BIOORGANIC CHEMISTRY 19, 274–282 (1991)

Interactions between 5,6-Dihydroxytryptamine and Cysteine

SATENDRA SINGH AND GLENN DRYHURST

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

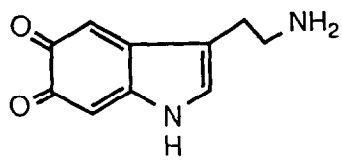
Received November 13, 1990

Autoxidation and enzyme-mediated oxidations of the serotonergic neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) generate the corresponding *ortho*-quinone (**1**) as the initial reactive intermediate. Autoxidation of 5,6-DHT in the presence of an excess of the cellular reductant/nucleophile cysteine results in a reaction where, initially, quinone **1** oxidizes cysteine to cystine with concomitant formation of 5,6-DHT. When the concentration of cysteine is sufficiently reduced **1** attacks, 5,6-DHT to give 2,7′-bi(5,6-dihydroxytryptamine) and, ultimately, an insoluble indolic melanin. When the oxidation of 5,6-DHT is catalyzed by tyrosinase, *o*-quinone **1** is attacked by cysteine to give 4-*S*-cysteinyl-5,6-dihydroxytryptamine (**A**). Methods have been devised to isolate **A** and evidence for its structure is presented. Conjugate **A** should be useful as a marker of enzyme-catalyzed oxidation of 5,6-DHT in the central nervous system.

INTRODUCTION

5,6-Dihydroxytryptamine (5,6-DHT) is one of a very small number of pharmacological tools which may be employed to selectively lesion serotonergic neurons in the mammalian central nervous system (*1–3*). The selectivity of 5,6-DHT derives from its active transport into serotonergic neurons by the membrane pump system. The mechanism(s) by which 5,6-DHT evokes its neurodegenerative action once inside the target neuron, however, remains an open question. It is widely believed that the neurotoxic properties of 5,6-DHT derive from its facile intraneuronal autoxidation (*4–11*). One theory proposes that this autoxidation reaction generates an electrophilic *o*-quinone which alkylates and cross-links neuronal proteins, thus compromising their function which leads, ultimately, to cell death. Another proposes that byproducts of the autoxidation are cytotoxic reduced oxygen species ($O_2^{\cdot-}$, H_2O_2, $HO\cdot$) which attack neuronal lipids, proteins, and other macromolecules. However, there is in fact little evidence to support the notion that 5,6-DHT is indeed oxidized *in vivo* and, until very recently, virtually nothing was known about the *in vitro* oxidation chemistry and biochemistry of the neurotoxin. In a recent report it was shown that at physiological pH 5,6-DHT is initially autoxidized to an *o*-quinone which is rapidly attacked by 5,6-DHT to give 2,7′-bi(5,6-dihydroxytryptamine) (*12*). Many of the mechanistic details of the autoxidation and enzyme-mediated autoxidations of 5,6-DHT have also been investigated (*13*). In all of the oxidation reactions studied the key intermediate is *o*-quinone, **1**. While this electron-deficient species would be expected to attack and

0045-2068/91 $3.00



1

alkylate protein nucleophiles, Nature has provided defense mechanisms within neurons to protect against such attack. Two such protective agents are glutathione and cysteine. Thus, evidence for the intraneuronal oxidation of 5,6-DHT might be provided by detection of the glutathione and/or cysteine conjugates of the indoleamine following administration of the drug. Glutathione is present in quite high concentrations within neurons (0.9–3.4 mM) (*14, 15*) compared to cysteine (ca. 0.1 mM) (*16, 17*) and, hence, it might be expected that the glutathionyl conjugates of 5,6-DHT might serve as the best marker of *in vivo* oxidation of the toxin. However, in a series of recent papers Fornstedt *et al.* (*18–21*) provided evidence for oxidation of dopamine and its metabolites in the brains of several mammalian species by detection of only their 5-*S*-cysteinyl conjugates. These observations might indicate that the addition rate of cysteine to putative catecholamine quinones is significantly greater than that of glutathione (*22, 23*) and/or that the glutathionyl conjugates are converted to cysteinyl conjugates by γ-peptidase and peptidase (*24, 25*). In our continuing efforts to understand the mechanisms by which 5,6-DHT expresses its neurodegenerative effects it will ultimately be necessary to ascertain that the indoleamine does indeed undergo oxidation in the central nervous system. Based upon the information summarized above a likely indicator of such reactions is the cysteinyl conjugate of 5,6-DHT. In this report, therefore, the interactions of cysteine with 5,6-DHT during its autoxidation and enzyme-assisted oxidation are described.

