

# Reactions of Cysteine and Cysteinyl Derivatives with Dopamine-*o*-quinone and Further Insights into the Oxidation Chemistry of 5-*S*-Cysteinyl dopamine: Potential Relevance to Idiopathic Parkinson's Disease

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Recent studies suggest that elevated rates of autoxidation of dopamine (DA) in the cytoplasm of neuromelanin-pigmented dopaminergic cell bodies in the substantia nigra (SN) and/or reactions of its proximate oxidation product, DA-*o*-quinone, with glutathione (GSH) or L-cysteine (CySH) might yield endotoxins that play roles in the pathogenesis of idiopathic Parkinson's Disease (PD). In this study the reactions between DA-*o*-quinone and CySH and some cysteine derivatives have been studied. The reactions between DA-*o*-quinone and CySH or cysteine methyl ester are rapid and give the corresponding 5-*S*-cysteinyl conjugates of DA as the predominant products. By contrast, the reaction between the sterically hindered D-penicillamine methyl ester (PME) and DA-*o*-quinone is much slower to give, initially, a mixture of the 2-*S*-, 5-*S*-, and 6-*S*-PME conjugates of DA. These conjugates are then further oxidized by unreacted DA-*o*-quinone to give a complex mixture of products which include 7-(2-aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic acid methyl ester (**13**). Large increases in the 5-*S*-cysteinyl dopamine (5-*S*-CyS-DA)/DA concentration ratio have been measured in the Parkinsonian SN and have been interpreted to reflect elevated rates of DA autoxidation in this structure that degenerates in PD. However, the present study reveals that at physiological pH 5-*S*-CyS-DA is not only more easily oxidized than DA but a bicyclic *o*-quinone imine (**5**) intermediate is formed that can chemically oxidize the former conjugate in a reaction that leads to 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (**6**). Compound **6** is lethal when administered into the brains of mice. However, this putative dihydrobenzothiazine endotoxin is even more easily oxidized than 5-*S*-CyS-DA in a rather complex reaction that ultimately forms 7-(2-aminoethyl)-5-hydroxy-1,4-benzothiazine-3-carboxylic acid (**15**). Although this benzothiazine could not be isolated it was identified by comparison of its electrochemical and spectroscopic properties in solution with those of **13**. The results of this study suggest that benzothiazine **15** might serve as a better analytical marker molecule than 5-*S*-CyS-DA for either elevated rates of DA autoxidation and/or for roles of GSH and CySH in the neurodegenerative mechanisms in the SN that contribute to PD. © 1995 Academic Press, Inc.

Parkinson's Disease (PD) is a neurodegenerative brain disorder that results in hypokinesia, rigidity, and tremor. These symptoms are caused in large part by a severe deficiency of the catecholaminergic neurotransmitter dopamine (DA) in the

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**3** that polymerizes to black indolic melanin. Residues derived from 5-S-CyS-DA result from the facile nucleophilic addition of L-cysteine (CySH) or glutathione (GSH) to DA-*o*-quinone. In the latter case 5-S-glutathionyl-dopamine (5-S-Glu-DA) is apparently hydrolyzed by intraneuronal peptidase enzymes to 5-S-CyS-DA (Scheme I) (10). *In vitro* experiments have demonstrated that oxidations of DA in aqueous solutions at physiological pH give black melanin polymer only when the concentrations of CySH or GSH are low relative to that of the neurotransmitter (10, 14). Such observations have led Carstam *et al.* (10) to conclude that when indolic residues are incorporated into insoluble SN neuromelanin both CySH and GSH must be virtually absent in dopaminergic cell bodies. Histochemical studies have also revealed that there is probably little or no GSH in neuronal cell bodies in any region of the brain and that this tripeptide is largely localized in glia, axons, and nerve terminals (15, 16).

Formation of indolic neuromelanin within SN cells implies that these cell bodies must possess a weak antioxidant defense system. Only under such conditions could DA-*o*-quinone escape reduction to DA and cyclize to **1** and 5,6-DHI, the precursors of neuromelanin. In fact, not only GSH (10, 17, 18) but also ubiquinols and  $\alpha$ -tocopherol levels (19) are low in the mammalian SN compared to other brain regions. Furthermore, the mouse SN, for example, has the highest levels of malondialdehyde (an index of lipid peroxidation) of any brain region (20). Such peroxidative damage would result from DA autooxidation in reactions that generate  $O_2^{\cdot -}$  (Scheme I) and thence  $H_2O_2$ , which, in the presence of trace levels of transition metal ions (e.g.,  $Fe^{2+}$ ), can produce the highly cytotoxic hydroxyl radical ( $HO^{\cdot}$ ) (21, 22). Indeed, such reactions have been suggested (23, 24) to contribute to the normal age-dependent degeneration of nigrostriatal dopaminergic neurons (25). However, it has been demonstrated that in the SN of PD patients there is a significant decrease in the neuromelanin content of surviving pigmented cells (26, 27). Furthermore, the more heavily neuromelanin-pigmented SN neurons appear to be more vulnerable to degeneration in the Parkinsonian brain (27, 28). These observations suggest that dopaminergic SN cells having the highest basal levels of DA autooxidation (and hence the heaviest neuromelanin pigmentation) are particularly vulnerable to degeneration in PD and that as the disease progresses the neuromelanin pathway is either blocked or diverted leading to depigmentation. Some potentially important insights into mechanisms that might contribute to this diversion can be drawn from measurements of the levels of 5-S-CyS-DA, formed by the reactions shown in Scheme I, in normal, aging, and Parkinsonian brains (29–31). For example, the 5-S-CyS-DA/DA concentration ratios measured in the striatum and SN of human brain tissue at autopsy have been found to increase with the degree of depigmentation and degeneration of SN cells (31). Furthermore, the 5-S-CyS-DA/DA ratio in the SN of a patient clinically diagnosed with PD was approximately an order of magnitude greater than that measured in patients without depigmentation and degeneration of the SN (31). These observations have been interpreted to indicate an increased rate of DA autooxidation in depigmenting and degenerating SN neurons, an effect that becomes particularly pronounced in the Parkinsonian brain (31). An increased rate of DA autooxidation in the SN, which normally contains little or no free GSH or CySH (10, 15, 16), would be expected to result in more rapid formation

