Hz, C6-H), 5.55 (bd, 1 H, J = 7 Hz, NH), 5.05 (d, 1 H, J = 1.8 Hz, C19-H), 4.21 (bt, 1 H, J = 15 Hz, CHN), 3.69 (s, 3 H, OCH_3), 3.09–2.01 (m, 6 H, 3 CH_2); IR (KBr) ν_{max} 3304, 1654, 1560, 1508, 1438, 1214, 1116 cm^{-1}; EIMS, m/e (relative *intensity*) 341 (M^+, 83), 309 (base), 282 (67), 265 (71), 253 (10), 240 (29), 226 (28), 212 (45), 197 (10); CIMS (isobutane), m/e (relative intensity) 342 (M^+ + H, base); EIHRMS, m/e 341.1264 ($C_{19}H_{19}NO_5$ requires 342.1263).

Methyl (9*S*)-4-methoxy-11-oxo-2-oxa-10-azatricyclo-[12.2.2.1^{3,7}]nonadeca-3,5,7(19),14,16,17-hexaene-9-carboxylate (19f): oil; $[\alpha]^{22}_D$ −7.14° (c 0.7, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz, ppm) 7.30 (dd, 1 H, J = 2.2, 8.3 Hz, C16-H or C17-H), 7.23 (dd, 1 H, J = 2.2, 8.2 Hz, C16-H or C17-H), 7.08 (dd, 1 H, J = 2.4, 8.3 Hz, C15-H or C18-H), 6.99 (dd, 1 H, J = 2.3, 8.2 Hz, C15-H or C18-H), 6.77 (d, 1 H, J = 8.3 Hz, C5-H), 6.58 (dd, 1 H, J = 2.1, 8.2 Hz, C6-H), 5.51 (d, 1 H, J = 7.2 Hz, NH), 5.08 (d, 1 H, J = 2.0 Hz, C19-H), 4.19 (ddd, 1 H, J = 1.5, 7.3, 10 Hz, C9-H), 3.94 (s, 3 H, $ArOCH_3$), 3.70 (s, 3 H, CO_2CH_3), 3.06–2.04 (m, 6 H, 3 CH_2); APT ^{13}C NMR ($CDCl_3$, 75 MHz, ppm) 172.7 (e), 172.2 (e), 156.9 (e), 152.8 (e), 146.9 (e), 138.5 (e), 132.4 (o), 130.0 (e), 129.9 (o), 124.9 (o), 124.8 (o), 121.4 (o), 114.8 (o), 111.9 (o), 56.3 (o), 53.9 (o), 52.7 (o), 41.0 (e), 34.7 (e), 32.1 (e); IR (neat) ν_{max} 3360, 3270, 1718, 1654, 1560, 1542, 1508, 1458, 1266, 1206, 1130 cm^{-1}; EIMS, m/e (relative intensity) 357 (M^+, 90), 341 (26), 324 (base), 297 (59), 283 (69), 280 (58), 268 (10); CIMS (isobutane), m/e (relative intensity) 358 (M^+ + H, base); EIHRMS, m/e 357.3876 ($C_{20}H_{21}NO_5$ requires 357.3876).

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Supplementary Material Available: Experimental details and full spectroscopic and physical characterization of **18a–f**, ^{1}H NMR spectra of **18a–f** and **19a–f**, and details of the single-crystal X-ray structure determination of **19b** (30 pages). Ordering information is provided on any current masthead page.

Reactions of the Serotonergic Neurotoxin 5,6-Dihydroxytryptamine with Glutathione

Satendra Singh and Glenn Dryhurst*

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019

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At physiological pH the serotonergic neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) catalyzes the oxidation of the cellular antioxidant glutathione (GSH) by molecular oxygen to give GSSG. At the stage when GSH is depleted, 5,6-DHT is then autoxidized to give first 2,7′-bi(5,6-dihydroxytryptamine) (**2**) and ultimately indolic melanin. In the presence of an excess of GSH and enzymes such as tyrosinase or ceruloplasmin, the oxidation of 5,6-DHT to its corresponding *o*-quinone (**1**) is greatly accelerated. Under such conditions, **1** is attacked by GSH to give 4-*S*-glutathionyl-5,6-dihydroxytryptamine (A), which is further oxidized to the corresponding quinone **4**. Further attack by GSH or A on **4** gives 4,7-diglutathionyl-5,6-dihydroxytryptamine (B) and 4,4′-di-*S*-glutathionyl-2,7′-bi(5,6-dihydroxytryptamine) (C), respectively. Reaction between **4** and B yields 4,7,4′-tri-*S*-glutathionyl-2,7′-bi(5,6-dihydroxytryptamine) (D).

5,6-Dihydroxygtryptamine (5,6-DHT) is a pharmacological tool used in neurobiology for the selective chemical lesioning of serotonergic neurons.[1] The selectivity of 5,6-DHT derives from the transport of the drug into a target neuron by the uptake mechanism normally used for the endogenous neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). The molecular mechanism(s) by which 5,6-DHT expresses its neurodegenerative effect is still an open question. However, it is widely believed that the neurotoxicity of 5,6-DHT results from an inherent chemical property, namely ease of oxidation by molecular oxygen, i.e., autoxidation. Two principal theories have been advanced to relate the autoxidation of 5,6-DHT to its neurodegenerative activity.[2-9] The first postulates that the autoxidation reaction converts the indolamine into an electrophilic *o*-quinone that alkylates and cross-links neuronal proteins. Such processes would be expected to compromise the normal functions of such proteins, leading to cell death. However, reactions between putative electrophilic intermediates generated upon autoxidation of 5,6-DHT with protein nucleophiles or even with model peptides have never been demonstrated. The second theory proposes that cytotoxic reduced oxygen species such as H_2O_2, $O_2^{\bullet-}$, and $HO^{\bullet}$ are formed as byproducts of autoxidation of 5,6-DHT and attack neuronal lipids and proteins or other susceptible structures.