EXPERIMENTAL

Chemicals

5,6-DHT (creatinine sulfate salt), cysteine, and tyrosinase (from mushroom, EC 1.14.18.1) 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 pH 6.5 were prepared according to Christian and Purdy (*26*).

Apparatus and Methods

High performance liquid chromatography (HPLC) employed a Bio-Rad gradient instrument equipped with dual Model 1300 pumps, an Apple II_e controller, a Rheodyne Model 7125 loop injector, and an Isco Model 226 UV detector (254 nm). For analytical studies a reversed phase HPLC column (Brownlee Laboratories, Santa Clara, CA; RP-18, 5-μm particle size, 25 × 0.2 cm) was used. This column

was protected with a short guard column (Brownlee, RP-18, 5 μm, OD-GU, 5 × 0.5 cm). Mobile phases were prepared as follows: Solvent A was prepared by adding 20 ml of HPLC grade methanol (MeOH) and 10 ml of concentrated ammonium hydroxide (NH_4OH) to 970 ml of deionized water. The pH of this solution was then adjusted to 3.25 by addition of 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 was adjusted to 3.25 with HCOOH. The gradient employed was as follows: 0–16 min, 100% solvent A at a flow rate of 1.5 ml min^{-1}; 16–35 min, a linear gradient to 5% solvent B and a concommitant increase of flow rate to 2.5 ml min^{-1}; 35–50 min, linear gradient to 60% solvent B and a linear increase of flow rate to 3.0 ml min^{-1}. The latter mobile phase was then maintained for 6 min with a linear 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, 0.8 ml of the reaction mixture was injected.

Low and high resolution fast atom bombardment-mass spectrometry (FAB-MS) was performed on a VG Instruments (Manchester, UK) Model ZAB-E spectrometer. NMR spectra were recorded on a Varian 300 XL spectrometer. Nuclear Overhauser effect difference FID's were obtained using a gated decoupling program (*27*). 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 32° was applied; the relaxation delay time was set at 4.0 s.

Ultraviolet–visible spectra were recorded on a Hewlett–Packard Model 8452A diode array spectrophotometer. Cyclic voltammetry was carried out on a Bioanalytical Systems (BAS, West Lafeyette, IN) Model 100A Electrochemistry System. Voltammograms were obtained at a pyrolytic graphite electrode (PGE, Pfizer Minerals, Pigments and Metals Division, Easton, PA) and were corrected for *iR* drop. The PGE had an approximate surface area of 3.6 mm^2. A conventional electrochemical cell containing a platinum counterelectrode and a saturated calomel reference electrode (SCE) was used. Potentials are referred to the SCE at ambient temperature (22 ± 2°C).

Autoxidation of 5,6-DHT in the Presence of Cysteine

5,6-DHT (16.12 mg; 2.0 mM) was stirred with cysteine (e.g., 14.54 mg; 6.0 mM) in 20 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 (typically 0.8 ml) and analysis by HPLC.

Tyrosinase-Catalyzed Oxidation of 5,6-DHT in the Presence of Cysteine

5,6-DHT (24.12 mg; 2.0 mM) and cysteine (36.36 mg; 10 mM or 72.72 mg; 20 mM) were dissolved in 25 ml of pH 6.5 phosphate buffer (μ = 0.1). A solution of tyrosinase (0.6 mg, 2058 units) in 5.0 ml of the same buffer was then added with stirring over the period of 2–3 min. The reaction solution became yellow. The course of the reaction was monitored by periodically removing a 0.8-ml aliquot

which was analyzed by HPLC. The maximum yield of **A** was obtained after 2 h under the conditions described above.

