± 5 mmHg to 108 ± 4 mmHg. Once the angiotensin II pressor response had stabilized (within 10 min), the β_2-adrenoceptor-mediated vasodepressor effect of each agonist was investigated by iv adminstration, and drug-induced decreases in diastolic blood pressure (mmHg) were recorded. Each rat received only one test compound.

Statistical Evaluation. Results are expressed as the mean ±SEM. Statistical differences between two means ($P < 0.05$) were determined by the student t-test for unpaired observations or by testing of overlap of 95% confidence limits.[24] All straight lines were drawn by linear regression[25] and tested, wherever possible, for deviations from linearity by analysis of variance in regression.[24]

Acknowledgment. We are grateful for support of this research by the National Institutes of Health (Grant GM29358).

Registry No. 1, 96826-13-0; 1·HCl, 96826-02-7; 2, 96826-14-1; 2·HCl, 96826-03-8; 3, 96826-15-2; 3·2HCl, 96826-04-9; 4, 96826-16-3; 4·2HCl, 96826-05-0; 5, 72143-18-1; 6, 1699-59-8; 7, 96826-06-1; 8·2HCl, 96826-07-2; 9, 96826-08-3; 9·2HCl, 96826-12-9; 10·HCl, 16694-46-5; 11·HCl, 96826-09-4; 12·HCl, 96826-10-7; 13, 1699-61-2; 14, 96826-11-8; ethylenediamine, 107-15-3.

(24) Sokal, R. A.; Rohlf, F. J. "Regression in Biometry"; Freeman: San Francisco, 1969; pp 404–493.

(25) Woolf, C. M. "Principles of Biometry: Statistics for Biologists"; van Nostrand: Princeton, NJ, 1968.

Molecular Mechanism of Action of 5,6-Dihydroxytryptamine. Synthesis and Biological Evaluation of 4-Methyl-, 7-Methyl-, and 4,7-Dimethyl-5,6-dihydroxytryptamines[1]

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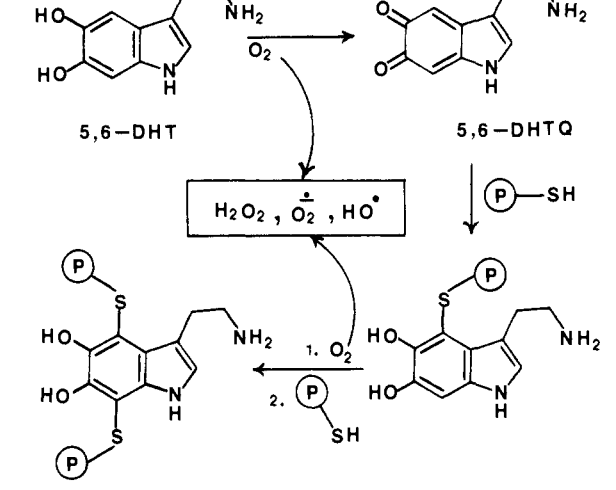
The major mechanism by which the serotonin neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) expresses its neurodegenerative action may involve alkylation of biological nucleophiles by the electrophilic quinoid autoxidation products. To determine the relative importance of various sites on these autoxidation products toward alkylation we have rationally designed and synthesized 4-Me-5,6-DHT (**16a**), 7-Me-5,6-DHT (**16b**), and 4,7-Me_2-5,6-DHT (**16c**). The indole nucleus of these analogues was constructed by the reductive cyclization of the corresponding 2,β-dinitrostyrenes, and the aminoethyl side chain was introduced via gramine methiodides. Redox data showed that all the analogues are more readily oxidized compared to 5,6-DHT. The biological activity was evaluated in differentiated neuroblastoma N-2a cells in culture. The order of inhibitory potency, as determined by measuring the inhibition of incorporation of [^{3}H]thymidine into DNA, was **16c** ≫ **16a** > 5,6-DHT ≃ **16b**. The order of affinity (expressed as IC_{50} values in μM) for serotonergic uptake as determined by measuring their inhibition of [^{3}H]-5-HT uptake was 5,6-DHT (4) > **16c** (20) > **16a** (23) > **16b** (52). The results of these studies established that these rationally designed C-methylated analogues of 5,6-DHT are suitable probes for elucidating the molecular mechanism of action of 5,6-DHT.

5,6-Dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) have become widely used pharmacological tools because of their ability to produce selective destruction of 5-hydroxytryptamine- (5-HT) containing nerve terminals.[2-7] The molecular mechanisms whereby 5,6-DHT and 5,7-DHT exert their neurodegenerative effects are still in question; however, the specific transport of these agents into serotonergic neurons by the appropriate neuronal membrane pumps is a prerequisite to their neurodegenerative effects. The chemical events that occur intraneuronally and eventually produce the destruction of the nerve terminals are initiated by the intraneuronal autoxidation of the neurotoxins. For 5,6-DHT two molecular theories have been proposed to explain the resulting cytotoxic effects:[4-6,8-12] (a) The quinone-like compound(s) generated by nonenzymatic autoxidation of 5,6-DHT may act as alkylating agent(s). (b) The H_2O_2, $O_2^{-}\cdot$, and HO·, which are also generated during autoxidation, may act as oxidizing agents. Although the relative importance of these two mechanisms is not known, neuronal degeneration is believed to be the result of alkylation of neuronal proteins by the quinone(s) together with oxidation of lipids and proteins by the reduced oxygen species.[4-6,8-12] Because of the complexity of the autoxidation reaction, it has not yet been possible to characterize the DHT-derived product(s), which has been

Chart I[a]



[a] P-SH is a protein with an exposed SH group.

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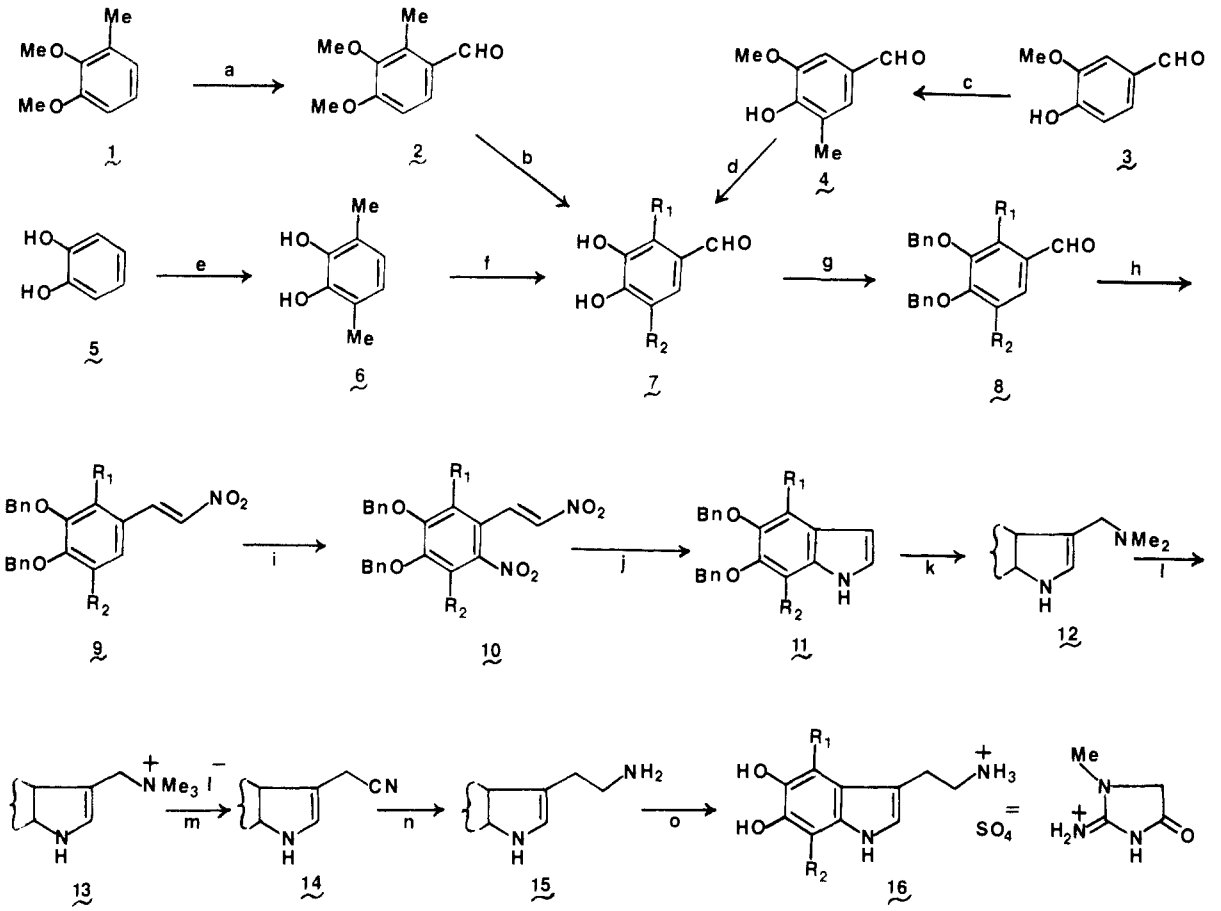
Scheme I[a]