In recent reports[10,11] we have shown that at physiological pH 5,6-DHT is indeed autoxidized to *o*-quinone **1** with concomitant formation of H_2O_2 as a byproduct (Scheme I). Nucleophilic attack by 5,6-DHT on **1** gives 2,7′-bi-(5,6-dihydroxytryptamine) (**2**), which is the major initial product of the autoxidation reaction. Dimer **2** is autoxidized to diquinone **3** with formation of 2 mol more of H_2O_2. Diquinone **3** can chemically oxidize 5,6-DHT to quinone **1** so that an autocatalytic cycle is established. The

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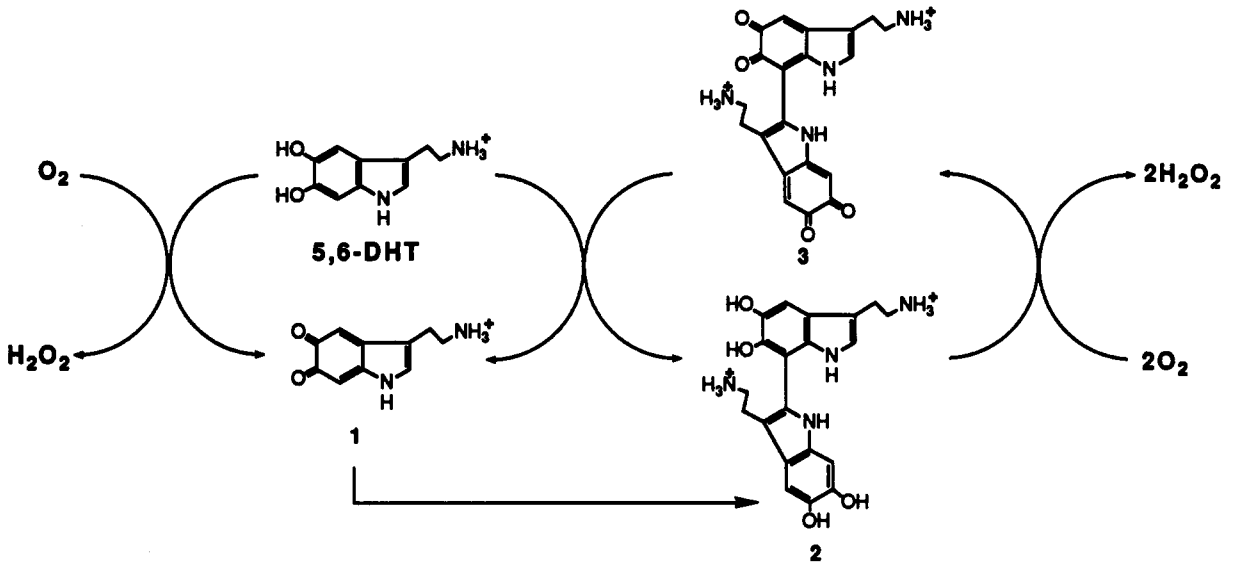
Scheme I



H_2O_2 formed as a result of the reactions outlined in Scheme I can undergo Fenton chemistry catalyzed by traces of transition-metal ions with resultant formation of the hydroxyl radical, HO•, which can directly oxidize 5,6-DHT so that a second autocatalytic cycle is established. Ultimately, diquinone **3** reacts with **2**, 5,6-DHT, and perhaps other minor autoxidation products by a sequence of coupling and oxidation reactions to give a black, insoluble indolic melanin. Oxidative enzyme systems such as tyrosinase/O_2, ceruloplasmin/O_2, peroxidase/H_2O_2, and rat brain mitochondria catalyze the oxidation of 5,6-DHT to form **2** and, ultimately, indolic melanin.

While such studies have provided important insight into the basic autoxidation chemistry of 5,6-DHT at physiological pH, they were carried out under conditions that are somewhat remote from those extant in an intact, living neuron. For example, Nature has developed defense mechanisms to protect cells against cytotoxic radicals (particularly HO•) deriving from H_2O_2 by means of enzymes such as glutathione peroxidase and catalase. Protection against electrophilic insult is provided by cellular sulfhydryl compounds, particularly the tripeptide glutathione (GSH).[12] Klemm et al.[6] have reported that cellular antioxidants such as GSH, cysteine, and ascorbate provide protection to 5,6-DHT against autoxidation as evidenced by the effects of the these compounds on the rate of oxygen consumption by the indolamine. The protective action of these antioxidants has been ascribed to their ability to maintain 5,6-DHT in its reduced form.[6] Such a process could imply that the antioxidant is progressively oxidized with resultant depletion. Furthermore, in the case of GSH it is also possible that in addition to acting simply as a reducing agent, i.e., converting **1** to 5,6-DHT, it might also act as a nucleophile, forming adducts with the indolamine. In this report we describe the interactions of the most important nonprotein cellular protective agent, GSH, with 5,6-DHT under physiological pH conditions in both the presence and absence of enzymes that catalyze the oxidation of the indolamine.

Results and Discussion

In the absence of GSH, complete autoxidation of a 2 mM solution of 5,6-DHT in pH 7.2 phosphate buffer (μ = 0.1) at room temperature to a black, insoluble, indolic melanin polymer requires approximately 4 h.[10,11] Figure 1 shows a series of chromatograms recorded throughout the autoxidation of 2 mM 5,6-DHT in the presence of a 5-fold molar excess of GSH under the same solution conditions. In these chromatograms peak a is due to creatinine (the creatinine sulfate salt of 5,6-DHT was used). The closely spaced peaks labeled GSSG are due to isomers of oxidized GSH. Peak 2 is due to 2,7′-bi(5,6-dihydroxytryptamine) (**2**). The products responsible for other minor unlabeled peaks have not been identified. GSH exhibits no significant absorption at 254 nm and hence does not give a chromatographic peak. During the first 20 h of the reaction the chromatographic peak of 5,6-DHT shows a small decrease (20–25%) while that of **2** shows a corresponding increase (Figure 1A–C). However, between 20 and 21.75 h (Figure 1C,D) there is a very rapid decline in the concentration of 5,6-DHT and an increase in **2**. Within another 45 min, all of the chromatographic peaks due to indolic products and 5,6-DHT have disappeared and a black precipitate of indolic melanin is formed. Between 0 and 20 h of reaction, the chromatographic peaks due to GSSG exhibit a large increase. Based upon calibration curves the concentration of GSSG after 20 h is 5 mM; i.e., at this stage of the reaction, GSH has been quantitatively oxidized to GSSG. Thus, the rapid autoxidation of 5,6-DHT is delayed until virtually all GSH is removed from the solution.

In the absence of 5,6-DHT, GSH is autoxidized at pH 7.2. HPLC analysis of a solution initially 10 mM in GSH showed that after 20 h the concentration of GSSG was ca. 2 mM; i.e., approximately 40% of GSH is autoxidized. Clearly, therefore, in the presence of 5,6-DHT the rate of oxidation of GSH to GSSG is more than doubled.