*4-S-Cysteinyl-5,6-dihydroxytryptamine (**A**)*

Compound **A** was isolated using a preparative reversed phase HPLC column (J.T. Baker, Bakerbond C_{18}, 25 × 2.12 cm, 10-μm particle size). Two mobile phase solvents were used. Solvent C was 1.1% HPLC grade acetonitrile (MeCN) in water adjusted to pH 2.10 with HCl. Solvent D was 60% MeOH and 10% MeCN in water. The following gradient program was used: 0–10 min, linear gradient from 4 to 10% solvent D and a linear increase of flow rate from 3.0 to 4.0 ml min^{-1}; 10–20 min, linear gradients to 15% solvent D and concommitant increase of flow rate to 5.0 ml min^{-1}; 20–25 min, linear gradient to 25% solvent D, flow rate 5.0 ml min^{-1}; 20–25 min, linear gradient to 25% solvent D, flow rate 5.0 ml min^{-1}; the latter conditions were then held constant for 5 min; 30–35 min, linear increase to 45% solvent D and flow rate to 6.0 ml min^{-1}; 35–37 min, linear increase to 60% solvent D; 37–39 min, latter conditions were held constant. The mobile phase was then linearly returned to 4% solvent D over a 3-min period and equilibrated for 5 min (3 ml min^{-1}) before another sample was injected. Typically 5 ml of the reaction solution was injected. Compound **A** eluted at a retention time (t_R) of ca. 26 min. The collected samples of **A** were degassed under vacuum and then were shell frozen and freeze-dried. **A** was obtained as a very pale orange solid.

In the analytical HPLC mobile phase (pH 3.25) **A** exhibited a characteristic uv spectrum: λ_{max} (nm), 312,302 sh.; and λ_{min} (nm), 262. FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 312.1001 (MH^+, 100%; $C_{13}H_{18}N_3O_4S$; calcd. m/e = 312.1018). Thus, **A** is a 1 : 1 adduct of cysteine and 5,6-DHT. ^1H NMR (D_2O): δ 7.18 (s, 1H, C(2)–H), 7.10 (s, 1H, C(7)–H), 3.96 (t, J = 6.0 Hz, C(a)–H), 3.52–3.31 (m, 4H, C(α)–H_2 and C(β)–H_2), 3.29 (d, J = 6.0 Hz, 2H, C(b)–H_2). In Me_2SO-d_6: δ 10.68 (d, $J_{1,2}$ = 2.1 Hz, N(1)–H), 9.45 (br s, 1H, OH), 8.56 (br s, ~3H, NH_3^+), 8.30 (br s, 1H, OH), 8.04 (br s, ~3H, NH_3^+), 6.99 (d, $J_{1,2}$ = 2.1 Hz, 1H, C(2)–H), 6.91 (s, 1H, C(7)–H), in addition to the other aliphatic protons expected from the 5,6-DHT and cysteine residues. These spectral data clearly indicate that **A** contains one cysteine residue linked to either the C(4)- or C(7)-positions of the 5,6-DHT residue. NOED experiments gave the most definitive results at 40–50°C (other conditions are given earlier). Upon saturation of the signal at 10.68 ppm (N(1)–H) significant positive enhancements of both signals at 6.99 ppm (C(2)–H) and 6.91 ppm (C(7)–H) were observed, confirming, therefore, that cysteine is linked to the C(4)-position of 5,6-DHT.