[a] a, R_1 = Me, R_2 = H; b, R_1 = H, R_2 = Me; c, R_1 = R_2 = Me. Reagents: a, $Cl_2CHOMe/TiCl_4/CH_2Cl_2$; b, 2 → 7a, BBr_3/CH_2Cl_2; c, ref 17; d, 4 → 7b, $AlCl_3$/pyridine/CH_2Cl_2; e, ref 18; f, 6 → 7c, $CH(OEt)_3/AlCl_3/PhH$; g, $PhCH_2Cl/KI/K_2CO_3$/acetone; h, $CH_3NO_2/NH_4OAc/HOAc$; i, HNO_3/HOAc for 9a,b → 10a,b and $Cu(NO_3)_2 \cdot 3H_2O/Ac_2O$ for 9c → 10c; j, Fe/HOAc/silica gel/$PhCH_3$; k, CH_2O/Me_2NH/HOAc/EtOH; l, MeI/EtOH/EtOAc; m, $KCN/Me_2NCHO/H_2O$; n, $LiAlH_4/PhH/Et_2O$; o, H_2SO_4; Pd/C/H_2/EtOH; creatinine.

postulated to be 5,6-DHTQ (Chart I), and it appears to be highly reactive. Thus, with radioactive 5,6-DHT it has been shown that the DHT-derived autoxidation product(s) (5,6-DHTQ) undergoes extensive covalent binding with protein nucleophiles both in vitro[12] (e.g., 17 mol of 5,6-DHTQ binds to 1 mol of bovine serum albumin) and in vivo.[10] However, the nature of this protein–quinone interaction including the relative importance of the postulated electrophilic sites (namely, 4- and 7-positions) of 5,6-DHTQ toward alkylation remains to be determined.

The objective of the present study was to design probes suitable for elucidating the nature of this protein–quinone interaction both in vitro and in vivo. Thus, we required analogues of 5,6-DHT that (1) will exhibit affinities similar to that of 5,6-DHT for the neuronal membrane pumps and (2) will undergo autoxidation at rates comparable to 5,6-DHT but (3) will generate upon autoxidation the corresponding 5,6-DHTQ's in which the 4- and 7-positions are either independently or simultaneously blocked with a suitable substituent so that nucleophilic attack cannot take place in the substituted positions. Our previous studies with 6-hydroxydopamine[13-15] suggested that methyl-substituted 5,6-DHT analogues with the methyl group in the 4- and/or 7-positions may satisfy all the above mentioned requirements. In this paper we report the synthesis of these methylated analogues, namely, 4-methyl-(4-Me-5,6-DHT, **16a**), 7-methyl-(7-Me-5,6-DHT, **16b**), and 4,7-dimethyl-5,6-dihydroxytryptamine (4,7-Me_2-5,6-DHT, **16c**) and the neurotoxic potency and neuronal uptake affinity of these analogues in neuroblastoma N-2a cells in culture.

(2) Baumgarten, H. G.; Bjorklund, A.; Lachenmayer, L.; Nobin, A.; Stenevi, U. *Acta Physiol. Scand. Suppl.* **1971**, *373*, 1. Baumgarten, H. G. *Prog. Histochem. Cytochem.* **1972**, *4*, 1. Baumgarten, H. G.; Bjorklund, A.; Lachenmayer, L.; Nobin, A. *Acta Physiol. Scand. Suppl.* **1973**, *391*, 1.

(3) Jacoby, J. H., Lytle, L. D., Eds. *Ann. N.Y. Acad. Sci.* **1978**, *305*, 1-702.

(4) Jonsson, G. *Ann. Rev. Neurosci.* **1980**, *3*, 169.

(5) Baumgarten, H. G.; Klemm, H. P.; Sievers, J.; Schlossberger, H. G. *Brain Res. Bull.* **1982**, *9*, 131.

(6) Baumgarten, H. G.; Jenner, S.; Bjorklund, A.; Klemm, H. P.; Schlossberger, H. G. In "Biology of Serotonergic Transmission"; Osborne, N. N., Ed.; Wiley: New York, 1982; Chapter 10.

(7) Jonsson, G. In "Handbook of Chemical Neuroanatomy: Methods in Chemical Neuroanatomy"; Bjorklund, A., Hokfelt, T., Eds.; Elsevier: Amsterdam, 1983; Vol. 1, Chapter XII.

(8) Cohen, G.; Heikkila, R. E. *Ann. N.Y. Acad. Sci.* **1978**, *305*, 74.

(9) Klemm, H. P.; Baumgarten, H. G.; Schlossberger, H. G. *J. Neurochem.* **1980**, *35*, 1400.

(10) Baumgarten, H. G.; Klemm, H. P.; Lachenmayer, L.; Bjorklund, A.; Lovenberg, W.; Schlossberger, H. G. *Ann. N.Y. Acad. Sci.* **1978**, *305*, 3.

(11) Klemm, H. P.; Baumgarten, H. G. *Ann. N.Y. Acad. Sci.* **1978**, *305*, 36.

(12) Creveling, C. R.; Rotman, A. *Ann. N.Y. Acad. Sci.* **1978**, *305*, 57.

(13) Borchardt, R. T.; Burgess, S. K.; Reid, J. R.; Liang, Y.-O.; Adams, R. N. *Mol. Pharmacol.* **1977**, *13*, 805.

(14) Borchardt, R. T.; Reid, J. R.; Thakker, D. R.; Liang, Y. O.; Wightman, R. W.; Adams, R. N. *J. Med. Chem.* **1976**, *19*, 1201.

(15) Borchardt, R. T.; Bhatia, P. *J. Med. Chem.* **1982**, *25*, 263.

Chemistry. Our general strategy for the synthesis of the C-methylated analogues **16a–c** was to transform appropriately substituted benzaldehydes to the corresponding indoles via 2,β-dinitrostyrenes followed by conversion of the indoles to the tryptamines (Scheme I). Initial studies revealed the unsuitability of methyl group as the protecting group for the phenolic hydroxyls. For example, 5,6-dimethoxy-4-methyltryptamine could not be satisfactorily O-demethylated to 4-Me-5,6-DHT under a variety of conditions. For this reason, phenolic hydroxyls were protected as their benzyl ethers in spite of the fact that these protecting groups rendered many of the intermediate steps more difficult to carry out when compared with the corresponding steps using methyl ethers.