Spectral changes that accompany the autoxidation of 5,6-DHT (0.2 mM) in the presence of a 5-fold molar excess of GSH are shown in Figure 2A. Thus, during the first 14 h the UV band of 5,6-DHT at 300 nm slowly shifts to longer wavelengths and then grows. This change largely reflects the slow conversion of 5,6-DHT to **2** (λ_{max} at pH

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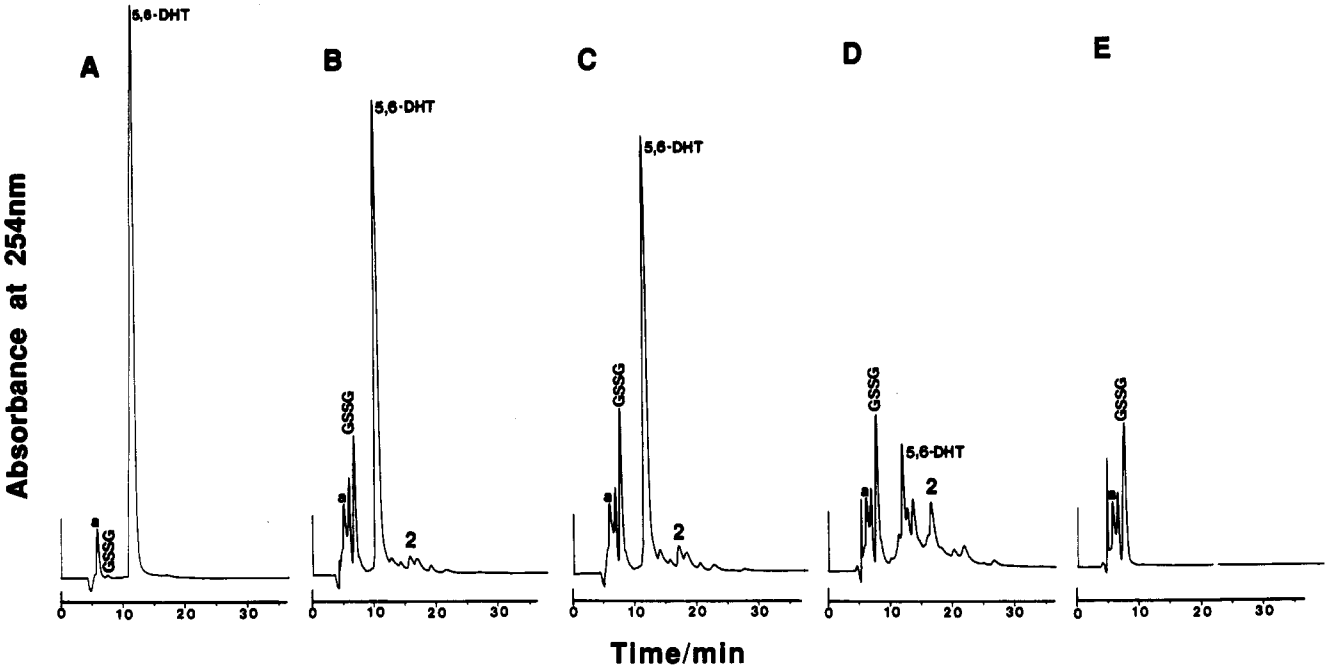


Figure 1. High-performance liquid chromatograms recorded at (A) 0 min, (B) 18 h, (C) 20 h, (D) 21 h 45 min, (E) 22 h 30 min during the autoxidation of 2.0 mM 5,6-DHT in the presence of 10 mM glutathione in pH 7.2 phosphate buffer (μ = 0.1) at room temperature. HPLC method I was employed. Injection volume: 0.80 mL. Peaks: a, creatinine; GSSG, oxidized glutathione.

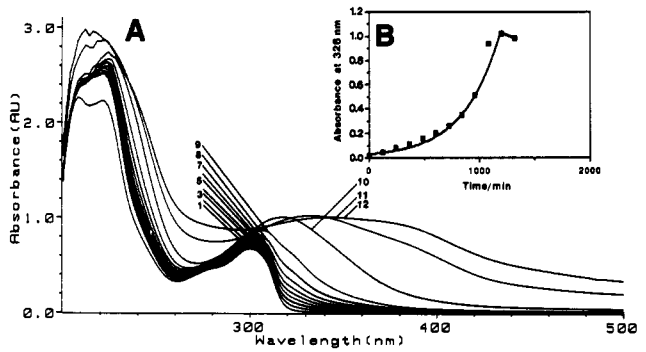
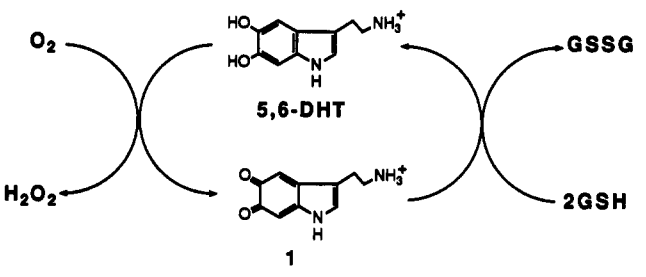


Figure 2. (A) Spectral changes during the autoxidation of 5,6-DHT (0.2 mM) in the presence of GSH (1.0 mM) in pH 7.2 phosphate buffer (μ = 0.1) at room temperature. Curve 1 is the spectrum of 5,6-DHT; curves 2–12 were recorded at 2-h intervals. (B) Absorbance at 328 nm versus time for the reaction described in (A). Experiments were carried out at room temperature.

7.2, 308 nm; λ_{min}, 253 nm). Other, as yet unidentified, minor autoxidation products of 5,6-DHT also contribute to this change because they exhibit absorption bands with λ_{max} values in the range 310–325 nm. At longer times there is a very rapid rise in absorbance corresponding to formation of 2 and other minor oxidation products. Between 18 and 22 h the general rise in absorbance over the entire spectral region is due to formation of black, indolic melanin. A plot of absorbance vs time (Figure 2B) indicates that the reaction exhibits characteristics of an autocatalytic process.

The above results reveal that 5,6-DHT is partially protected against autoxidation in the presence of an excess of GSH. However, the rate of oxidation of GSH to GSSG is accelerated in the presence of 5,6-DHT. The formal potential for the 5,6-DHT/*o*-quinone 1 couple is +0.088 V vs SCE[11] whereas that for the GSSG/GSH couple is −0.582 V.[16] Thus, the simplest explanation of the above observations is that *o*-quinone 1 formed upon autoxidation

Scheme II



of 5,6-DHT is rapidly reduced by GSH, forming GSSG as conceptualized in Scheme II.

On the basis of HPLC analysis only a relatively small amount 1 escapes this redox cycle to react with 5,6-DHT and to form dimer 2 as long as there is free GSH available. However, 2 is more rapidly autoxidized than 5,6-DHT[11] to give diquinone 3 (Scheme I). It is likely therefore that formation of 2 and hence, upon autoxidation, 3 contributes to some extent to the conversion of GSH to GSSG. In an earlier report[11] it was demonstrated that the H_2O_2 formed as a byproduct of autoxidation of 5,6-DHT (and of 2) could participate in Fenton chemistry catalyzed by trace transition-metal ion contaminants generating the hydroxyl radical, HO•. While no attempts have been made to detect HO• in the present study, it is likely that this strong oxidant[17] plays a role in oxidizing both GSH in the early stages of the reaction and of 5,6-DHT and 2 in the later stages when GSH is depleted.