of indolic neuromelanin polymer and hence increased pigmentation of degenerating SN neurons. However, in the Parkinsonian SN precisely the opposite effects occur, i.e., the more heavily pigmented cells appear to be preferentially vulnerable to degeneration (27, 28) and as they degenerate they become progressively depigmented (26, 27). It has recently been reported that the activity of only one GSH-related enzyme,  $\gamma$ -glutamyltranspeptidase, is significantly elevated in the Parkinsonian SN (32). One function of this enzyme is to translocate extracellular GSH into cells, including neurons, via the  $\gamma$ -glutamylcycle (33). Furthermore, in the Parkinsonian brain only the SN is depleted of GSH and this depletion is not accompanied by a corresponding increase in the oxidized form of the tripeptide, i.e., GSSG (32, 34, 35). Additionally, levels of GSH in the SN of patients with incidental Lewy body disease, an early presymptomatic stage of PD (36), are decreased to the same extent as those in patients with advanced PD without any corresponding increase in GSSG (34). Thus, decreased nigral GSH levels appear to be a very early component of the pathological processes that underlie PD. The decrease in nigral GSH levels in PD does not appear to be related to decreased activity of  $\gamma$ -glutamylcysteine synthetase, the rate-limiting enzyme for the biosynthesis of the tripeptide, or to alterations in GSH peroxidase, GSSG reductase, or GSH transferase activities (32, 37, 38). Thus, in the Parkinsonian SN, the biochemical systems for GSH biosynthesis and for conversion of GSSG to GSH do not appear to be compromised, suggesting, perhaps, that the tripeptide is being siphoned off by reaction with some exogenous or endogenous species. Based upon these observations we have recently proposed (14) that a key step in the pathoetiology of PD might involve the translocation of GSH (or CySH), biosynthesized in glial cells (39, 40), into the cytoplasm of neuromelanin-pigmented SN cell bodies. This GSH (or CySH) would be expected to scavenge DA-*o*-quinone, the proximate autoxidation product of DA and precursor of indolic neuromelanin, leading to an increase in the 5-S-CyS-DA/DA ratio and diversion of the neuromelanin pathway (10, 14), hence accounting for the depigmentation of SN neurons. However, 5-S-CyS-DA is significantly more easily oxidized than DA (14). Thus, under conditions where cytoplasmic DA is autoxidized in neuromelanin-pigmented SN cell bodies, 5-S-CyS-DA would also be expected to be even more readily oxidized. Some insights into the oxidation chemistry of 5-S-CyS-DA have been obtained in a recent *in vitro* study in which DA was electrochemically oxidized in the presence of a large (i.e., five-fold) molar excess of CySH at physiological pH. In this reaction DA-*o*-quinone is avidly scavenged by CySH to give 5-S-CyS-DA, which is further oxidized to *o*-quinone **4** (Scheme I). The latter *o*-quinone then undergoes a very rapid intramolecular cyclization to the bicyclic *o*-quinone imine **5**, which appears to be a key precursor of many secondary products. Thus, in the presence of a large excess of free CySH, **5** is apparently partially reduced to 7-(2-aminoethyl)-3,4-dihydro-5-*hydroxy*-2*H*-1,4-benzothiazine-3-carboxylic acid (**6**) or is attacked by CySH to give the 6-S- and 8-S-cysteinyl conjugates of **6**, i.e., **7** and **8**, respectively (Scheme I). Further facile oxidations of **6**–**8** in the presence of free CySH ultimately results in the formation of a very large number of products, most of which remain to be identified. Dihydrobenzothiazine **6** and its 6-S-(**7**) and 8-S-(**8**) cysteinyl conjugates are lethal when administered into the brains of laboratory mice having LD<sub>50</sub> values of 14, 17, and