For the synthesis of aldehyde **8a** (precursor to 4-Me-5,6-DHT) the key intermediate **2** was synthesized by the formylation[16] of **1**, prepared[15] from 3-methylcatechol. The position of the formyl functionality was confirmed from the ^{1}H NMR spectrum of **2**, which exhibited characteristic ortho coupling of the two aromatic protons (J = 8 Hz). O-Demethylation of **2** with BBr_3 followed by O-benzylation of the resulting catechol **7a** gave aldehyde **8a**. Synthesis of aldehyde **8b** (precursor to 7-Me-5,6-DHT) was accomplished readily from **4** by O-demethylation followed by O-benzylation. The aldehyde **4**, in turn, was synthesized[17] from vanillin (**3**) in two steps without requiring protection of either the phenolic hydroxyl or the formyl function of **3**. Synthesis of aldehyde **8c** (precursor to 4,7-Me_2-5,6-DHT) started with catechol **5**, which was first converted[18] to the dimethylcatechol **6**. Formylation of **6** with $CH(OEt)_3$ in benzene in the presence of $AlCl_3$, followed by O-benzylation, furnished **8c** in high overall yield.

The sequence of reactions that were used for the conversion of aldehydes **8** to the corresponding tryptamines **16** was modeled after that reported for related compounds.[19–21] Thus, the aldehydes were condensed with CH_3NO_2 to give the nitrostyrenes **9**. The most satisfactory procedures for the nitration of **9** involved treatment with HNO_3/HOAc at 25 °C for **9a**, HNO_3/HOAc at 70 °C for **9b**, and $Cu(NO_3)_2 \cdot 3H_2O$ in Ac_2O at 65 °C for **9c**. Attempted reductive cyclization of **10** to the corresponding indoles **11** with Fe/HOAc gave very poor yields. This led us to modify this method of reductive cyclization.[22] By the modified procedure, which involved use of Fe/HOAc in toluene in the presence of silica gel, indoles **11a–c** were produced in 96, 85, and 90% yields, respectively. The aminoethyl side chains were then introduced in the 3-position of indoles **11** in such a way that the same reaction sequences could be adapted without modification for the incorporation of a ^{14}C label using a readily available ^{14}C reagent (e.g., K^{14}CN) for future studies. Thus, although there are simpler and more efficient ways of introducing the aminoethyl side chain,[21] we used the well-established

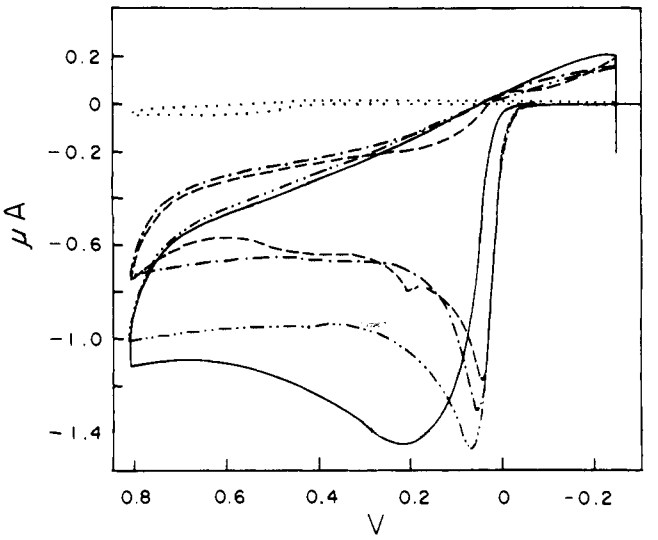


Figure 1. Cyclic voltammograms of 5,6-DHT and its methylated analogues in phosphate buffer containing 0.9% NaCl at pH 7.2 at a scan rate of 50 mV/s: —, 5,6-DHT; –·–, 4-Me-5,6-DHT (**16a**); –··–, 7-Me-5,6-DHT (**16b**); ---, 4,7-Me_2-5,6-DHT (**16c**); ···, background.

Table I. Effect of 5,6-DHT's on the Uptake of [^{3}H]-5-HT into Differentiated N-2a Cells[a]

drug	IC_{50}, μM	drug	IC_{50}, μM
5,6-DHT	6.0 ± 2.0	7-Me-5,6-DHT (**16b**)	51.8 ± 13.6
4-Me-5,6-DHT (**16a**)	23.0 ± 11.2	4,7-Me_2-5,6-DHT (**16c**)	19.7 ± 12.0

[a] The results are expressed as mean ± SD of three independent determinations in duplicate. For the details of procedure see the Experimental Section.

but relatively lengthy sequence involving indoles (**11**) → gramines (**12**) → gramine methiodides (**13**) → indole-3-acetonitriles (**14**) → tryptamine (**15**). Catalytic O-debenzylation of the hydrogen sulfates of **15** followed by treatment with creatinine furnished the target ring C-methylated dihydroxytryptamines **16a–c**.

Cyclic Voltammetry. As mentioned earlier, the neurotoxic action of 5,6-DHT is intimately related to its autoxidizability. Thus, it was of interest to determine and compare the redox potentials of the C-methylated 5,6-DHT's with that of 5,6-DHT. The redox potentials were determined by cyclic voltammetry with the standard three-electrode configuration[23] with a carbon-paste electrode as the working electrode (Figure 1). The irreversible oxidation potentials vs. Ag/AgCl observed at pH 7.2 were (in mV) 225 for 5,6-DHT, 57 for 4-Me-5,6-DHT, 72 for 7-Me-5,6-DHT, and 50 and 210 for 4,7-Me_2-5,6-DHT. These results suggest that the introduction of a methyl group increases the ease of electrochemical oxidation, which is expected[14] due to the increased electron density of the methyl-substituted indole rings. It is not clear why the second peak at 210 mV was present for 4,7-Me_2-5,6-DHT. As would be expected from the results of electrochemical studies, substitution by methyl groups also increases the rates of autoxidation. Thus, qualitative observations on the rate of formation of colored (quinoidal) products at pH 7.4 from 5,6-DHT and its methylated analogue suggest that all the methylated derivatives autoxidize considerably faster than does 5,6-DHT.

Biology and Discussion. Previously,[24] our laboratory has shown that neuroblastoma clone N-2a cells in culture

(16) Rieche, A.; Gross, H.; Hoft, E. *Chem. Ber.* **1960**, *93*, 88. "Organic Syntheses"; Wiley: New York, 1973; Collect. Vol. V, p 49.
(17) Sinhababu, A. K.; Borchardt, R. T. *Synth. Commun.* **1983**, *13*, 677.
(18) Sinhababu, A. K.; Borchardt, R. T. *Synth. Commun.* **1982**, *12*, 983.
(19) Schlossberger, H. G.; Kuch, H. *Chem. Ber.* **1960**, *93*, 1318; *Liebigs Ann. Chem.* **1963**, *662*, 132.
(20) Benigni, J. D.; Minnis, R. L. *J. Heterocycl. Chem.* **1965**, *2*, 387. Lee, F. G. H.; Dickson, D. E.; Suzuki, J.; Zirnis, A.; Manian, A. A. *Ibid.* **1973**, *10*, 649.
(21) Spande, T. F. In "Indoles"; Houlihan, W. J., Ed.; Wiley: New York, 1979; Part 3, Chapter 1.
(22) Sinhababu, A. K., Borchardt, R. T. *J. Org. Chem.* **1983**, *48*, 3347.
(23) Adams, R. N. "Electrochemistry at Solid Electrodes"; Marcel Dekker: New York, 1969.
(24) Yoffe, J. R.; Borchardt, R. T. *Mol. Pharmacol.* **1982**, *21*, 362.