Enzymes such as tyrosinase, ceruloplasmin, and peroxidase and rat brain mitochondria greatly accelerate the oxidation of 5,6-DHT to 2 and, ultimately, to indolic melanin.[11] However, in the presence of tyrosinase or ceruloplasmin and GSH the oxidation of 5,6-DHT yields quite different products. Figure 3 shows a series of chromatograms recorded periodically during the oxidation of 2 mM 5,6-DHT in the presence of 10 mM GSH and

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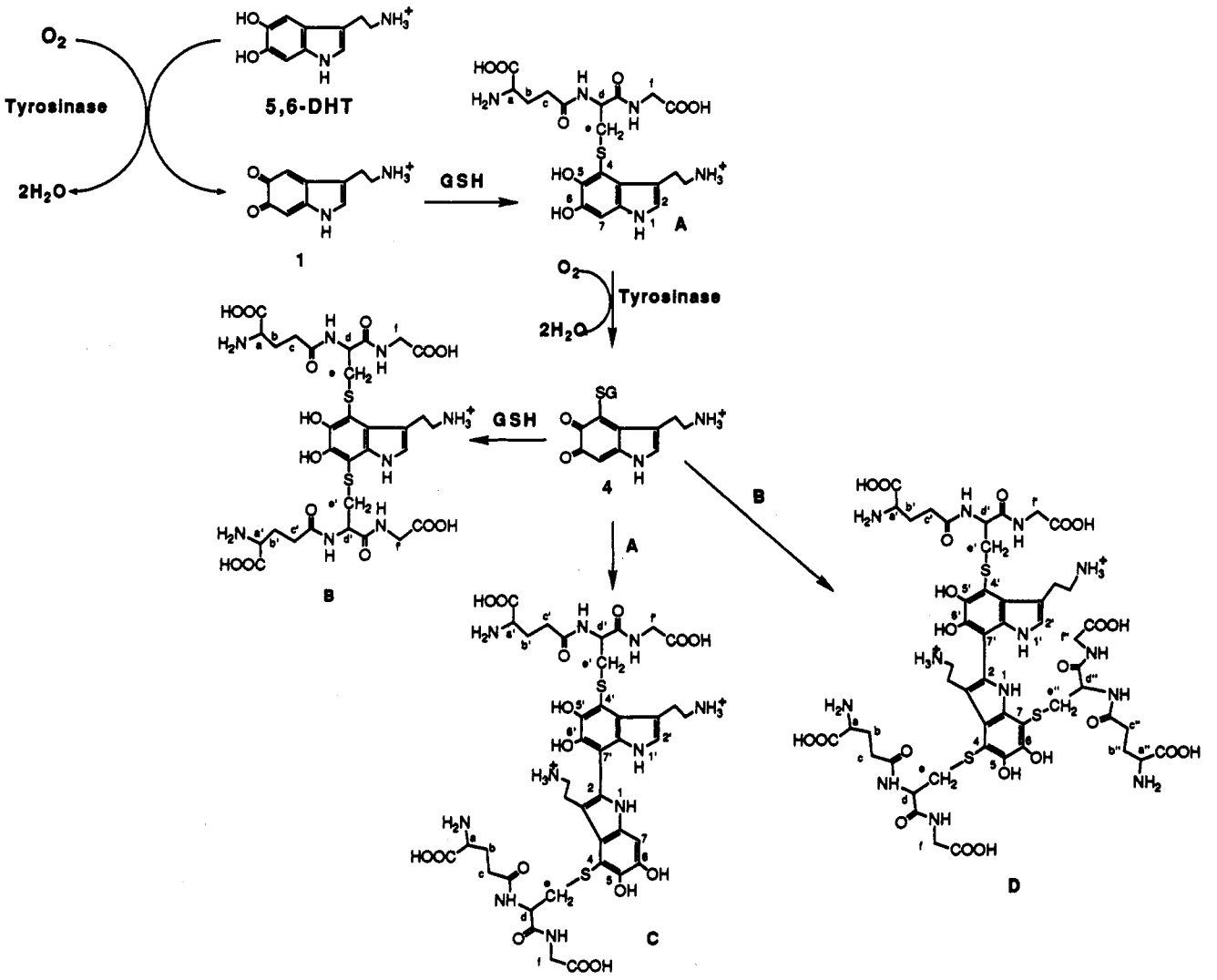
Scheme III



tyrosinase. After less than 1 h, 5,6-DHT has disappeared and a large peak corresponding to 4-*S*-glutathionyl-5,6-dihydroxytryptamine (A) appears along with smaller peaks corresponding to glutathione adducts B–D. The broad, distorted peak at ca. 24 min corresponds to the overlapping elution of creatinine and GSSG (HPLC method II). At longer reaction times the yield of A decreases whereas those of B–D increase (Figure 3). This trend continues with time such that after 3–4 h monomer B, which contains two GSH residues, and dimer D, which contains three GSH residues, are the major products. HPLC analyses of product solutions generated in the tyrosinase-assisted oxidations of 5,6-DHT in the presence of GSH failed to detect the presence of even traces of dimer **2**. Chromatograms similar to those shown in Figure 3 were obtained in the ceruloplasmin-assisted oxidation of 5,6-DHT in the presence of GSH. In the absence of 5,6-DHT tyrosinase exerts only a very minor catalytic effect on the oxidation of GSH to GSSG. For example, following autoxidation of GSH (10 mM) in pH 6.5 phosphate buffer (μ = 0.1) for 5 h at room temperature, the concentration of GSSG was 0.35 mM. In the presence of tyrosinase (0.02 mg ml^{-1}) under exactly the same conditions, the concentration of GSSG was 0.5 mM.