70  $\mu\text{g}$  (administered in 5.0  $\mu\text{l}$  of isotonic saline solution), respectively (14). These observations suggest that increasing substitutions of CySH residues into **6** decrease the toxicity of this dihydrobenzothiazine. Indeed, the 6,8-bi-*S*-cysteinyl conjugate of **6** is not lethal at doses of 100  $\mu\text{g}$  (14). Based on these results we have speculated that **6**, in particular, might be an endotoxin formed as a result of an influx of GSH or CySH into pigmented SN cells that contributes to the degeneration of nigrostriatal dopaminergic neurons leading ultimately to PD (14). Because of the very large number ( $>60$ ) of products formed when DA is oxidized in the presence of large molar excesses of CySH it was difficult to fully elucidate mechanistic pathways. Accordingly, preliminary studies have been carried out in which DA was oxidized in the presence of lower molar excesses of CySH (e.g., equimolar DA and CySH) at physiological pH. These studies revealed that significantly fewer products are formed although dihydrobenzothiazine **6** continued to be formed in the initial stages of the reaction in relatively high yields. This observation was rather surprising in view of the fact that **6** is appreciably more easily oxidized than DA or 5-*S*-CyS-DA (14). Subsequently, it was found that oxidation of 5-*S*-CyS-DA in the absence of free CySH also gave **6** as a major initial product along with one other major unidentified compound. Oxidation of 5-*S*-CyS-DA via *o*-quinone **4** would, as demonstrated previously (14), give bicyclic *o*-quinone imine **5** (Scheme I) not dihydrobenzothiazine **6**. Formation of **6**, therefore, implies that even in the absence of free CySH there must be an alternative pathway to convert **5** to this lethal dihydrobenzothiazine. Unfortunately, insights into the mechanisms underlying this chemistry were hampered by the fact that the second major product of oxidation of 5-*S*-CyS-DA could not be identified owing to its facile decomposition during the isolation procedures employed. In this communication it will be demonstrated that the reaction of a highly substituted analog of CySH, D-penicillamine methyl ester (PME), with DA-*o*-quinone, the proximate oxidation product of DA, leads to products which, based on a comparison of their spectroscopic and electrochemical properties with those of the previously unidentified product of oxidation of 5-*S*-CyC-DA, permit an unequivocal identification of the latter compound. Furthermore, reaction pathways have been elucidated which explain formation of dihydrobenzothiazine **6** as an initial oxidation product of 5-*S*-CyS-DA at physiological pH.

## EXPERIMENTAL

### *Chemicals*

Dopamine hydrochloride (DA.HCl), L-cysteine hydrochloride (CySH.HCl), L-cysteine methyl ester (CME) hydrochloride, and D-penicillamine methyl ester (PME; D-2-amino-3-mercapto-3-methylbutanoic acid, hydrochloride salt) were obtained from Sigma (St. Louis, MO) and were used without additional purification.

### *Apparatus and Methods*

Voltammograms were obtained at a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 4 mm<sup>2</sup>. A conventional three-electrode voltammetric cell was used with a

platinum wire counter electrode and a saturated calomel reference electrode (SCE). Linear sweep and cyclic voltammograms were obtained using a BAS-100A (Bioanalytical Systems, West Lafayette, IN) electrochemical analyzer. All voltammograms were corrected for  $iR$  drop. The PGE was resurfaced prior to recording each voltammogram as described previously (41). Controlled potential electrolyses employed a Princeton Applied Research Corp. (Princeton, NJ) model 173 potentiostat. The working electrode consisted of several plates of pyrolytic graphite having a total surface area of approximately 430 cm<sup>2</sup>. A three-compartment cell was used in which the working, counter, and reference electrode compartments were separated by a Nafion membrane (type 117, DuPont Co., Wilmington, DE). The working electrode compartment had a capacity of 80 ml. The counter electrode consisted of several plates of pyrolytic graphite suspended into a solution of the supporting electrolyte. The solution in the working electrode compartment was continuously deoxygenated with a vigorous stream of N<sub>2</sub>. All potentials are referred to the SCE at ambient temperature (22 ± 2°C).

<sup>1</sup>H NMR spectra were recorded on a Varian XL-300 spectrometer. High and low resolution fast atom bombardment mass spectrometry (FAB-MS) employed a VG Instruments (Manchester, UK) model ZAB-E spectrometer. Thermospray mass spectrometry was performed with a Kratos (Manchester, UK) model MS 25/RFA instrument equipped with a thermospray source. Samples analyzed by thermospray mass spectrometry were collected from a conventional high performance liquid chromatography (HPLC) instrument and then introduced into the mass spectrometer via a Rheodyne (Cotati, CA) model 7125 injector equipped with a 2.0-ml sample loop. The solvent was 0.1 M ammonium acetate in deionized water adjusted to pH 4.1 with acetic acid. A flow rate of 1.0 ml/min was used. The thermospray capillary was maintained at 150°C and the source at 250°C. uv-visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

HPLC employed a Gilson (Middleton, WI) gradient system equipped with model 305 and 306 pumps (25-ml pump heads) and a Holochrome detector set at 254 nm. A Rheodyne model 7125 loop injector was used to introduce samples. Two reversed phase columns were employed; a Spherisorb (Phase Sep, Clwyd, UK) S<sub>10</sub>, ODS-2, 25 × 2.0-cm column and a Regis (Morton Grove, IL) ODS, 10-μm particle size, 25 × 2.1-cm column. A short guard column (1 × 5-cm) packed with Bakerbond C<sub>18</sub> (J.T. Baker, Phillipsburg, NJ, 10-μm particle size) was used to protect these preparative HPLC columns. In order to separate the products formed in the reaction systems studied for analytical or preparative purposes, six binary gradient procedures were employed which will be referred to as HPLC methods I–VI. For HPLC method I solvents A and B were employed. Solvent A was prepared by adding 30 ml of concentrated ammonium hydroxide solution (NH<sub>4</sub>OH) to 4 liters of deionized water; the pH of the resulting solution was adjusted to 3.0 by addition of concentrated trifluoroacetic acid (TFA). Solvent B was prepared by adding 30 ml of NH<sub>4</sub>OH to 2 liters of HPLC grade methanol (MeOH) and 2 liters of deionized water; the pH of this solution was adjusted to 3.0 with TFA. The following gradient was employed; 0–2 min, 100% solvent A; 2–35 min, linear gradient to 100% solvent B; 35–55 min, 100% solvent B. The flow rate was 7 ml/min.