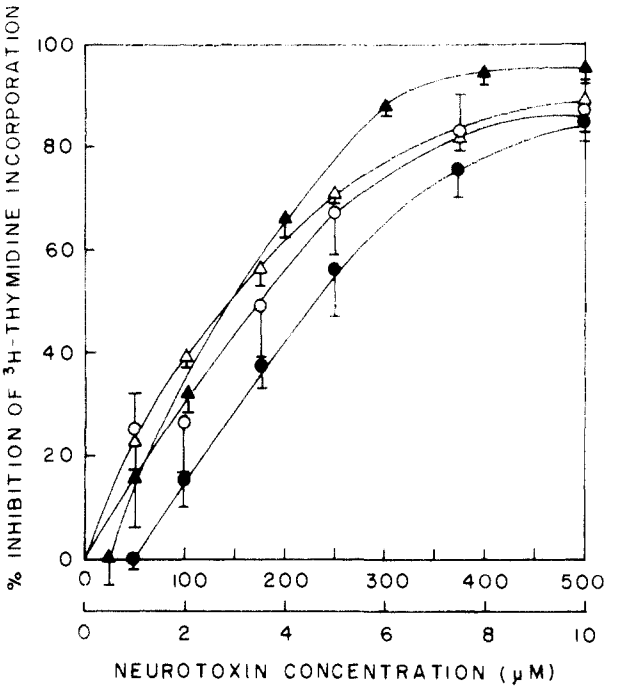


Figure 2. Cytotoxicity of 5,6-DHT's toward differentiated N-2a cells as a function of concentration. The final concentrations for 5,6-DHT (△), 4-Me-5,6-DHT (**16a**; ○), and 7-Me-5,6-DHT (**16b**; ●) were 50–500 μM while those for 4,7-Me_2-5,6-DHT (**16c**; ▲) were 0.5–10 μM. For details see the Experimental Section. The bars denote mean ± SD of at least three independent measurements in duplicate.

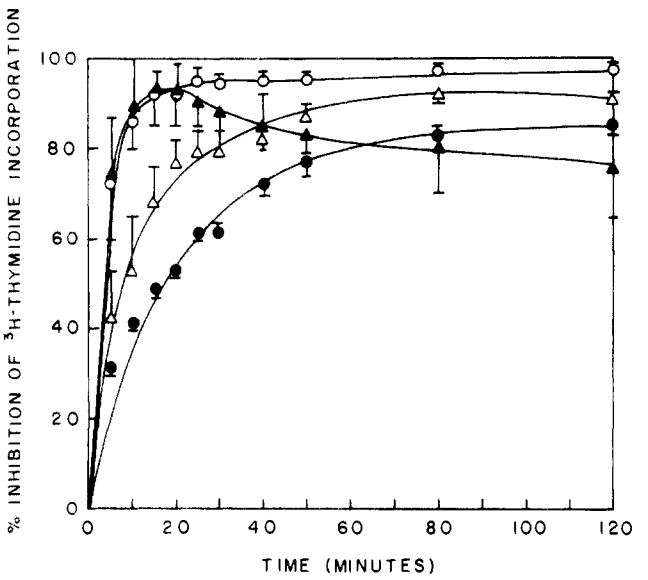


Figure 3. Cytotoxicity of 5,6-DHT's toward differentiated N-2a cells as a function of time. The cells were incubated for up to 120 min with either 5,6-DHT (500 μM; △), 4-Me,5,6-DHT (**16a**, 500 μM; ○), 7-Me-5,6-DHT (**16b**, 500 μM; ●), or 4,7-Me_2-5,6-DHT (**16c**, 10 μM; ▲). For details see the Experimental Section. The bars denote mean ± SD of at least three independent measurements in duplicate.

have characteristics of serotonergic neurons, making them simple and adequate in vitro models for studying the mechanism of action of serotonergic neurotoxins such as 5,6-DHT. These clonal cells, differentiated by serum deprivation, have been shown to take up [^{3}H]-5-HT from external medium with high affinity. This uptake is saturable, temperature dependent, partially sodium dependent, and follows Michaelis–Menten kinetics.

The cytotoxicities of 5,6-DHT and its methylated analogues **16a–c** were evaluated by measuring the inhibition of incorporation of [^{3}H]thymidine into the DNA of the differentiated N-2a cells[24] as a function of both concentration (Figure 2) and time (Figure 3). The affinities of 5,6-DHT and its methylated analogues **16a–c** for the 5-HT uptake system were determined by measuring the inhibition of [^{3}H]-5-HT uptake in the presence or absence of 5,6-DHT and its analogues (Table I).

The results presented in Figure 2 indicate that, in terms of cytotoxicity, the dimethyl analogue **16c** is at least 50 times more cytotoxic than 5,6-DHT or **16a,b**. According to the alkylation theory (see preliminary paragraphs) of cytotoxicity, the vastly greater potency of the dimethyl analogue **16c** is surprising as both of the more reactive electrophilic sites that will be generated upon autoxidation are blocked by the methyl groups. However, **16c** is also the analogue that does not undergo time-dependent degradation by polymerization to any detectable extent in solution in contrast to 5,6-DHT and the two monomethylated analogues **16a,b** that formed black precipitates extensively under the assay conditions. Thus, more of the dimethyl analogue **16c** will be available to the cells from a given concentration compared to 5,6-DHT and **16a,b**. A higher rate of autoxidation, together with no loss of **16c** due to precipitation by polymerization, also means that proportionally greater amounts of O_2-derived toxic species may be formed compared to 5,6-DHT and **16a,b**. Finally, increased lipophilicity due to the presence of two methyl groups may enhance transport of **16c** into the cell compared to 5,6-DHT and **16a,b**.

In terms of onset of cytotoxic action, all the compounds express cytotoxicity relatively rapidly (Figure 3). When tested at the concentration of highest cytotoxic action for each compound, the dimethyl analogue **16c** and the 4-methyl analogue **16a** exhibited maximum cytotoxic potential within 10–15 min whereas 5,6-DHT and the 7-methyl analogue **16b** required 60 min. The time-dependent behavior of **16c** is slightly different from that of 5,6-DHT and **16a,b** as it shows a decrease in observed toxicity with time. The significance of this small but reproducible decrease is not clear at the present time.

The results of uptake studies (Table I) show that substitution by methyl groups reduces the affinity for the 5-HT uptake system. Compared to 5,6-DHT, this reduction in affinity is 3–4-fold for **16a**, 8–10-fold for **16b**, and 2–4-fold for **16c**. It should be noted that none of the present studies can differentiate whether the observed toxicity is due to the action of the toxins inside the N-2a cell or on the cell surface. To determine whether the toxins are indeed transported inside the neuronal cell, it will be necessary to use radiolabeled derivatives of the methylated analogues. These studies as well as the evaluation of cytotoxic potential of the methylated derivatives in rat brain in vivo are in progress.

In summary, a series of methyl-substituted analogues of 5,6-DHT have been synthesized so that it would be possible to block either simultaneously or independently with methyl group the 4- or 7-positions of 5,6-DHTQ from nucleophilic attack. This substitution by methyl groups does not have any deleterious effect on the cytotoxic potential as measured in vitro in N-2a cells in culture but does reduce the uptake affinity compared to 5,6-DHT.

Experimental Section

Chemistry. General Methods. Infrared spectra were determined on a Beckman IR-33 spectrophotometer and are reported in reciprocal centimeters. ^{1}H NMR spectra were determined in the indicated solvent on Varian T-60 or FT-80A spectrometers, and chemical shifts are reported in δ units downfield from internal Me_4Si. For compounds whose spectra were recorded in D_2O,

chemical shifts were measured with respect to *p*-dioxane (Me_4Si δ 3.56) as the internal standard. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