The above results indicate that the primary product of the enzyme-assisted oxidation of 5,6-DHT in the presence of GSH is A. Previous studies[11] have shown that tyrosinase catalyzes the oxidation of 5,6-DHT to quinone 1. Tyrosinase, however, exerts only a minor catalytic effect on the oxidation of GSH. Thus, it may be concluded that the influence of tyrosinase on the products formed by oxidation of 5,6-DHT in the presence of GSH derives from the rapid formation of significant concentrations of *o*-quinone 1. Under such conditions GSH can function both as a reducing agent and, more significantly, as a strong nucleophile. The initial product of nucleophilic attack of GSH on quinone 1 is the 4-*S*-glutathionyl adduct A (Scheme III). The HPLC traces recorded throughout the tyrosinase-assisted oxidation of 5,6-DHT in the presence of GSH (Figure 3) clearly indicate the rapid conversion of 5,6-DHT to A followed by further conversion of A to B, C, and D. We propose that autoxidation and/or enzymatic oxidation of A yields quinone **4** (Scheme III). In view of the products deriving from A, quinone **4** represents a key intermediate. Nucleophilic attack by GSH on the C(7) position of **4** yields the 4,7-diglutathionyl adduct of 5,6-DHT B, which is the second major product formed in the early stages of the oxidation reaction. Alternatively, **4** can be attacked by A to yield the 4,4′-diglutathionyl adduct of dimer **2**, i.e., C. It is not possible to completely exclude the possibility that **2** is formed and oxidized to diquinone **3**, which is then attacked by GSH to give C. However, HPLC analyses provide no evidence for the presence of even traces of dimer **2** at any stage of the reaction. Quinone **4** can also be attacked by B to yield D as conceptualized in Scheme III.

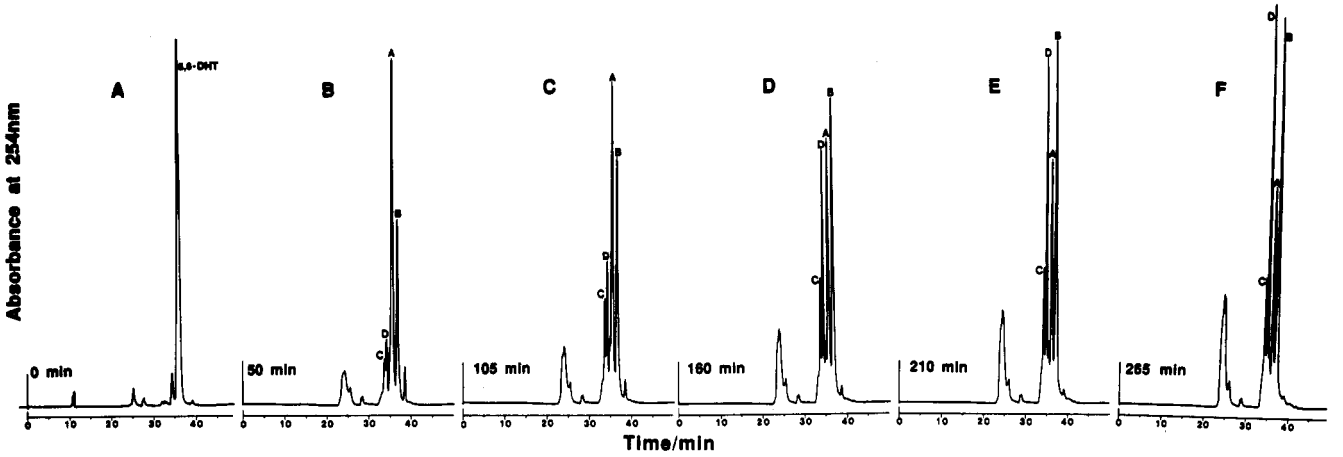


Figure 3. High-performance liquid chromatograms recorded at various times during the oxidation of 5,6-DHT (2.0 mM) in the presence of glutathione (10 mM) and tyrosinase (20 μg mL^{-1}; 68.6 units mL^{-1}) in pH 6.5 phosphate buffer (μ = 0.1) at room temperature. HPLC method II was used. Injection volume: 3.0 mL. Times are shown in the figure.

The chromatogram shown in Figure 3 after 265 min in fact represents only a stage in the reaction at which there are significant concentrations of glutathione conjugates A–D. Indeed, at this point in the reaction, B and D are the major products. However, at longer reaction times additional products appear. For example, the 4,7-diglutathionyl adduct B is replaced by another major product. Unfortunately, it has not been possible to obtain a mass spectrum on this new product. However, the absence of any aromatic proton resonances in its ^{1}H NMR spectrum suggests that it is probably the 2,2′-dimer of B. At even later stages of the reaction a fine gray/black precipitate appears in the solution, suggesting that oligomers/polymers are formed.

Conclusions

The results reported indicate that in the absence of a suitable oxidative enzyme 5,6-DHT can act as a catalyst to convert GSH to GSSG. The catalytic effect of 5,6-DHT autoxidation on the oxidation of GSH might conceivably play an important role in the neurodegenerative action of 5,6-DHT. Thus, once the indolamine is sequestered within a target serotonergic neuron, it could, over a period of time, act to deplete the endogenous concentration of the cellular antioxidant GSH, particularly if the rate of 5,6-DHT-catalyzed autoxidation of GSH is competitive with its rate of synthesis (replenishment). At an advanced stage of the reaction, GSH could be reduced to such a low level that autoxidation of 5,6-DHT would occur. This is an autocatalytic reaction resulting from the reactions outlined in Scheme I and due to the effects of $O_2^{\bullet-}$ and HO$^\bullet$ deriving from transition-metal ion assisted Fenton chemistry of H_2O_2. The hydroxyl radical is a known potent cytotoxin.[18,19] Previous work[11] has demonstrated that rat brain mitochondria catalyze the oxidation of 5,6-DHT, and it is also conceivable that other intraneuronal oxidative enzyme systems may also potentiate such oxidation chemistry. Under such conditions oxidation of 5,6-DHT should lead to the formation of glutathione conjugates such as A–D. On the basis of results reported here, it is unlikely, in the absence of a suitable catalyst, that electrophilic quinone 1 would alkylate protein sulfhydryl residues. The latter reaction is widely believed to be an important mechanism by which 5,6-DHT expresses its neurodegenerative properties.[2-9] However, in the event that the oxidation of 5,6-DHT is potentiated by one or more endogenous enzymes, then alkylation and cross-linking of neuronal proteins are likely. Isolated conjugates such as B–D serve as important models for potential cross-linking reactions in vivo.

Clearly, further insight into the in vivo oxidation reactions of 5,6-DHT could be provided by investigating the fate of the toxin. Detection of dimer 2 would provide evidence for in vivo autoxidation whereas detection of GSH conjugates A–D would provide evidence for an enzyme-potentiated oxidation of 5,6-DHT. We hope to report on the in vivo oxidation products of 5,6-DHT in the near future.