For HPLC method II, solvents C and D were employed. Solvent C was prepared

by adding 30 ml of  $\text{NH}_4\text{OH}$  to 4 liters of deionized water; the pH was then adjusted to 7.0 with TFA. Solvent D was prepared by adding 30 ml of  $\text{NH}_4\text{OH}$  to 1 liter of HPLC grade acetonitrile (MeCN), 1 liter of MeOH, and 2 liters of deionized water; the pH was adjusted to 7.0 with TFA. The following gradient was employed: 0–2 min, 100% solvent C; 2–15 min, linear gradient to 30% solvent D; 15–40 min, linear gradient to 40% solvent D; 40–50 min, linear gradient to 100% solvent D; 50–60 min, 100% solvent D. The flow rate was 7.0 ml/min.

HPLC method III employed solvents A and B and the following gradient: 0–2 min, 100% solvent A; 2–10 min, linear gradient to 60% solvent B; 10–30 min, linear gradient to 100% solvent B; 30–40 min, 100% solvent B. The flow rate was 7 ml/min.

For HPLC methods IV–VI solvents E and F were used. Solvent E was prepared by adding TFA to deionized water until the pH was 3.0. Solvent F was prepared by adding TFA to 5% MeCN in deionized water into the pH was 3.0. The following gradients were used. HPLC method IV: 0–2 min, 100% solvent E; 2–20 min, linear gradient to 10% solvent F; 20–25 min, linear gradient to 100% solvent F; 25–30 min, 100% solvent F. HPLC method V: 0–2 min, 100% solvent E; 2–30 min, linear gradient to 100% solvent F; 30–40 min, 100% solvent F. HPLC method VI: 0–2 min, 100% solvent E; 2–15 min, linear gradient to 40% solvent F; 15–20 min, linear gradient to 100% solvent F; 20–30 min, 100% solvent F. The flow rate for methods IV–VI was 7 ml/min. For HPLC methods I–III the Spherisorb column was used; for methods IV–VI the Regis column was used.

### *Synthesis of DA-*o*-Quinone*

DA.HCl (30 mg; 2.0 mM) was dissolved in 80 ml of 0.1 M HCl and electro-oxidized at pyrolytic graphite electrodes for 30–50 min at 1.0 V. The reaction solution turned from colorless to bright yellow, characteristic of DA-*o*-quinone. HPLC analysis (method I) revealed that during the latter period of reaction DA was converted ( $\geq 80\%$ ) to DA-*o*-quinone and no other significant products were formed. The uv-visible spectrum, thermospray mass spectrum, and cyclic voltammetry of DA-*o*-quinone were all in agreement with those reported previously (11).

### *Reactions of DA-*o*-Quinone with Cysteine, Cysteine Methyl Ester (CME), and D-Penicillamine Methyl Ester (PME)*

CySH.HCl (160 mg), or CME hydrochloride (180 mg), or PME hydrochloride (200 mg) was added to 80 ml of DA-*o*-quinone (ca. 2 mM) in 0.1 M HCl electrochemically synthesized as described in the preceding paragraph. The reactions were carried out with  $\text{N}_2$  gas bubbling vigorously through the solution. Upon addition of CySH or CME the reaction solution changed immediately from bright yellow to a very pale yellow color. Chromatography (HPLC method I) revealed that the major product of reaction of DA-*o*-quinone with CySH (i.e., 5-S-CyS-DA) had a retention time ( $t_R$ ) of 24.8 min. The product formed between DA-*o*-quinone and CME (i.e., **9**) eluted at  $t_R = 27.2$  min. The solutions eluted under these peaks were collected and freeze-dried. The resulting solids were dissolved in the minimum volume of deionized water and the solutions desalted and purified using HPLC method IV. 5-S-CyS-DA eluted at  $t_R = 10.0$  min; **9** eluted at  $t_R = 14.0$  min. The solutions

containing desalted and purified 5-S-CyS-DA and **9** were freeze-dried. The spectroscopic properties of 5-S-CyS-DA ( $^1\text{H}$  NMR, FAB-MS, uv) have been reported previously (11, 14).

Addition of PME.HCl to DA-*o*-quinone caused the initially bright yellow solution to turn to a dull yellow color over the course of 20–30 min. HPLC analysis (method I) at the end of this time showed five major chromatographic peaks of products having  $t_R$  values of 36.5, 40.5, 44, 50, and 56.5 min, in addition to a large peak of DA and several peaks corresponding to minor, unidentified products. The compounds eluted under the major chromatographic peaks were collected individually. These solutions were then desalted and purified using either HPLC methods VI (compound **10**,  $t_R$  = 17 min) or method V (**11**,  $t_R$  = 24 min; **12**,  $t_R$  = 23 min; **13**,  $t_R$  = 26 min; **14**,  $t_R$  = 23 min). The desalted eluents containing these compounds were then shell frozen and freeze-dried.