3,4-Dihydroxy-2-methylbenzaldehyde (7a). To a stirred solution of 2,3-dimethoxytoluene (30.4 g, 200 mmol; prepared[15] from 3-methylcatechol) in 200 mL of CH_2Cl_2 at 0–5 °C protected from moisture was added $TiCl_4$ (60.8 g, 320 mmol) followed by dropwise addition of a solution of Cl_2CHOCH_3 (23.6 g, 205 mmol) in 50 mL of CH_2Cl_2. The mixture was stirred at 0–5 °C for 15 min and then at 25 °C for 2 h. The mixture was then poured into crushed ice and was stirred for 30 min. The organic layer was separated, washed with $NaHCO_3$ solution and then with brine, dried (Na_2SO_4), and evaporated in vacuo to give 33 g (91.5%) of 3,4-dimethoxy-2-methylbenzaldehyde (**2**) as an oil. A portion was recrystallized from benzene–pentane: mp 48–50 °C (lit.[15] mp 48–50 °C). To a stirred solution of crude **2** (18 g, 100 mmol) in 200 mL of CH_2Cl_2 at −78 °C in an N_2 atmosphere was added BBr_3 (50 g, 200 mmol) dropwise. The mixture was stirred at −78 °C for 30 min and then at 25 °C for 2 h. Excess BBr_3 was decomposed by slowly adding MeOH (50 mL). The mixture was then diluted with 200 mL of saturated NaCl solution and 300 mL of Et_2O. After vigorous shaking, the organic layer was collected and the aqueous layer was extracted with Et_2O (3 × 50 mL). The combined organic layer was washed with brine, dried (Na_2SO_4), and then evaporated in vacuo to give 14.8 g (97%) of **7a** as a brown solid that was used in the next step without further purification. A portion was recrystallized from EtOAc–hexane: mp 150–152 °C; IR (Nujol) 3460, 1648 cm^{-1}; NMR (acetone-d_6) δ 2.55 (s, 3, Me), 6.87 (d, $J_{5,6}$ = 8 Hz, 1, H-5), 7.27 (d, $J_{5,6}$ = 8 Hz, 1, H-6), 7.80 (br s, 2, OH), 10.03 (s, 1, CHO). Anal. ($C_8H_8O_3$) C, H.

4,5-Dihydroxy-3-methylbenzaldehyde (7b). Compound **7b** was prepared from 4-hydroxy-5-methoxy-3-methylbenzaldehyde (**4**)[17] by Lange's[25] demethylation procedure ($AlCl_3$/pyridine/CH_2Cl_2) in 76% yield (220-mmol scale). An analytical sample was prepared by recrystallization from EtOAc–cyclohexane: mp 186–188 °C; IR (Nujol) 3360, 3170, 1645 cm^{-1}; NMR (acetone-d_6) δ 2.28 (s, 3, Me), 7.26 (s, 2, H-2 and H-6), 8.13 (br, s, 1, OH), 9.00 (br, s, 1, OH), 9.55 (s, 1, CHO). Anal. ($C_8H_8O_3$) C, H.

3,4-Dihydroxy-2,5-dimethylbenzaldehyde (7c). To a stirred solution of 3,6-dimethylcatechol[18] (**6**; 24.6 g, 178 mmol) and $CH(OEt)_3$ (178 mL) in 300 mL of dry benzene in an N_2 atmosphere was added anhydrous $AlCl_3$ (49.9 g, 374 mmol) over a period of 20 min while keeping the temperature of the mixture at 60 °C. After stirring the mixture for 3 h at ambient temperature, 400 mL of 3 N HCl was added dropwise with cooling and the resulting mixture was stirred at 25 °C for 1 h. The mixture was diluted with 500 mL of Et_2O and then filtered. The precipitate was dissolved in a minimum volume of acetone and combined with the organic layer of the filtrate. The combined organic solution was washed with brine, dried ($MgSO_4$), clarified with neutral charcoal, and then evaporated in vacuo to dryness. The residue was dissolved in a minimum volume of acetone, and then 50 g of silica gel was added. After evaporation of solvent, the mixture was applied to a column of silica gel (70 g) in Et_2O. Elution with Et_2O and evaporation of solvent gave a crude solid that was recrystallized twice from benzene, yielding 19 g (64%) of **7c** as an off-white solid: mp 178–179 °C; IR (Nujol) 3400, 1655 cm^{-1}; NMR (acetone-d_6) δ 2.30 (s, 3, Me), 2.55 (s, 3 Me), 7.27 (s, 1, H-6), 8.03 (br, s, 2, OH), 10.03 (s, 1, CHO). Anal. ($C_9H_{10}O_3$) C, H.

O-Benzylation of Catechols 7a–c. General Procedure. A mixture of catechol **7a–c** (100 mmol), $PhCH_2Cl$ (27.7 g, 220 mmol), KI (3.32 g, 20 mmol), K_2CO_3 (41.4 g, 300 mmol) and acetone (400 mL) was refluxed in an N_2 atmosphere for 7 h. The mixture was cooled and filtered, and the filtrate was evaporated in vacuo to give a syrup that was dissolved in CH_2Cl_2. After it was washed with water and then with brine, the CH_2Cl_2 solution was evaporated to dryness. The syrupy residue was stirred with hexane for 30 min, and the mixture was filtered (or decanted in the case of **8c**, which was a syrup). The solid residue (or the syrup) was dissolved in CH_2Cl_2 and passed through a column of silica gel (40 g) in CH_2Cl_2. Further elution with CH_2Cl_2 and evaporation of solvent gave products that were almost pure. Aldehyde **8a** was recrystallized from benzene–hexane, yielding 25.2 g (76%): mp 83–84 °C; IR (Nujol) 1670 cm^{-1}; NMR ($CDCl_3$) δ 2.60 (s, 3, Me), 4.96 (s, 2, OCH_2), 5.20 (s, 2, OCH_2), 6.95–7.60 (m, 12, H-5, H-6, and Ph), 10.16 (s, 1, CHO). Anal. ($C_{22}H_{20}O_3$) C, H. Aldehyde **8b** was recrystallized from benzene–hexane, yielding 24.6 g (74%): mp 73–76 °C; IR (Nujol) 1690 cm^{-1}; NMR ($CDCl_3$) δ 2.27 (s, 3, Me), 5.13 (s, 2, OCH_2), 5.19 (s, 2, OCH_2), 7.23–7.53 (m, 12, H-2, H-6, and Ph), 9.83 (s, 1, CHO). Anal. ($C_{22}H_{20}O_3$) C, H. Aldehyde **8c** was obtained as a syrup (32 g, 92%): IR (neat) 1690 cm^{-1}; NMR ($CDCl_3$) δ 2.22 (s, 3, Me), 2.51 (s, 3, Me), 4.95 (s, 2, OCH_2), 5.08 (s, 2, OCH_2), 7.22–7.48 (m, 11, H-6 and Ph), 10.10 (s, 1 CHO). Anal. ($C_{23}H_{22}O_3$) C, H.

Synthesis of β-Nitrostyrenes 9a–c. General Procedure. A mixture of aldehyde **8a–c** (50 mmol), CH_3NO_2 (9.3 g, 150 mmol), dry NH_4OAc (11.55 g, 150 mmol), and 150 mL of HOAc was refluxed for 1.5 h (or until the reaction was complete as judged by TLC in CH_2Cl_2) protected from moisture. The mixture was then cooled to 25 °C and poured into water (1 L). The precipitated solid was collected by filtration, washed with water, and then dissolved in CH_2Cl_2. The CH_2Cl_2 solution was washed with $NaHCO_3$ solution, dried (Na_2SO_4), and then passed directly through a column of silica gel (40 g) in CH_2Cl_2. Further elution with CH_2Cl_2 and evaporation of solvent gave almost pure yellow solids that were recrystallized from benzene–hexane.

3,4-Bis(benzyloxy)-2-methyl-β-nitrostyrene (**9a**): yield 16.1 g (86%); mp 142–143.5 °C; NMR ($CDCl_3$) δ 2.31 (s, 3, Me), 4.98 (s, 2, OCH_2), 5.18 (s, 2, OCH_2), 6.90 (d, $J_{5,6}$ = 8.5 Hz, 1, H-5), 7.21–7.53 (m, 12, H-6, H_α and Ph), 8.23 (d, $J_{\alpha,\beta}$ = 15 Hz, 1, H_β). Anal. ($C_{23}H_{21}NO_4$) C, H, N.