RESULTS

In the absence of cysteine the autoxidation of 2 mM 5,6-DHT to a black, insoluble indolic melanin is complete within 4 h (*12*). However, in the presence of a threefold molar excess of cysteine, complete oxidation of 5,6-DHT requires ca. 8 h. Increasing excesses of cysteine retard the rate of the autoxidation even further. HPLC analyses reveal that in the presence, for example, of a threefold molar

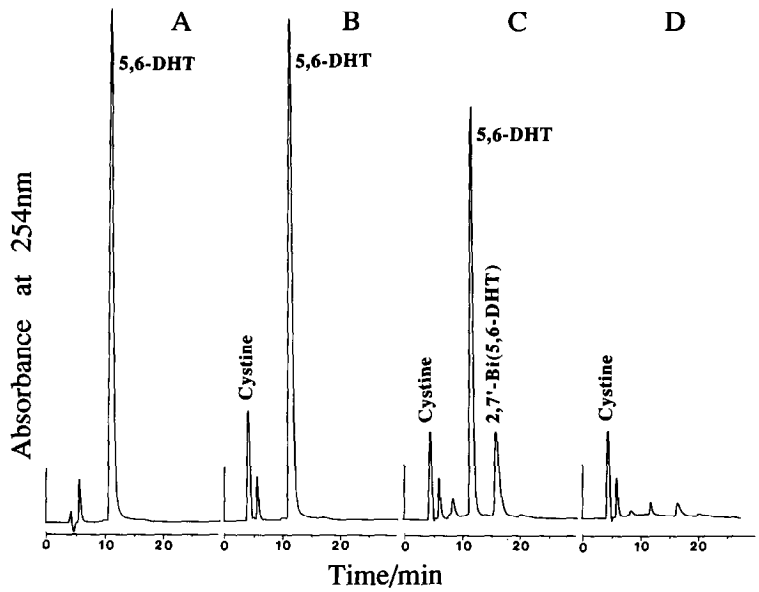
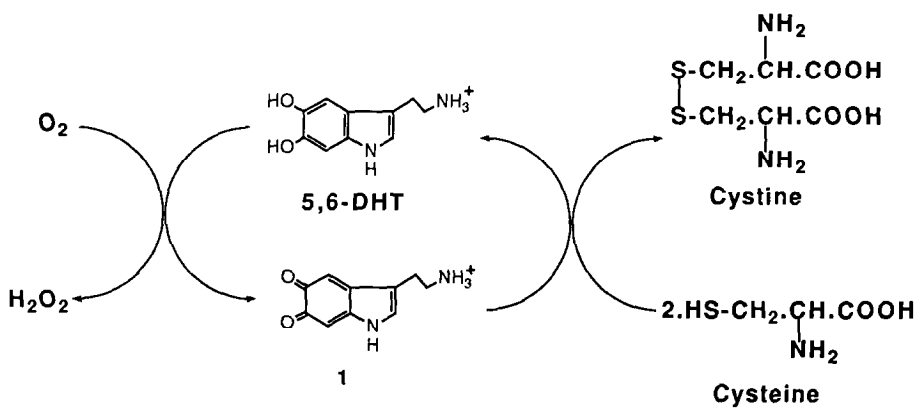


FIG. 1. HPLC chromatograms recorded during the autoxidation of 5,6-DHT (2 mM) in the presence of cysteine (6 mM) in pH 7.2 phosphate buffer (μ = 0.1) at room temperature (22 ± 2°C). Time: (A) 0, (B) 2 h, (C) 6 h, (D) 8 h. Analytical chromatographic conditions are given under Experimental. Injection volume: 0.8 ml.

excess of cysteine very little 5,6-DHT (≤3%) is oxidized during the first 2–3 h but that the oxidized form of cysteine, i.e., the dimer cystine, is formed (Fig. 1B). In the absence of 5,6-DHT very little (≤5%) cysteine is autoxidized to cystine. Thus, in the initial stages of the reaction it appears that 5,6-DHT is autoxidized to its *o*-quinone (**1**) which in turn oxidizes cysteine to cystine, hence regenerating 5,6-DHT, as conceptualized in Scheme 1. At a point in the reaction where cysteine is