#### *Oxidation of 5-S-CyS-DA*

5-S-CyS-DA (22 mg; 1.0 mM) was dissolved in 80 ml of pH 7.4 phosphate buffer ( $\mu$  = 0.2) and electrolyzed at 66 mV for 20 min. HPLC analysis (method II) indicated that two major products were formed (**6**,  $t_R$  = 22 min; **15**,  $t_R$  = 25 min). For preparative purposes the entire volume of the reaction solution (80 ml) was pumped onto the HPLC column and separated using method II. Compound **6** and unreacted 5-S-CyS-DA coeluted and were collected together; the eluent containing **15** was also collected. These solutions were further separated, purified, and desalted using HPLC method IV (5-S-CyS-DA,  $t_R$  = 10 min; **6**,  $t_R$  = 30 min; **15**,  $t_R$  = 22 min). The purified and desalted solutions were then freeze-dried. Compound **15** decomposed during the freeze-drying process to give a complex mixture of products which remain to be identified. Dihydrobenzothiazine **6** was initially isolated as a pale yellow solid although even when stored in a vacuum dessicator it gradually darkened and ultimately became black. The spectroscopic and chromatographic properties and cyclic voltammetry of **6** were identical to those reported previously for 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (14).

#### *Oxidation of 5-S-Cysteinyldopamine Methyl Ester (9)*

Compound **9** (11.4 mg; 0.5 mM) was dissolved in 80 ml of pH 7.4 phosphate buffer ( $\mu$  = 0.2) and electro-oxidized at 16 mV for 30 min. HPLC (method I) indicated that two products were formed (**16**,  $t_R$  = 31.5 min; **17**,  $t_R$  = 35.5 min). For preparative purposes the total volume of the reaction solution was introduced onto the HPLC column and products separated using method III. The eluent solutions containing **16** and **17** were collected and then desalted and purified using HPLC method V. The solutions containing **16** and **17** were freeze-dried. During this process **17** decomposed to several secondary products that remain to be identified.

#### *5-S-Cysteinyldopamine Methyl Ester (9)*

Compound **9** was isolated as a white solid. In pH 7.4 phosphate buffer ( $\mu$  = 1.0) **9** exhibited a uv spectrum with bands at  $\lambda_{\text{max}}$ , nm ( $\log \epsilon_{\text{max}}$ ,  $\text{M}^{-1} \text{cm}^{-1}$ ) 294 (3.08), 255 sh(3.23), 230 (3.52). FAB-MS (3-nitrobenzyl alcohol matrix) gave  $m/e$  = 287.1075



(MH<sup>+</sup>, 100%, C<sub>12</sub>H<sub>19</sub>N<sub>2</sub>O<sub>4</sub>S; calcd m/e = 287.1066). <sup>1</sup>H NMR (D<sub>2</sub>O) gave δ 6.94 (d, *J*<sub>2,6</sub> = 2.1 Hz, 1H, C(6)-H), 6.84 (d, *J*<sub>2,6</sub> = 2.1 Hz, 1H, C(2)-H), 4.34 (t, *J* = 4.8 Hz, 1H, C(b)-H), 3.70 (dd, *J* = 15.6 Hz, *J* = 4.8 Hz, 1H, C(a)-H), 3.32 (dd, *J* = 15.6 Hz, *J* = 4.8 Hz, 1H, C(a)-H), 3.19 (t, *J* = 7.3 Hz, 2H, C(β)-H<sub>2</sub>), 2.84 (t, *J* = 7.3 Hz, 2H, C(α)-H<sub>2</sub>). 2D-correlated spectroscopy (COSY) experiments revealed that the following resonances were coupled: 6.94 and 6.84; 4.34 and 3.70; 4.23 and 3.32; 3.70 and 3.32; 3.19 and 2.84.

*N*-[4-(2-Aminoethyl)-2-[(2-amino-3-methoxy-1,1-dimethyl-3-oxopropyl)thio]-6-hydroxyphenyl]-3-mercapto-valine Methyl Ester (**10**)