Experimental Section

5,6-Dihydroxytryptamine (creatinine sulfate salt), glutathione (GSH), oxidized glutathione (GSSG), tyrosinase (mushroom, EC 1.14.8.1), and ceruloplasmin (human type X) were obtained from Sigma (St. Louis, MO) and were used without further purification. Phosphate buffers of known ionic strength (μ) at pH 7.2 and 6.5 were prepared according to Christian and Purdy.[13]

In order to monitor the autoxidation of 5,6-DHT in the presence of GSH, but in the absence of an enzyme, HPLC method I was employed. For this method a semipreparative reversed-phase column (Brownlee laboratories, RP-18, 5-μm particle size, 25 × 0.7 cm) was employed, which was protected by a short guard column (Brownlee, RP-18, 5 μm, OD-DU, 50 × 5 mm). Two mobile phase solvents were employed: Solvent A was prepared by adding 20 mL of HPLC-grade methanol (MeOH, Fisher) and 10 mL of concentrated ammonium hydroxide solution (NH_4OH) to 970 mL of deionized water. The pH of this solution was adjusted to 3.25 with concentrated formic acid (HCOOH). Solvent B was prepared by adding 400 mL of MeOH and 10 mL of NH_4OH to 590 mL of water. The pH of this solution was adjusted to 3.25 by addition of HCOOH. The gradient employed was as follows: 0–16 min, 100% solvent A, flow rate 1.5 mL min^{-1}; 16–35 min, linear gradient to 5% solvent B and a corresponding linear increase of flow rate to 2.5 mL min^{-1}; 35–50 min, linear gradient to 60% solvent B, linear increase of flow rate to 3.0 mL min^{-1}; 50–56 min, 60% solvent B, increase of flow rate to 3.5 mL min^{-1}. The mobile phase was then linearly returned to 100% solvent A over 4 min. The column was equilibrated with solvent A for 10 min (1.5 mL min^{-1}) before another sample was injected. Typically, 2.0 mL of sample solution was injected.

In order to monitor the progress of the enzyme-catalyzed oxidation of 5,6-DHT in the presence of GSH and to isolate reaction products, HPLC method II was used. For this method a preparative reversed-phase HPLC column (J. T. Baker, Bakerbond C_{18}, 25 × 2.12 cm, 10-μm particle size) was used, which was protected by a guard column (5.0 × 0.95 cm) containing the same packing material. Two mobile phases were used. Solvent C was 1.1% HPLC-grade acetonitrile (MeCN) in deionized water adjusted to pH 2.1 with concentrated HCl. Solvent D was prepared

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(19) Kontos, H. A. *Chem. Biol. Interact.* **1989**, *72*, 229–255.

by mixing 600 mL of HPLC-grade MeOH, 100 mL of MeCN, and 300 mL of water. The gradient employed was as follows: 0–10 min, linear gradient from 4% to 10% solvent D, linear increase of flow rate from 3.0 to 4.0 mL min^{-1}; 10–20 min, linear gradient to 15% solvent D, concomitant increase of flow rate to 5.0 mL min^{-1}; 20–25 min, linear gradient to 25% solvent D, flow rate 5 mL min^{-1}; 25–30 min, 25% solvent D, flow rate 5 mL min^{-1}; 30–35 min, linear gradient to 45% solvent D, increase of flow rate to 6.0 mL min^{-1}; 35–40 min, linear gradient to 60% solvent D, flow rate 6 mL min^{-1}; 40–42 min, 60% solvent D, flow rate 6 mL min^{-1}. The mobile phase composition was then returned to 4% solvent D over 3 min (6 mL min^{-1}), and the column was equilibrated under the latter conditions for 5 min before another sample was injected.

Nuclear Overhauser effect difference FID's were obtained with a gated decoupling program.[14] For each measurement, 512 scans with irradiation were subtracted from those with irradiation on resonance. A decoupler amplitude of up to 73.8 Hz was employed, and a flip angle of about 45° was applied.

Voltammograms were obtained at a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate geometric surface area of 3.6 mm^2. Voltammograms were corrected for iR drop. A conventional electrochemical cell containing a platinum counter electrode and a saturated calomel reference electrode (SCE) was used. All potentials were referenced to the SCE at ambient temperature (22 ± 2 °C). Test solutions were thoroughly deaerated with a vigorous stream of nitrogen gas for about 5 min before voltammograms were recorded.

Autoxidation of 5,6-DHT in the Presence of Glutathione. 5,6-DHT (16.12 mg; 2 mM) was stirred with GSH (36.84 mg, 6 mM; or 61.4 mg, 10 mM) in 20.0 mL of pH 7.2 phosphate buffer (μ = 0.1) in a round-bottomed flask open to the atmosphere at room temperature. The reaction was monitored by periodically removing aliquots of the reaction solution and analysis with HPLC method I.

Oxidation of 5,6-DHT in the Presence of Glutathione and Tyrosinase. Glutathione (92.1 mg, 10 mM) and 5,6-DHT (24.18 mg, 2.0 mM) were dissolved in 25 mL of pH 6.5 phosphate buffer. Then a solution of tyrosinase (0.6 mg, 2058 units) in 5.0 mL of the same buffer was added with stirring over a period of 2–3 min. After approximately 15 min, the colorless reaction solution became pale yellow, and this color intensified as the reaction proceeded. The course of the reaction was monitored by periodically removing a 3.0-mL aliquot of the solution and anlysis by HPLC method II. The four major products were most conveniently isolated by preparative HPLC after the reaction had proceeded for 3.5 h. Collected products dissolved in the HPLC mobile phase were degassed under vacuum before freezing. The frozen solutions were then freeze-dried.

4-*S*-Glutathionyl-5,6-dihydroxytryptamine (A). This compound was eluted under HPLC peak A (see Figure 3). In the HPLC mobile phase (pH ≈ 2.2) A showed a characteristic UV spectrum with λ_{max} = 312 nm and λ_{min} = 263 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave an intense pseudomolecular ion (MH^+) at m/e = 498.1693 (30.7%; $C_{20}H_{27}N_5O_8S$; calcd m/e = 498.1658). Thus, A is a 1:1 adduct of 5,6-DHT and GSH. ^1H NMR spectrum (D_2O): δ 7.09 (s, 1 H, C(2)-H), 7.01 (s, 1 H, C(7)-H), 4.34 (dd, J = 7.7 Hz, 4.2 Hz, 1 H, C(d)-H), 3.74 (s, 2 H, C(f)-H_2), 3.66 (t, J = 6.6 Hz, 1 H, C(a)-H), 3.28 and 3.14 (dd, J = 17.4 Hz, 4.5 Hz, 1 H and dd, J = 17.4 Hz, 8.7 Hz, 1 H, C(e)-H_2), 3.26 (t, J = 5.4 Hz, 2 H, C(β)-H_2), 3.24 (t, J = 6.6 Hz, 2 H, C(α)-H_2), 2.20–2.01 (m, 2 H, C(c)-H_2), 1.99–1.82 (m, 2 H, C(b)-H_2). Assignment of the various resonances in the spectrum of A was based initially upon comparisons with the ^1H NMR spectrum of GSH in D_2O and homonuclear selective decoupling experiments. The appearance of only two resonances in the aromatic region of the spectrum for A indicates that GSH is linked to position C(2), C(4), or C(7). ^1H NMR in Me_2SO-d_6, in addition to the expected resonances due to the glutathionyl residue, showed three singlets at δ 10.59 (1 H, N(1)-H), 6.96 (1 H), and 6.84 (1 H). Preirradiation of the signal at δ 10.59 (N(1)-H) caused enhancements of the signals at δ 6.96 (4.9%) and 6.84 (3.4%) in NOED experiments. Accordingly, it may be concluded that the resonance at δ 6.96 corresponds to C(2)-H and that at δ 6.84 to C(7)-H and, therefore, that the glutathionyl residue is attached to the 5,6-DHT residue at the C(4) position.

