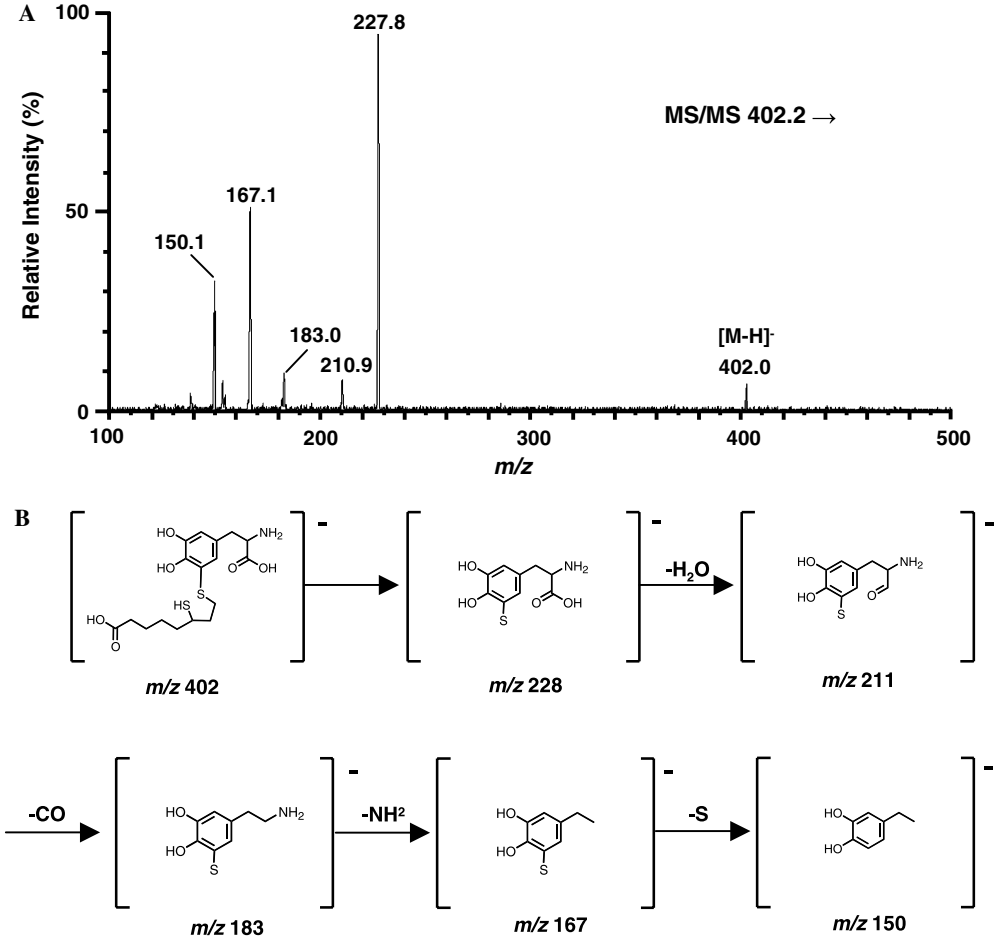


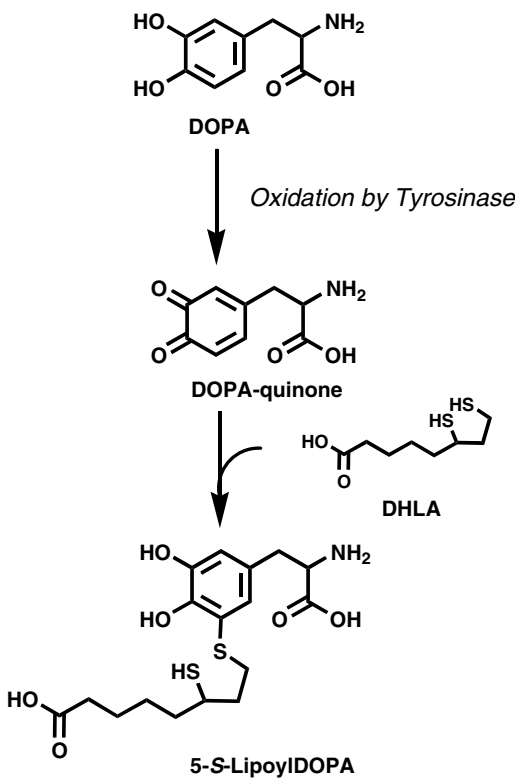

Fig. 4. Collision-induced MS/MS spectra (ESI) for the molecular ion $m/z$ 402 $[M - H]^-$ of peak III (A). Fragmentation route proposed for peak III (B).