Compound **10** was isolated as a very pale yellow solid. In pH 7.4 phosphate buffer **10** exhibited uv bands at λ<sub>max</sub> 320(sh), 302, and 216 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 458.1787 [(M-2H) H<sup>+</sup>, 10%, C<sub>20</sub>H<sub>32</sub>N<sub>3</sub>O<sub>5</sub>S<sub>2</sub>; calcd m/e = 458.1783]. <sup>1</sup>H NMR (D<sub>2</sub>O) gave δ 7.03 (d, *J*<sub>3,5</sub> = 2.1 Hz, 1H, C(3)-H), 6.99 (d, *J*<sub>3,5</sub> = 2.1 Hz, 1H, C(5)-H), 4.18 (s, 1H, C(b)-H), 3.90 (s, 1H, C(b')-H), 3.90 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.81 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.24 (t, *J* = 7.5 Hz, 2H, C(β)-H<sub>2</sub>), 2.90 (t, *J* = 7.5 Hz, 2H, C(α)-H<sub>2</sub>), 1.55 (s, 3H, C(a)-CH<sub>3</sub>), 1.54 (s, 3H, C(a)-CH<sub>3</sub>), 1.43 (s, 3H, C(a')-CH<sub>3</sub>), 1.41 (s, 3H, C(a')-CH<sub>3</sub>). Cyclic voltammetry of **10** in pH 7.4 phosphate buffer at a sweep rate (*v*) of 100 mVs<sup>-1</sup> showed three oxidation peaks having peak potentials (*E*<sub>p</sub>) at 28, 132, and 608 mV on the initial anodic sweep. After sweep reversal, small indistinct reduction peaks were observed at ca. 100 and -48 mV. At *v* = 10 Vs<sup>-1</sup> the first two oxidation peaks merged to give one peak at *E*<sub>p</sub> = 147 mV; after scan reversal a reversible reduction peak, *E*<sub>p</sub> = 99 mV, appeared having the same peak current (*i*<sub>p</sub>) as the initial oxidation peak.

8-(2-Aminoethyl)-3,7-dihydro-5-hydroxy-2,2-dimethyl-7-oxo-2H-1,4-benzothiazine-3-carboxylic Acid Methyl Ester (**11**)

Compound **11** was isolated as a blue solid. In pH 7.4 phosphate buffer **11** exhibited uv-visible bands at λ<sub>max</sub> 572, 314(sh), 260, and 226 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 311.1059 (MH<sup>+</sup>, 100%, C<sub>14</sub>H<sub>19</sub>N<sub>2</sub>O<sub>4</sub>S; calcd m/e = 311.1066). <sup>1</sup>H NMR (D<sub>2</sub>O) gave δ 6.58 (s, 1H, C(6)-H), 4.28 (s, 1H, C(3)-H), 3.78 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.21 (t, *J* = 7.2 Hz, 2H, C(β)-H<sub>2</sub>), 2.81 (t, *J* = 7.2 Hz, 2H, C(α)-H<sub>2</sub>), 1.44 (s, 3H, C(2)-CH<sub>3</sub>), 1.37 (s, 3H, C(2)-CH<sub>3</sub>). Cyclic voltammograms (*v* = 100 mVs<sup>-1</sup>) of **11** in pH 7.4 phosphate buffer exhibited a reduction peak at *E*<sub>p</sub> = -262 mV on the initial cathodic sweep and, following scan reversal, a reversible oxidation peak of equal height at *E*<sub>p</sub> = -244 mV.

7-(2-Aminoethyl)-6-[(2-amino-3-methoxy-1,1-dimethyl-3-oxopropyl)thio]-3,4-dihydro-5-hydroxy-2,2-dimethyl-2H-1,4-benzothiazine-3-carboxylic Acid Methyl Ester (**12**)

Compound **12** was isolated as a yellow solid. In pH 7.4 phosphate buffer **12** exhibited uv bands at λ<sub>max</sub> 324 and 244 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 458.1769 (MH<sup>+</sup>, 93%, C<sub>20</sub>H<sub>32</sub>N<sub>3</sub>O<sub>5</sub>S<sub>2</sub>; calcd m/e 458.1783). <sup>1</sup>H NMR (D<sub>2</sub>O) gave δ 6.78 (s, 1H, C(8)-H), 4.42 (s, 1H, C(3)-H), 4.14 (s, 1H, C(b)-H), 3.83

(s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.73 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.3–2.8 (m, 4H, C(α)-H<sub>2</sub> and C(β)-H<sub>2</sub>), 1.48–1.46 (m, 12H, 2 × C(2)-CH<sub>3</sub> and 2 × C(a)-CH<sub>3</sub>). Cyclic voltammetry ( $\nu = 100 \text{ mVs}^{-1}$ ) of **12** in pH 7.4 phosphate buffer showed an oxidation peak at  $E_p = 130 \text{ mV}$  followed by a shoulder at  $E_p = 200 \text{ mV}$  and a further broad oxidation peak at  $E_p \approx 590 \text{ mV}$  on the initial anodic sweep. After scan reversal a small, broad reduction peak appeared at  $E_p = 120 \text{ mV}$ .

*7-(2-Aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic Acid Methyl Ester (13)*

Compound **13** was isolated as a yellow solid. In pH 7.4 phosphate buffer **13** exhibited uv bands at  $\lambda_{\text{max}}$  340, ca. 300 sh, 242 and 230 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave  $m/e = 295.1115$  (MH<sup>+</sup>, 100%, C<sub>14</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub>S; calcd  $m/e = 295.116$ ). <sup>1</sup>H NMR (D<sub>2</sub>O) gave  $\delta$  6.82 (d,  $J_{6,8} = 2.0 \text{ Hz}$ , 1H, C(8)-H), 6.75 (d,  $J_{6,8} = 2.0 \text{ Hz}$ , 1H, C(6)-H), 3.93 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.24 (t,  $J = 7.28 \text{ Hz}$ , 2H, C(β)-H<sub>2</sub>), 2.91 (t,  $J = 7.28 \text{ Hz}$ , 2H, C(α)-H<sub>2</sub>), 1.50 (s, 6H, 2 × C(2)-CH<sub>3</sub>). Cyclic voltammetry ( $\nu = 100 \text{ mVs}^{-1}$ ) of **13** in pH 7.4 phosphate buffer exhibited a single, irreversible oxidation peak at  $E_p = 612 \text{ mV}$  on the initial anodic sweep.