SCHEME 1

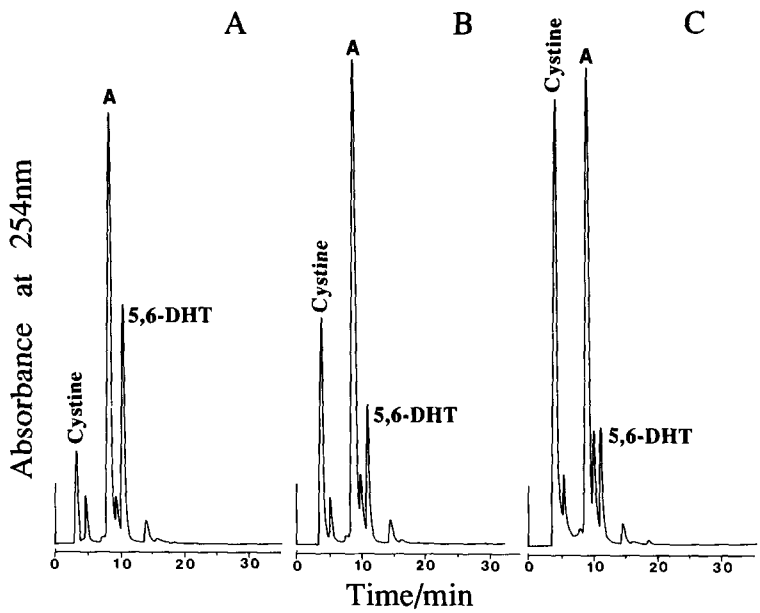
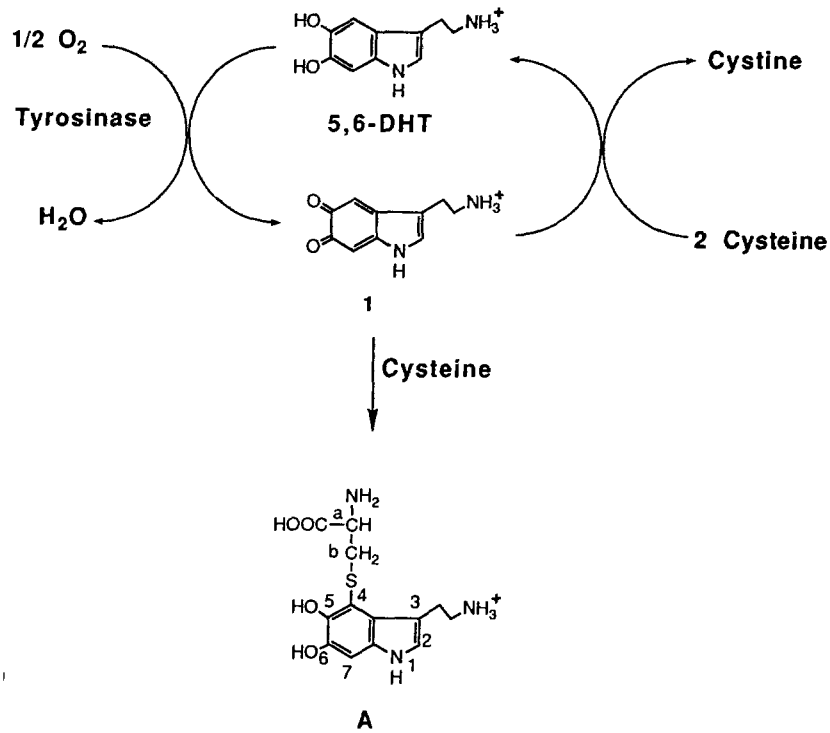


FIG. 2. HPLC chromatograms recorded during the oxidation of 5,6-DHT (2 mM) in the presence of cysteine (20 mM) and tyrosinase (0.02 mg ml^{-1}; 68.6 units ml^{-1}) in pH 6.5 phosphate buffer (μ = 0.1) at room temperature after (A) 1 h, (B) 2 h, and (C) 3 h. Analytical chromatographic conditions are given under Experimental. Injection volume: 0.8 ml.

largely depleted, i.e., the chromatographic peak corresponding to cystine no longer increases (Fig. 1C), then quinone **1** is attacked by 5,6-DHT to give initially 2,7'-bi(5,6-dihydroxytryptamine) (**2**) (Fig. 1C) and thence indolic melanin by the reaction pathways discussed in earlier reports (*12, 13*). A major byproduct of autoxidation of 5,6-DHT is H_2O_2 (*13*).