3,4-Bis(benzyloxy)-5-methyl-β-nitrostyrene (**9b**): yield 13.5 g (72%); mp 130–130.5 °C; NMR ($CDCl_3$) δ 2.20 (s, 3, Me), 5.07 (s, 2, OCH_2), 5.12 (s, 2, OCH_2), 6.96 (s, 2, H-2 and H-6), 7.20–7.53 (m, 11, H_α and Ph), 7.87 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_β). Anal. ($C_{23}H_{21}NO_4$) C, H, N.

3,4-Bis(benzyloxy)-2-5-dimethyl-β-nitrostyrene (**9c**): yield 12.5 g (64%); mp 98–99 °C; NMR ($CDCl_3$) δ 2.20 (s, 3, Me), 2.29 (s, 3, Me), 4.98 (s, 2, OCH_2), 5.08 (s, 2, OCH_2), 7.13 (s, 1, H-6), 7.40 (s, 10, Ph), 7.43 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_α), 8.23 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_β).

3,4-Bis(benzyloxy)-2-methyl-6,β-dinitrostyrene (10a). To a stirred solution of nitrostyrene **9a** (9.4 g, 25 mmol) in 150 mL of HOAc was added 25 mL of 90% fuming HNO_3 at such a rate that the temperature did not rise above 40 °C. After the addition was complete, the mixture was stirred at ambient temperature for 2.5 h and then poured into 1 L of water. The solid was collected by filtration, washed thoroughly with water, and then dissolved in CH_2Cl_2. The CH_2Cl_2 solution was washed with $NaHCO_3$ solution, dried (Na_2SO_4), and then passed directly through a column of silica gel (20 g) in CH_2Cl_2. Further elution with CH_2Cl_2 and evaporation of solvent gave almost pure product that was recrystallized from EtOH, yielding 8.61 g (82%): mp 115–116 °C; NMR ($CDCl_3$) δ 2.23 (s, 3, Me), 5.13 (s, 2, OCH_2), 5.23 (s, 2, OCH_2), 6.97 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_α), 7.33 (s, 5, Ph), 7.43 (s, 5, Ph), 7.70 (s, 1, H-5), 8.22 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_β). Anal. ($C_{23}H_{20}N_2O_6$) C, H, N.

4,5-Bis(benzyloxy)-3-methyl-2,β-dinitrostyrene (10b). To a stirred solution of nitrostyrene **9b** (7.5 g, 20 mmol) in 200 mL of HOAc at 70 °C was added 20 mL of fuming 90% HNO_3 over a period of 5 min. The mixture was then stirred at 70 °C for 10 min, cooled to 25 °C, and then poured into water. The product was isolated as described for **10a**. Recrystallization from benzene and then from EtOH gave a yellow solid (5.2 g, 62%): mp 192–194 °C; NMR ($CDCl_3$) δ 2.23 (s, 3, Me), 4.99 (s, 2, OCH_2), 5.11 (s, 2, OCH_2), 7.12 (s, 1, H-6), 7.21–7.56 (m, 11, H, Ph), 7.89 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_β). Anal. ($C_{23}H_{20}N_2O_6$) C, H, N.

4,5-Bis(benzyloxy)-3,6-dimethyl-2,β-dinitrostyrene (10c). To a stirred solution of nitrostyrene **9c** (3.89 g, 10 mmol) in 40 mL of Ac_2O (freshly distilled from P_2O_5) at 60–65 °C protected from moisture was added $Cu(NO_3)_2{\cdot}3H_2O$ (3.87 g, 16 mmol) in small portions over a period of 30 min. The mixture was then stirred at 60–65 °C for 1.25 h, cooled to 25 °C, and then poured into water (400 mL). The product was isolated as described above for **10a**. Recrystallization of the crude product from benzene–cyclohexane and then from EtOH gave 2.1 g (48%) of **10** as a yellow solid: mp 124–125 °C; NMR ($CDCl_3$) δ 2.13 (s, 3, Me), 2.23 (s, 3, Me), 5.07 (s, 2, OCH_2), 5.10 (s, 2, OCH_2), 7.16 (d, $J_{\alpha,\beta}$ = 14

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Hz, 1, H_α), 7.38 (s, 10, Ph), 8.00 (d, $J_{\alpha,\beta}$ = 14 Hz, 1, H_β). Anal. ($C_{24}H_{22}N_2O_6$) C, H, N.

Synthesis of Indoles 11a–c. General Procedure.[22] A mixture of dinitrostyrene **10a–c** (4 mmol), silica gel (70–270 mesh, 10 g), HOAc (24 mL), reduced Fe powder (electrolytic grade IX, Mallinckrodt; 4 g, 71 mmol), and toluene (40 mL) was refluxed under an N_2 atmosphere for 1 h. The mixture was then cooled to 25 °C, diluted with CH_2Cl_2, and then filtered. The filter cake was washed thoroughly with CH_2Cl_2, and the combined filtrates were washed, in order, with $NaHSO_3$ solution, water, and $NaHCO_3$ solution, dried (Na_2SO_4), and then evaporated in vacuo to dryness. The residue was chromatographed on a column of silica gel (15 g) with 9:1 CH_2Cl_2–hexane as eluent. Evaporation of solvent gave essentially pure indoles that were recrystallized from cyclohexane–benzene.

5,6-Bis(benzyloxy)-4-methylindole (**11a**): yield 1.31 g (96%); mp 91 °C; NMR ($CDCl_3$) δ 2.45 (s, 3, Me), 5.00 (s, 2, OCH_2), 5.06 (s, 2, OCH_2), 6.47 (distorted t, $J_{1,3} = J_{2,3}$ = 3 Hz, 1, H-3), 6.77 (s, 1, H-7), 6.99 (t, $J_{1,2} = J_{2,3}$ = 3 Hz, 1, H-2), 7.25–7.57 (m, 10, Ph), 8.22 (br, s, 1, H-1). Anal. ($C_{23}H_{21}NO_2$) C, H, N.

5,6-Bis(benzyloxy)-7-methylindole (**11b**): yield 1.17 g (85%); mp 97–99 °C; NMR ($CDCl_3$) δ 2.25 (s, 3, Me), 4.98 (s, 2, OCH_2), 5.10 (s, 2, OCH_2), 6.37 (t, $J_{1,3} = J_{2,3}$ = 2.5 Hz, 1, H-3), 7.46–7.80 (m, 12, H-2, H-4, and Ph), 7.80 (br s, 1, H-1). Anal. ($C_{23}H_{21}NO_2$) C, H, N.

5,6-Bis(benzyloxy)-4,7-dimethylindole (**11c**): yield 1.28 g (90%); mp 88–89 °C; NMR ($CDCl_3$) δ 2.20 (s, 3, Me), 2.43 (s, 3, Me), 4.97 (s, 4, OCH_2), 6.40 (t, $J_{1,3} = J_{2,3}$ = 3 Hz, 1, H-3), 6.8 (t, $J_{2,3} = J_{1,2}$ = 3 Hz, 1, H-2), 7.30 (s, 10, Ph), 7.70 (br s, 1, H-1). Anal. ($C_{24}H_{23}NO_2$) C, H, N.

Indole-3-acetonitriles 14a–c. To a stirred mixture of HOAc (10 mL), EtOH (5 mL), 37% aqueous CH_2O (400 mg, 5 mmol), and 40% aqueous Me_2NH (600 mg, 5 mmol) at 0–5 °C was added a solution of the indole (**11a–c**; 2 mmol) in 5 mL of EtOH. After stirring at 0–5 °C for 2 h and then at 25 °C for 12 h, the mixture was diluted with water (100 mL) and then made strongly basic (pH >10) with 4 N NaOH while cooling in an ice bath. The mixture was stirred for 30 min, and the precipitated solid (or gum) was collected by filtration (or decantation). The precipitate was dissolved in CH_2Cl_2, and the resulting solution was washed with water and brine, dried (K_2CO_3), and then evaporated in vacuo to dryness to give the gramines as gums that were used in the next step without further purification.