*8-[(1,1-Dimethyl-2-amino-2-carboxyethyl methyl ester)thio]-7-(2-aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic Acid Methyl Ester (14)*

Compound **14** was isolated as a yellow solid. In pH 7.4 phosphate buffer **14** exhibited uv bands at  $\lambda_{\text{max}}$  354, 232, and 216 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave  $m/e = 456.1617$  (MH<sup>+</sup>, 100%, C<sub>20</sub>H<sub>30</sub>N<sub>3</sub>O<sub>5</sub>S<sub>2</sub>; calcd  $m/e = 456.1627$ ). <sup>1</sup>H NMR (D<sub>2</sub>O) gave  $\delta$  7.06 (s, 1H, C(6)-H), 4.11 (s, 1H, C(b)-H), 3.97 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.84 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.65–3.06 (m, 4H, C(α)-H<sub>2</sub> and C(β)-H<sub>2</sub>), 1.57 (s, 6H, 2 × CH<sub>3</sub>), 1.44 (s, 3H, CH<sub>3</sub>), 1.40 (s, 3H, CH<sub>3</sub>). Cyclic voltammetry ( $\nu = 100 \text{ mVs}^{-1}$ ) of **14** in pH 7.4 phosphate buffer exhibited two overlapping irreversible oxidation peaks ( $E_p = 590$  and  $680 \text{ mV}$ ) on the initial anodic sweep.

*7-(2-Aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic Acid Methyl Ester (16)*

Compound **16** was obtained as a very pale yellow solid. In pH 7.4 phosphate buffer **16** exhibited uv bands at  $\lambda_{\text{max}}$  304 and 234 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave  $m/e = 269.0974$  (MH<sup>+</sup>, 100%, C<sub>12</sub>H<sub>17</sub>N<sub>2</sub>C<sub>3</sub>S; calcd  $m/e = 269.0960$ ). <sup>1</sup>H NMR (D<sub>2</sub>O) gave  $\delta$  6.56 (d,  $J_{6,8} = 1.8 \text{ Hz}$ , 1H, C(8)-H), 6.54 (d,  $J_{6,8} = 1.8 \text{ Hz}$ , 1H, C(6)-H), 4.63 (t,  $J = 4.2 \text{ Hz}$ , 1H, C(3)-H), 3.74 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.34 (dd,  $J = 13.2 \text{ Hz}$ ,  $J = 4.2 \text{ Hz}$ , 1H, C(2)-H), 3.27 (dd,  $J = 13.2 \text{ Hz}$ ,  $J = 4.2 \text{ Hz}$ , 1H, C(2)-H), 3.16 (t,  $J = 14.1 \text{ Hz}$ , 2H, C(β)-H<sub>2</sub>), 2.77 (t,  $J = 14.1 \text{ Hz}$ , 2H, C(α)-H<sub>2</sub>).

## RESULTS

### *Reactions of CySH, CME, and D-PME with DA-o-Quinone*

DA-o-quinone is the proximate product of the electrochemical oxidation of DA over a wide range of pH (II). However, at physiological pH DA-o-quinone is unstable and rapidly cyclizes to 5,6-dihydroxyindoline (**1**) the precursor of black

indolic melanin polymer (Scheme I) (11–13). Thus, in order to study the direct interactions between DA-*o*-quinone and CySH, CME, and PME, it was necessary to oxidize DA in solution at low pH (0.1 M HCl), conditions under which the quinone is relatively stable (11). HPLC (method I) of a solution of DA (2.0 mM) in 0.1 M HCl both before and after controlled potential electro-oxidation (1.0 V; 30 min) revealed that the neurotransmitter was almost quantitatively transformed into DA-*o*-quinone. The latter compound was identified on the basis of its characteristic uv-visible spectrum, thermospray mass spectrum, and cyclic voltammetry as described previously (11).

Addition of a sixfold molar excess of CySH to a thoroughly deoxygenated solution of DA-*o*-quinone (ca. 2 mM) in 0.1 M HCl resulted in the initially bright yellow solution becoming almost colorless. HPLC (method I) of the product solution revealed that the chromatographic peak of DA-*o*-quinone had disappeared and that one major peak was present. The compound responsible for this peak was isolated and structurally identified as 5-*S*-CyS-DA. Reaction of CME with DA-*o*-quinone under the same experimental conditions also gave one major product identified as 5-*S*-CyS-DA methyl ester (9). A number of minor products were also formed as a result of the reactions between DA-*o*-quinone and CySH and CME although these were not isolated and identified.