In the presence of tyrosinase the oxidation of 5,6-DHT to **1** is considerably accelerated (*12*). HPLC analysis of the reaction mixture when this enzyme-mediated reaction is carried out in the presence of excess cysteine reveals that **A** is the major product although a considerable amount of cystine is also formed (Fig. 2). The other minor products responsible for the small chromatographic peaks have not been identified. Incubations of cysteine with tyrosinase in the absence of 5,6-DHT showed only a small conversion to cystine. Thus, owing to the increased rate of oxidation of 5,6-DHT in the presence of tyrosinase *o*-quinone **1** can undergo two interactions with cysteine, nucleophilic attack to give **A** and reduction to regenerate 5,6-DHT and cystine (Scheme 2).

Cyclic voltammetry of **A** at a PGE at pH 7.2 shows an apparently irreversible oxidation peak with a peak potential (E_p) at +0.103 ± 0.012 V (0.2 mM, sweep rate 1000 mV s^{-1}). Under the same conditions E_p for 5,6-DHT is +0.092 V. Thus, electrochemically, **A** is slightly more difficult to oxidize than 5,6-DHT. Autoxidation of **A** (1.0 mM) at pH 7.2 is complete in ca. 3.5 h to give a black, insoluble precipitate. However, HPLC analyses throughout such a reaction showed only a



SCHEME 2

systematic decrease of the chromatographic peak due to **A**; no product peaks appeared. This probably reflects a rather rapid polymerization of the putative *o*-quinone derived from **A**.

DISCUSSION

The results reported here indicate that autoxidation of 5,6-DHT in the presence of cysteine does not result in the formation of a cysteinyl conjugate of the neurotoxin. Rather, 5,6-DHT, via its rather slow autoxidation reaction to *o*-quinone **1**, catalyzes the oxidation of cysteine to cystine with comcommitant formation of H_2O_2 by the reactions shown in Scheme 1. Because cysteine does not form a conjugate with 5,6-DHT under such circumstances it seems unlikely that **1** could alkylate and cross-link cellular proteins in which the most nucleophilic sites are provided by cysteinyl residues. This, therefore, implies that in the absence of any catalysis of the oxidation of 5,6-DHT its neurodegenerative properties must result from formation of H_2O_2 and thence, via Fenton-type chemistry (*13*), the highly cytotoxic hydroxyl radical. It is known, however, that the oxidation of 5,6-DHT to *o*-quinone **1** is greatly accelerated in the presence of brain mitochondria (*8*).

Under such circumstances the concentration of **1** generated during the oxidation of 5,6-DHT is increased such that not only is cysteine oxidized to cystine but also nucleophilic attack by cysteine on **1** leads to conjugate **A**. Such behavior suggests that it might be conceivable that **1** could alkylate nucleophilic residues of cellular proteins.

It is still not known definitively if 5,6-DHT is oxidized within serotonergic neurons or whether such oxidations are responsible for the neurotoxicity of this drug. These questions can only be addressed by central administration of 5,6-DHT followed by a search for a marker of *in vivo* oxidation. Compound **A** represents such a marker although its detection in the CNS could be taken to indicate that the oxidation of 5,6-DHT is catalyzed by an intraneuronal enzyme. Conjugate **A** could also be formed as a result of peptidase-catalyzed degradation of 4-glutathionyl-5,6-DHT.

ACKNOWLEDGMENTS

This work was supported by NIH Grant No. GM-32367. Additional support was provided by the Vice Provost for Research Administration at the University of Oklahoma.