Gramine **12a**: NMR ($CDCl_3$) δ 2.26 (s, 6, NMe_2), 2.63 (s, 3, Me), 3.63 (s, 2, CH_2N), 4.92 (s, 4, OCH_2), 6.63 (br s, 1, H-2), 6.83 (s, 1, H-7), 7.30 (s, 10, Ph), 8.77 (br s, 1, H-1).

Gramine **12b**: NMR ($CDCl_3$) δ 2.60 (s, 9, NMe_2 and Me), 3.57 (s, 2, CH_2N), 4.93 (s, 2, OCH_2), 5.05 (s, 2, OCH_2), 6.93–7.40 (m, 12, H-2, H-4, and Ph), 8.63 (br s, 1, H-1).

Gramine **12c**: NMR ($CDCl_3$) δ 2.18 (s, 3, Me), 2.25 (s, 6, NMe_2), 2.68 (s, 3, Me), 3.53 (s, 2, CH_2N), 4.87 (s, 2, OCH_2), 4.97 (s, 2, OCH_2), 6.78 (d, $J_{1,2}$ = 2 Hz, 1, H-2), 7.30 (s, 10, Ph), 7.93 (br s, 1, H-1).

To a stirred solution of CH_3I (10 g, 70 mmol) in 10 mL of EtOH at 0–5 °C, protected from moisture, was added a solution of the crude gramine **12a–c** (1 mmol), obtained in the previous step, in 10 mL of EtOAc dropwise. The mixture was refrigerated for 24 h and then evaporated in vacuo at 25 °C to dryness in a hood. The gummy residue was triturated with a 9:1 mixture of EtOAc–hexane and refrigerated for a few hours. The precipitated solid was collected by filtration and then dried under vacuum. The crude methiodides were used in the next step without further purification.

A solution or suspension of crude methiodide **13a–c** (1 mmol) in 10 mL of Me_2NCHO was placed in a water bath at 75 °C, and a solution of KCN (520 mg, 8 mmol) was added immediately with stirring. After stirring at 75 °C for 1 h, the mixture was cooled to 25 °C and then diluted with water (100 mL). The mixture was kept at 0 °C for 1 h and the precipitated solid (or gum) was collected by filtration (or decantation). The precipitate was dissolved in CH_2Cl_2, and the CH_2Cl_2 solution was washed with water and brine, dried (Na_2SO_4), and then evaporated in vacuo to dryness. Chromatography of the residue on a column of silica gel (20 g) using 9:1 CH_2Cl_2–hexane and evaporation of eluents gave nearly pure solids that were recrystallized from benzene–cyclohexane. Yields (based on respective indoles) and physical properties of the nitriles are described below.

Nitrile **14a**: yield 290 mg (38%); mp 179 °C; IR (Nujol) 2230 cm^{-1}; NMR ($CDCl_3$) δ 2.43 (s, 3, Me) 3.95 (s, 2, CH_2CN), 4.95 (s, 2, OCH_2), 5.10 (s, 2, OCH_2), 6.77 (s, 1, H-7), 7.07 (d, $J_{1,2}$ = 2 Hz, 1, H-2), 7.38 (s, 10, Ph), 7.98 (br s, 1, H-1). Anal. ($C_{25}H_{22}N_2O_2$) C, H, N.

Nitrile **14b**: yield 367 mg (48%); mp 163 °C; IR (Nujol) 2230 cm^{-1}; NMR ($CDCl_3$) δ 2.23 (s, 3, Me), 3.72 (s, 2, CH_2CN), 4.97 (s, 2, OCH_2), 5.12 (s, 2, OCH_2), 6.97 (br s, 2, H-2 and H-4), 7.13–7.50 (m, 10, Ph), 8.18 (br s, 1, H-1). Anal. ($C_{25}H_{22}N_2O_2$) C, H, N.

Nitrile **14c**: yield 324 mg (41%); mp 181–182 °C; IR (Nujol) 2225 cm^{-1}; NMR ($CDCl_3$) δ 2.27 (s, 3, Me), 2.56 (s, 3, Me), 3.94 (s, 2, CH_2CN), 4.93 (s, 2, OCH_2), 4.99 (s, 2, OCH_2), 7.11 (d, $J_{1,2}$ = 3 Hz, 1, H-2), 7.34 (s, 10, Ph), 7.90 (br s, 1, H-1). Anal. ($C_{26}H_{24}N_2O_2$) C, H, N.

Reduction of Nitriles 14a–c. General Procedure.[19] To a stirred suspension of $LiAlH_4$ (570 mg, 15 mmol) in 30 mL of anhydrous Et_2O under an N_2 atmosphere was added a solution of nitrile **14a–c** (1 mmol) in 30 mL of anhydrous benzene gradually, and the mixture was then refluxed for 6 h. After the mixture was cooled to 5–10 °C, excess $LiAlH_4$ was decomposed by carefully adding water. The organic solution was collected by decantation (or filtration) of the mixture and then was washed with water and brine, dried (K_2CO_3), and evaporated in vacuo to dryness to give the bis(benzyloxy)tryptamines **15a–c** in greater than 90% yield in each case. These tryptamines (pure by NMR, see below) were utilized in the next step without further purification.

Tryptamine **15a**: mp 93–96 °C; NMR ($CDCl_3$) δ 1.37 (br s, 2, NH_2), 2.58 (s, 3, Me), 2.94 (br s, 4, CH_2CH_2), 4.93 (s, 4, OCH_2), 6.60 (br s, 2, H-2 and H-7), 7.33 (s, 10, Ph), 8.67 (br s, 1, H-1).

Tryptamine **15b**: NMR ($CDCl_3$) δ 1.45 (br s, 2, NH_2), 2.31 (s, 3, Me), 2.90 (br s, 4, CH_2CH_2), 5.02 (s, 2, OCH_2), 5.15 (s, 2, OCH_2), 6.90 (d, $J_{1,2}$ = 2 Hz, 1, H-2), 7.03 (s, 1, H-4), 7.22–7.51 (m, 10, Ph), 8.12 (br s, 1, H-1).

Tryptamine **15c**: NMR ($CDCl_3$) δ 1.33 (br s, 2, NH_2), 2.27 (s, 3, Me), 2.60 (s, 3, Me), 3.00 (s, 4, CH_2CH_2), 4.97 (s, 2, OCH_2), 5.02 (s, 2, OCH_2), 6.87 (d, $J_{1,2}$ = 2 Hz, 1, H-2), 7.33 (s, 10, Ph), 8.23 (br s, 1, H-1).

Dihydroxytryptamine Creatinine Sulfates 16a–c. General Procedure. To a solution of bis(benzyloxy)tryptamine **15a–c** (1 mmol) in 50–75 mL of deoxygenated 95% EtOH was added 1 M H_2SO_4 (1 mL, 1 mmol) and 10% Pd/C (200 mg). The mixture was shaken in a Parr shaker at 40 psi of H_2 for 6 h at ambient temperature. [All the operations described below were conducted, as far as practicable, in a positive N_2 atmosphere. During the isolation of **16c** strict exclusion of O_2 was essential since this DHT in solution undergoes extremely rapid autoxidation to a purple material in the presence of O_2]. The mixture was then filtered under gravity, and to the filtrate was added a solution of creatinine (113 mg, 1 mmol) in 1 mL of deoxygenated water. The resulting cloudy mixture was evaporated in vacuo at <40 °C to dryness. The residue was dissolved in deoxygenated water and filtered. To the filtrate was added deoxygenated acetone until precipitation of some solid had begun, and the mixture was then stored at −20 °C overnight. White solid was obtained in each case and was collected by filtration and dried under vacuum.

5,6-Dihydroxy-4-methyltryptamine creatinine sulfate (4-Me-5,6-DHT, **16a**): yield 346 mg (83%): mp 232–234 °C dec; NMR (D_2O) δ 2.30 (s, 3, Me), 2.90 (s, 3, NMe), 3.05 (s, 4, CH_2CH_2), 4.04 (s, 2, CH_2 of creatinine), 6.88 (s, 2, H-2 and H-7). Anal. ($C_{15}H_{23}N_5O_7S)\cdot 2H_2O$ C, H, N.