levels for reactions without DHLA or with LA (Fig. 3). The other two peaks with the same UV spectra have slopes similar to peak III (results not shown), suggesting that the time courses of the formation of these products correlated remarkably well with the depletion of DOPAchrome formation. Based on these results, we anticipated that these three compounds would be the DOPA-quinone conjugation products, and that the binding of DHLA to DOPA-quinone suppressed DOPAchrome formation via the withdrawal of DOPA-quinone from the reaction of the mixture, since conjugations of DOPA with sulfhydryl compounds such as L-cysteine and GSH can occur via nucleophilic attacks by thiol groups on DOPA-quinone [9,10].

These adduct peaks were well resolved under semipreparative HPLC conditions. In particular, peak III was prominent enough to be isolated by HPLC. The fraction of peak III was collected and processed without further purification. The structural identification of the adduct isolated from treatment of DHLA with DOPA was made by $FAB^-$-MS, $ESI^-$-MS, $ESI^-$-MS/MS, and $^1H$ NMR, and 2D-COSY experiment.

The results for an $FAB^-$-MS analysis of peak III coincided with the results for DOPA-quinone conjugation products (See Materials and methods). The collision-induced MS/MS spectra of $[M-H]^-$ showed five product ions, at $m/z = 150.1$, 167.1, 183.0, 210.9, and 227.9 (Fig. 4A). The predominant production was $m/z = 227.8$, interpreted as the thiol-DOPA ion formed in the breakage of the bond between the thiol and the carbon of the modified *S*-terminal LA (Fig. 4B). In the $^1H$ NMR spectrum of peak III, signals of the thiol moiety were assigned by comparison to spectra for DOPA and DHLA, and confirmed in all cases by 2D-COSY experiment, indicating that the coupling of 1.7 Hz between protons in the aromatic region corresponds to the meta positions of these protons. Based on these chemical analyses, we identified peak III as 5-*S*-lipoyl-DOPA. Peaks I and II may be isomers arising from the addition of the DHLA thiol group to either the 2 or 6 position of the DOPA-quinone, since the fragmentations of peak I and II overlapped those of 5-*S*-lipoyl-DOPA ($m/z$ 402.2 $[M-H]^- \rightarrow$ 227.9, 167.1, and 150.1) in the LC-$ESI^-$-MS/MS experiment (result not shown).

These catechol-thiol conjugates may derive from nonenzymatic reactions between catecholamine *o*-quinone and DHLA, in which these covalent reactions are likely to occur under both enzymatic and nonenzymatic conditions, since tyrosinase in this reaction oxidizes DOPA to DOPA-quinone (Scheme 1). In neuro-pigmentation, neuromelanin is formed by nonenzymatic autoxidation of cytoplasmic DA to DA-quinone that subsequently cyclizes and oxidatively polymerizes [20,21]. Several investigators have suggested that the effect of L-cysteine and GSH on scavenging DA-quinone diverts or blocks the oxidation of DA to insoluble black melanin polymer, forming a soluble cysteinyl or glutathionyl conjugates with DA [22,23]. Spencer et al. [17] have shown that DHLA, but not LA,




Scheme 1. Formation of 5-*S*-lipoyl-DOPA.

inhibits the formation of the 5-*S*-cysteinyl- and 5-*S*-glutathionyl-catecholamine adducts in a system generating $O_2^{\cdot-}$, proposing this as a model of oxidative stress for nervous system in general, and suggesting that DHLA may achieve these inhibitory effects by competing with either L-cysteine or GSH for the catecholamine *o*-quinone species.

We show here that DHLA, not LA, significantly inhibited the formation of DOPAchrome in the presence of tyrosinase, based on the observation that DHLA reacts readily with DOPA in the presence of tyrosinase to form lipoyl adducts based on LC/ESI-MS in Q1-MS scans. We characterize one of the main adducts, 5-*S*-lipoyl-DOPA, as a novel thiol conjugation product. We believe the conjugated reactions may also help regulate melanin quantity and quality by inhibiting DOPAchrome formation, which leads to eumelanin production.

The cultural demand for effective skin lightening in various regions of the world suggests that further study of DHLA's inhibitory effects, potentially leading to practical application, is in order.

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