REFERENCES

1. BAUMGARTEN, H. G., BJORKLUND, A., LACHENMEYER, L., NOBIN, A., AND STENEVI, U. (1975) *Acta Physiol. Suppl.* **373,** 1.
2. BAUMGARTEN, H. G., LACHENMEYER, L., AND SCHLOSSBERGER, H. G. (1972) *Z. Zellforsch.* **125,** 553.
3. JACOBY, J. H., LYTLE, L. D. Eds., (1978) *Ann. N.Y. Acad. Sci.* **305,** 1.
4. JONSSON, G. (1980) *Annu. Rev. Neurosci.* **3,** 169.
5. BAUMGARTEN, H. G., JENNER, S. BJORKLUND, A., KLEMM, H. P., AND SCHLOSSBERGER, H. G. (1983) Biology of Serotonergic Neurotransmission (Osborne, N.N. Ed.), Ch. 10, Wiley, New York.
6. BAUMGARTEN, H. G., KLEMM, H. P., SIEVERS, J., AND SCHLOSSBERGER, H. G. (1982) *Brain Res. Bull.* **9,** 131.
7. COHEN, G., AND HEIKKILA, R. E. (1978) *Ann. N.Y. Acad. Sci.* **305,** 74.
8. KLEMM, H. P., BAUMGARTEN, H. G., AND SCHLOSSBERGER, H. G. (1980) *J. Neurochem.* **35,** 1400.
9. BAUMGARTEN, H. G., KLEMM, H. P., LACHENMEYER, L., BJORKLUND, A., LOVENBERG, W., AND SCHLOSSBERGER, H. G. (1978) *Ann. N.Y. Acad. Sci.* **305,** 3.
10. KLEMM, H. P., AND BAUMGARTEN, H. G. (1978) *Ann. N.Y. Acad. Sci.* **305,** 36.
11. CREVELING, C. R., AND ROTMAN, A. (1978) *Ann. N.Y. Acad. Sci.* **305,** 57.
12. SINGH, S., JEN, J-F., AND DRYHURST, G. (1990) *J. Org. Chem.* **55,** 1484.
13. SINGH, S., AND DRYHURST, G. (1990) *J. Med. Chem.* **33,** 3035.
14. MESINA, J. E., PAGE, R. H., HETZEL, F. W., AND CHOPP, M. (1989) *Brain Res.* **478,** 181.
15. SLIVKA, A., MYTILINOU, C., AND COHEN, G. (1987) *Brain Res.* **409,** 275.
16. JARA, J. R., AROCA, P., SOLANO, F., MARTINEZ, J. H., AND LOZANO, J. A. (1988) *Biochim. Biophys. Acta.* **967,** 296.
17. YEUNG, J. M., BAKER, G. B., AND COUTTS, R. T. (1986) *J. Chromatogr.* **378,** 293.
18. FORNSTEDT, B., ROSENGREN, E., AND CARLSSON, A. (1986) *Neuropharmacology* **25,** 451.
19. FORNSTEDT, B., AND CARLSSON, A. (1989) *J. Neural Transm.* **76,** 155.
20. FORNSTEDT, B., BRUN, A., ROSENGREN, E., AND CARLSSON, A. (1989) *J. Neural Transm.* [*P-D Section*] **1,** 279.

21. FORNSTEDT, B., BERGH, I., ROSENGREN, E., AND CARLSSON, A. (1990) *J. Neurochem.* **54,** 578.
22. TSE, D. C., MCCREERY, R. L., AND ADAMS, R. N. (1976) *J. Med. Chem.* **19,** 37.
23. AGRUP, G., HANSSON, C., RORSMAN, H., AND ROSENGREN, E. (1982) *Arch. Dermatol. Res.* **272,** 103.
24. AGRUP, G. FALCK, B., KENNEDY, B-M., RORSMAN, H. ROSENGREN, A.-M., AND ROSENGREN, E. (1975) *Acta Derm. Venereol.* **55,** 1.
25. AGRUP, G., FALCK, B., RORSMAN, H., ROSENGREN, A-M., AND ROSENGREN, E. (1977) *Acta Derm. Venereol.* **57,** 221.
26. CHRISTIAN, G., AND PURDY, W. C. (1962) *J. Electroanal. Chem. Interfacial Electrochem.* **3,** 363.
27. SANDERS, J. K. M., AND MARSH, J. D. (1982) *Progr. NMR Spectrosc.* **15,** 353.