5,6-Dihydroxy-7-methyltryptamine creatinine sulfate (7-Me-5,6-DHT, **16b**): yield 362 mg (87%); mp softening and color change at 192 °C, 255 °C dec; NMR (D_2O) δ 2.15 (s, 3, Me), 2.85–3.08 (m, 7, CH_2CH_2 and NMe), 3.99 (s, 2, CH_2 of creatinine), 6.77 (s, 1, H-2), 6.96 (s, 1, H-4). Anal. ($C_{15}H_{23}N_5O_7S)\cdot 2H_2O$ C, H, N.

5,6-Dihydroxy-4,7-dimethyltryptamine creatinine sulfate (4,7-Me_2-5,6-DHT, **16c**): yield 336 mg (78%); mp 196 °C softened, 230 °C dec; NMR (D_2O) δ 2.11 (s, 3, Me), 2.29 (s, 3, Me), 2.90 (s, 3, NMe), 3.06 (s, 4, CH_2CH_2), 4.04 (s, 2, CH_2 of creatinine), 6.93 (s, 1, H-2). Anal. ($C_{16}H_{25}N_5O_7S)\cdot 2H_2O$ C, H, N.

Cyclic Voltammetry. Irreversible oxidation potentials were measured at 25 °C by cyclic voltammetry using the standard three-electrode configuration[23] (Figure 1). A carbon-paste electrode was used as the working electrode along with a platinum

auxiliary electrode and a standard Ag/AgCl reference electrode. The carbon paste was prepared by mixing Ultracarbon (Ultracarbon Corp.) and hexadecane in a ratio of 2:1 by weight. The voltammograms were generated, using a freshly prepared carbon-paste electrode each time. The electrolyte was dissolved in O_2 free 0.1 M phosphate buffer containing 0.9% NaCl at pH 7.2. The concentration of the compounds were approximately 10^{-4} M in each case. The voltammograms were recorded, while maintaining an N_2 atmosphere at a scan rate of 50 mV/s on an IBM EC 225 voltammetric analyzer. The pseudo-reversible cyclic voltammogram of 4-methylcatechol, also generated under identical conditions, displayed anodic (oxidation) potential at +365 mV and cathodic (reduction) potential at −90 mV vs. Ag/AgCl.

Biology: Materials. Buffer A was an isotonic Dulbecco's phosphate-buffered saline containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.3 mM KH_2PO_4. Buffer B was prepared fresh by supplementing buffer A with 0.63 mM $CaCl_2 \cdot 2H_2O$, 0.74 mM $MgSO_4$, 5.3 mM glucose, and 0.1 mM ascorbic acid. [1,2-^{3}H]-5-HT creatinine sulfate (23.4–26.7 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA) and diluted with buffer B to give a final concentration of 0.1 μM in the incubation mixture. Thymidine (methyl-^{3}H) (20 Ci/mmol) was from Research Products International Corp. (Elk Grove Village, IL). 5,6-DHT creatinine sulfate was from Sigma Chemical Co. (St. Louis, MO).

Cell Culture, Incubation Conditions, and Procedures. Starter cultures of neuroblastoma cells, clone N-2a, were kindly provided by Dr. X. O. Breakfield (Yale University School of Medicine). Conditions for growing and differentiating cells were same as described earlier.[24] All experiments to determine the cytotoxicity and inhibition of [^{3}H]-5-HT uptake were conducted on cell suspensions rather than monolayer cultures. All experiments were done with differentiated N-2a cells that were suspended in buffer B by trituration, washed three times using a Dynac centrifuge, and resuspended in the same buffer. Cell viability, as determined by trypan blue exclusion, was found to be more than 85%. An aliquot of 10^6 cells per tube, counted by using a hemocytometer, was used for all experiments.

Cytotoxicity of 5,6-DHT's. Cytotoxicity was determined by measuring the inhibition of [^{3}H]thymidine incorporation into the DNA of the N-2a cells. The cells (10^6) were incubated at 37 °C in an incubation mixture of 1 mL of DMEM containing the different DHT's of varied concentrations or at fixed concentration for varied times as indicated in the legends to Figures 2 and 3. Solutions containing the neurotoxins were made in DMEM immediately before addition to the incubation mixture. The cells were separated by centrifugation and washed with buffer A. Finally, the cell pellets were suspended in 0.5 mL of DMEM containing 50 nM [^{3}H]thymidine (0.5 μCi) and incubated at 37 °C for an additional 90 min. The incubation was terminated by adding 2.5 mL of cold 10% TCA and kept for 12 h at 0–4 °C. The TCA-precipitated fraction was collected by filtration on GF/C glass fiber filters in a Millipore manifold previously wetted with TCA and washed three times with the same TCA solutions followed by two washes each with 2.5 mL of 95% EtOH. The filter papers containing the precipitates were then carefully transferred to the scintillation vials, dried in an oven at 60–65 °C for 15 min, and then digested with 0.5 mL of NCS tissue solubilizer (Amersham) for 30 min. To each sample was added 10 mL of complete counting cocktail, 3a70B (Research Products International Corp.), and the samples were counted, after overnight storage in the dark, on a Beckman LS-7500 scintillation counter. Cytotoxicity is expressed (mean ± SD of three sets of duplicates) as percentage inhibition of [^{3}H]thymidine incorporation with respect to the cells not incubated with the neurotoxins.

Inhibition of [^{3}H]-5-HT uptake was determined by coincubation of cell suspension (10^6) and the respective neurotoxins either at 0–4 °C or at 37 °C for 2 min in an incubation mixture of 1 mL of buffer B containing 0.1 μM [^{3}H]-5-HT (4 μCi). Neurotoxin solutions were made in buffer B immediately before addition to the incubation mixture to give a final concentration of 1–100 μM. The uptake experiment was stopped with the addition of 2.5 mL of cold buffer A followed by immediate filtration through GF/C glass fiber filters in a Millipore manifold and washing (5 × 2.5 mL) with the same cold buffer. The filter papers containing the cells were then carefully transferred to the scintillation vials, and the radioactivity was counted as described above. The active uptake is expressed (mean ± SD of three sets of duplicates) as the total accumulation of radioactivity at 37 °C minus the accumulation at 0–4 °C (passive diffusion). The degree of potency of the neurotoxins in inhibiting the [^{3}H]-5-HT uptake was determined by comparing the concentrations required to give 50% inhibition of active uptake (IC_{50} values) measured at 37 °C. These were calculated by plotting the percentage inhibition of active uptake vs. concentration and using regression analysis (Table I).

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Registry No. **2**, 51234-09-4; **4**, 32263-14-2; **6**, 2785-78-6; **7a**, 88122-12-7; **7b**, 82628-68-0; **7c**, 97073-42-2; **8a**, 97073-43-3; **8b**, 97073-44-4; **8c**, 97073-45-5; **9a**, 97073-46-6; **9b**, 97073-47-7; **9c**, 97073-48-8; **10a**, 97073-49-9; **10b**, 97073-50-2; **10c**, 97073-51-3; **11a**, 97102-11-9; **11b**, 97073-52-4; 11c, 97073-53-5; **12a**, 97073-54-6; **12b**, 97073-55-7; **12c**, 97073-56-8; **14a**, 97073-57-9; **14b**, 97073-58-0; **14c**, 97073-59-1; **15a**, 97073-60-4; **15b**, 97073-61-5; **15c**, 97073-62-6; **16a**, 97073-66-0; **16b**, 97073-69-3; **16c**, 97073-72-8; CH_3NO_2, 75-52-5; Cl_2CHOCH_3, 4885-02-3; $CH(OEt)_3$, 122-51-0; $PhCH_2Cl$, 100-44-7; Me_2NH, 124-40-3; 2,3-dimethoxytoluene, 4463-33-6.