The reaction between DA-*o*-quinone (ca. 2 mM) and a sixfold molar excess of PME in 0.1 M HCl was considerably slower than that observed with CySH or CME and required, based upon spectroscopic measurements and HPLC analysis (method I), about 20–30 min to reach completion (i.e., complete removal of DA-*o*-quinone). At the end of this time HPLC of the resulting solution revealed the presence of more than nine products in addition to a significant peak for DA. The five major reaction products were isolated and spectroscopically identified as *N*-[4-(2-aminoethyl)-2-[(2-amino-3-methoxy-1,1-dimethyl-3-oxopropyl)thio]-6-hydroxyphenyl]-3-mercapto-valine methyl ester (10), 8-(2-aminoethyl)-3,7-dihydro-5-hydroxy-2,2-dimethyl-7-oxo-2*H*-1,4-benzothiazine-3-carboxylic acid methyl ester (11), 7-(2-aminoethyl)-6-[2-amino-3-methoxy-1,1-dimethyl-3-oxopropylthio]-3,4-dihydro-5-hydroxy-2,2-dimethyl-2*H*-1,4-benzothiazine-3-carboxylic acid methyl ester (12), 7-(2-aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic acid methyl ester (13), and 8-[(1,1-dimethyl-2-amino-2-carboxyethyl methyl ester)thiol]-7-(2-aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic acid methyl ester (14) (see Experimental).

#### *Oxidation of 5-S-CyS-DA and 5-S-CyS-DA Methyl Ester (9)*

At a slow sweep rate ( $v = 10 \text{ mVs}^{-1}$ ) the primary voltammetric oxidation peak of 5-*S*-CyS-DA (1.0 mM) in pH 7.4 phosphate buffer had a peak potential ( $E_p$ ) of 69 mV. Following controlled potential electro-oxidation of 5-*S*-Cys-DA (1.0 mM; pH 7.4 phosphate buffer) at 66 mV for 20 min HPLC analysis (method II) showed formation of two major products. The compound eluting at  $t_r = 22$  min was isolated and spectroscopically identified ( $^1\text{H}$  NMR, FAB-MS, uv spectra) as 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (6) (see *Experimental*). The second major product of this reaction (15;  $t_R = 25$  min; method

II) was relatively stable in buffered solution at pH 7.4 and in the HPLC mobile phase (pH 7.0). However, attempts to isolate this compound were unsuccessful owing to its facile transformation into a mixture of unidentified secondary products during the final freeze-drying step. With longer electrolysis times the chromatographic peak of dihydrobenzothiazine **6** decreased and ultimately disappeared. Correspondingly, the peak of **15** increased.

The primary voltammetric oxidation peak of **9** (0.5 mM; 10 mVs<sup>-1</sup>) at pH 7.4 occurs at  $E_p = 16$  mV. HPLC (method I) of the solution formed following controlled potential electro-oxidation of **9** (1.0 mM) at 16 mV for 20 min in pH 7.4 phosphate buffer revealed that two major products were formed having  $t_R$  values of 31.5 and 35.5 min. The product eluting at  $t_R = 31.5$  min was isolated and spectroscopically identified as 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid methyl ester (**16**). The second major product (**17**) could not be isolated owing to its decomposition to several unidentified products during the final freeze-drying step. When **9** was electrolyzed for times greater than 20 min, HPLC analysis showed that the chromatographic peak for **16** decreased and disappeared and that for **17** correspondingly increased.

The structures of **15** and **17**, the major ultimate products of controlled potential electro-oxidation of 5-*S*-CyS-DA and **9**, respectively, were assigned by comparisons of their uv spectra, thermospray mass spectra, and voltammetric behaviors with those of 7-(2-aminoethyl)-5-hydroxy-2,2-dimethyl-1,4-benzothiazine-3-carboxylic acid methyl ester (**13**), a stable product of the reaction between DA-*o*-quinone and PME in 0.1 M HCl. Thus, the thermospray mass spectra of **15**, **17**, and **13** exhibited intense pseudomolecular ions (MH<sup>+</sup>) at  $m/e = 253$  (100%), 267 (100%), and 295 (100%), respectively. The uv spectra of **15** ( $\lambda_{max} = 344$ , ca. 300 sh, 246 nm), **17** ( $\lambda_{max} = 360$ , ca. 300 sh, 244 nm), and **13** ( $\lambda_{max} = 340$ , ca. 300 sh, 242 nm) at pH 7.4 were also very similar. Voltammograms of **15**, **17**, and **13** ( $v = 100$  mVs<sup>-1</sup>) at pH 7.4 exhibited single irreversible oxidation peaks at  $E_p = 612$ , 582, and 612 mV, respectively. Based upon such comparisons it was concluded that **15** is 7-(2-aminoethyl)-5-hydroxy-1,4-benzothiazine-3-carboxylic acid and **17** its methyl ester. Furthermore, controlled potential electro-oxidations of **6** (66 mV) or **16** (16 mV) in pH 7.4 phosphate buffer resulted in formation of benzothiazines **15** or **17**, respectively, as the major reaction products along with additional minor unidentified compounds.

## REACTION PATHWAYS

In strongly acidic solution (e.g., 0.1 M HCl) the exocyclic amino group of DA-*o*-quinone is fully protonated such that its intramolecular cyclization to 5,6-dihydroxyindoline (**1**) is effectively blocked (11–13). Thus, under such acidic conditions reactions between DA-*o*-quinone and CySH, CME, and PME can be studied directly. The reaction between CySH and CME with DA-*o*-quinone at low pH is rapid and results in the formation of 5-*S*-CyS-DA and **9**, respectively (Scheme II). Additional minor products are formed in these reactions but only in low yields and hence no attempts were made to identify these compounds. The particular