4,7-Di-*S*-glutathionyl-5,6-dihydroxytryptamine (B). This compound eluted under HPLC peak B and was isolated as a white solid. In the HPLC mobile phase (pH ≈ 2.2) B exhibited a UV spectrum with λ_{max} = 322 nm and λ_{min} = 273 nm. FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 803.2335 (MH^+, 9%; $C_{30}H_{43}N_8O_{14}S_2$; calcd m/e = 803.2340). Thus, B consists of two glutathionyl residues and one residue of 5,6-DHT. ^1H NMR (D_2O): δ 7.13 (s, 1 H, C(2)-H), 4.30 (dd, J = 8.7 Hz, 4.2 Hz, 1 H, C(d)-H), 4.20 (dd, J = 8.4 Hz, 4.8 Hz, 1 H, C(d′)-H), 3.65 (t, J = 6.6 Hz, 1 H, C(a)-H), 3.60 (t, J = 6.6 Hz, 1 H, C(a′)-H), 3.57 (s, 2 H, C(f)-H_2), 3.44 (s, 2 H, C(f′)-H_2), 3.32 and 3.19 (dd, J = 15.0 Hz, 4.5 Hz, 1 H; and dd, J = 15.0 Hz, 6.9 Hz, 1 H (C(e)-H_2), 3.29 and 3.15 (dd, J = 14.4 Hz, 4.5 Hz, 1 H; and dd, J = 14.4 Hz, 9.1 Hz, 1 H, C(e′)-H_2), 3.29 (t, J = 5.1 Hz, 2 H, C(β)-H_2), 3.27 (t, J = 5.7 Hz, 2H, C(α)-H_2), 2.33–2.17 (m, 4 H, C(c)-H_2 and C(c′)-H_2), 2.09–1.85 (m, 4 H, C(b)-H_2 and C(b′)-H_2. ^1H NMR (Me_2SO-d_6): δ 10.65 (s, 1 H, N(1)-H), 7.07 (s, 1 H, C(2)-H), in addition to the expected resonances due to the glutathionyl residues.

In Me_2SO-d_6 the C(2)-H resonances observed in the spectra of 5,6-DHT, **2**,[10] and all of their glutathionyl conjugates occur at δ ≥6.90 whereas C(7)-H resonances occur upfield at δ ≤6.84, suggesting that the signal at δ 7.07 ppm for B corresponds to C(2)-H. In NOED experiments preirradiation of the signal at δ 10.65 (N(1)-H) of B caused an enhancement of that at δ 7.07 by 5.14%. Preirradiation of the N(1)-H signal in A and the N(1′)-H signal in **2** caused enhancement of the C(2)-H and C(2′)-H resonances by 4.92% and 5.65%, respectively. The enhancement of the C(7)-H signals in the latter compounds were significantly lower (3.40% and 3.25%, respectively). Taken together, the chemical shift and NOED experiment data indicate that in B the glutathionyl residues are connected to the 5,6-DHT residue at the C(4) and C(7) positions.

4,4′-Di-*S*-glutathionyl-2,7′-bi(5,6-dihydroxytryptamine) (C). This compound eluted under HPLC peak C by method II. After freeze-drying, C was isolated as a white solid. In the HPLC mobile phase (pH ≈ 2.2) C exhibited UV bands at λ_{max} = 328 nm and λ_{min} = 268 nm. FAB-MS (dithiothreitol/dithioerythritol matrix) gave m/e = 993 (MH^+, 17%) and 1015 (MNa^+, 12%). Thus, C consists of two glutathionyl residues and two residues of 5,6-DHT. ^1H NMR (D_2O): δ 7.17 and 7.12 (both s, 1 H, C(2′)-H), 7.09 and 7.08 (both s, 1 H, C(7)-H), 4.50–4.44 (m, 1 H, C(d′)-H), 4.34–4.25 (m, 1 H, C(d)-H), 3.89–3.84 (m, 2 H, C(a′)-H and C(a)-H), 3.80 (s, 2 H, C(f′)-H_2), 3.78 (s, 2 H, C(f)-H_2), 3.47–3.10 (br m, 12 H, C(e′)-H_2, C(e)-H_2, C(α′)-H_2, C(α)-H_2, C(β′)-H_2, C(β)-H_2), 2.43–2.35 (m, 4 H, C(c′)-H_2, C(c)-H_2), 2.10–1.98 (m, 4 H, C(b)-H_2, C(b′)-H_2). When the temperature of the sample was increased to 65–80 °C, the pair of peaks at δ 7.17 and 7.12 (C(2′)-H) and the pair of peaks at δ 7.09 and 7.08 (C(7)-H) collapsed together to give two singlet resonances. The splitting of these peaks probably derives from the restricted rotation about the C(2)–C(7′)–bond at low temperature, which is eliminated at higher temperature.[15] ^1H NMR (Me_2SO-d_6): δ 10.725 and 10.720 (both s, 1 H, N(1)-H), 10.16 and 10.13 (both s, 1 H, N(1′)-H), 6.92 and 6.89 (both s, 1 H, C(2′)-H), 6.84 and 6.83 (both s, 1 H, C(7)-H), in addition to the signals expected for the two glutathionyl residues. Autoxidation of 4-*S*-glutathionyl-5,6-dihydroxytryptamine (A) (0.5 mM) in pH 7.2 phosphate buffer (μ = 0.1) gave C as the major product as evidenced by spectral data and HPLC retention time. This observation confirms both the sites of addition of the glutathionyl residues in C and the linkage sites between the two 5,6-DHT residues.

4,7,4′-Tri-*S*-glutathionyl-2,7′-bi(5,6-dihydroxytryptamine) (D). This compound eluted under HPLC peak D and, after freeze-drying, was isolated as a white solid. In the HPLC mobile phase (pH ≈ 2.2) D showed a UV spectrum with λ_{max} = 326 nm and λ_{min} = 273 nm. FAB-MS (dithiothreitol/dithioerythritol matrix) gave m/e = 1298.3797 (MH^+, 15%; $C_{50}H_{68}N_{13}O_{22}S_3$; calcd m/e = 1298.3764). Thus, D consists of three glutathionyl residues and two residues of 5,6-DHT. ^1H NMR (D_2O): δ 7.17 and 7.14 (both s, 1 H, C(2′)-H), 4.51 (m, 1 H, C(d′)-H), 4.26 (m, 1 H, C(d)-H), 3.99 (m, 1 H, C(d″)-H), 3.88 (t, J = 5.7 Hz, 2 H, C(a)-H and C(a′)-H), 3.84 (t, J = 6.3 Hz, 1 H, C(a″)-H), 3.81 (s, 4 H, C(f)-H_2 and C(f′)-H_2), 3.70 (s, 2 H, C(f″)-H_2, 3.49–2.90 (br m, 14 H, C(e)-H_2, C(e′)-H_2, C(e″)-H_2, C(α)-H_2, C(α′)-H_2, C(β)-H_2, C(β′)-H_2), 2.44–2.31 (m, 6 H, C(c)-H_2, C(c′)-H_2, C(c″)-H_2), 2.13–1.98 (m, 6 H, C(b)-H_2, C(b′)-H_2, C(b″)-H_2). The splitting of the single

aromatic proton resonance (1 H) could be eliminated by increasing the temperature of the sample to ca. 80 °C, indicating restricted rotation about the C(2)–C(7′) bond. ^{1}H NMR (Me_2SO-d_6): δ 10.713 and 10.636 (both s, 1 H, N(1)-H), 10.268 and 10.193 (both s, 1 H, N(1′)-H), 6.947 and 6.917 (both s, 1 H, C(2′)-H), in addition to the expected signals for three glutathionyl residues. The splitting of all of these signals also indicates restricted rotation about the C(2)–C(7′) bond. Assignment of the signals at δ 6.947 and 6.917 of D to C(2′)-H is based on comparisons of the chemical shifts for its precursor C, i.e., δ 6.92 and 6.89 assigned to C(2′)-H and δ 6.84 and 6.83 to C(7)-H.

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Cembrane and Pseudopterane Diterpenoids of the Octocoral *Pseudopterogorgia acerosa*

Wilfred R. Chan,[†] Winston F. Tinto,[†] Richard S. Laydoo,[‡] Percy S. Manchand,[§] William F. Reynolds,[∥] and Stewart McLean*[,∥]

Department of Chemistry, University of the West Indies, St. Augustine, and Institute of Marine Affairs, Hilltop Lane, Chaguaramas, Trinidad and Tobago, Chemistry Research Department, Hoffman-La Roche Inc., Nutley, New Jersey 07110, and Department of Chemistry, University of Toronto, Toronto M5S 1A1, Canada

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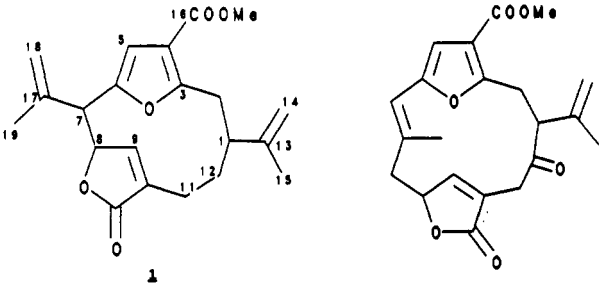
The diterpenoid metabolites isolated from *Pseudopterogorgia acerosa* collected off the coast of Tobago have been investigated. Collections made in the month of March showed a different distribution of these metabolites from a collection made in July. The dominant constituents in March were acidic diterpenoids which were converted by reaction with diazomethane to deoxypseudopterolide (1), a pseudopterane, and acerosolide (2), a cembrane. Esters 1 and 2 were isolated directly from the July collection, which also afforded the novel nitrogenous pseudopteranoid tobagolide (3). Tobagolide undergoes a novel reaction with acetic anhydride that leads to the displacement of Me_2N by OAc. Tobagolide reacts with diazomethane to form a pyrazoline. Structural assignments have depended heavily on 2-D NMR spectroscopy; in particular, the value of our FLOCK pulse sequence for establishing two-bond and three-bond ^{13}C–^{1}H shift correlations is illustrated.

Secondary metabolites of the gorgonian corals in the genus *Pseudopterogoria* have attracted much interest in recent years both for their chemical complexity and for their biological activity; some are of potential pharmacological value as cytotoxic or antiinflammatory agents.[1] The isolation of pseudopterolide from the Caribbean sea whip, *Pseudopterogoria acerosa*, was reported in 1982, and its remarkable structure was established.[2] It proved to be the first example of a rare group of diterpenoids having the pseudopterane skeleton incorporating a 12-membered carbocyclic framework. It has been suggested that the pseudopterane skeleton is formed biosynthetically by the rearrangement of a precursor with the cembrane skeleton that incorporates a 14-membered carbocyclic ring. Cembranoids are common in other gorgonian genera, but their occurrence in the genus *Pseudopterogoria* appears to be limited to very few species.[1]

We have investigated the constituents of *P. acerosa*, which occurs abundantly on reefs off the coast of Tobago, and we report here on the isolation and structure elucidation of three new metabolites of this species. Collections made in the month of March (1984 and 1985) provided two acidic diterpenes, a pseudopteranoid and a cembranoid, which were converted by treatment with diazomethane to their methyl esters, deoxypseudopterolide (1) and acerosolide (2), to facilitate isolation. A collection made in the month of July (1987) had a completely different distribution of metabolites; the principal constituent was the novel nitrogenous pseudopteranoid tobagolide (3), which we have described in a preliminary communication.[3] Small amounts of deoxypseudopterolide and acerosolide were isolated directly (i.e., without diazomethane treatment) from the July specimen.



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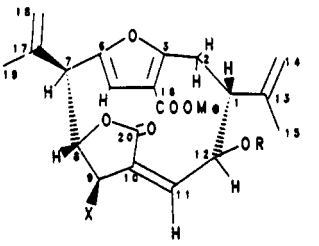

3 X = NMe_2; R = H
4 X = OAc; R = Ac

Tobagolide was isolated from the extract (without the use of diazomethane) as white crystals, mp 165–166 °C, $[\alpha]_D$ –135°, and the formula $C_{23}H_{29}O_6N$ was established by HRMS. The spectroscopic characteristics of tobagolide, collected in a routine manner, indicated that it had an

[†] University of the West Indies.
[‡] Institute of Marine Affairs.
[§] Hoffman-La Roche.
[∥] University of Toronto.

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