In Vivo and in Vitro Studies on the Neurotoxic Potential of 6-Hydroxydopamine Analogs

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In an attempt to determine which physical and biological properties could best be correlated with neurotoxic potential, seven analogs of 1-(2,4,5-trihydroxyphenyl)-2-aminoethane (**1**), better known as 6-hydroxydopamine, were synthesized and compared to **1** in a variety of ways both in vivo and in vitro. The analogs, in combination with the standard **1**, include all eight of the 2,4,5-trisubstituted-phenyl derivatives of phenethylamine and α-methylphenethylamine in which the substitution is of the trihydroxy or aminodihydroxy form. Low (60 nmol) and high (300 nmol) intracerebroventricular doses of all analogs produced long-term (7 day) reduction of mouse whole brain norepinephrine (NE) and lesser depletions of dopamine (DA), and effects on serotonin were varied. The analog 1-(5-amino-2,4-dihydroxyphenyl)-2-aminopropane (**8**) was both more complete and more selective than the standard **1** in depleting NE. Using a histofluorometric glyoxylic acid method and Fink–Heimer silver degeneration stain, it was determined that overt neural degeneration was produced by **8**. In vitro, the ease of oxidation of the eight analogs was found to be represented by a formal potential range of −130 to −212 mV vs SCE. However, there was no obvious relationship between ease of oxidation and the extent of monoamine depletion from mouse brain. Using kinetic analysis of synaptosomal accumulation of [^{3}H]NE and [^{3}H]DA, it was found that the standard **1** is more potent in its interaction with the DA uptake site (K_i = 12 ± 0 μM) than the NE uptake site (K_i = 51 ± 1 μM). A correlation analysis was used to determine that differences in NE and DA depletion by each analog could not be explained by differences in potency for in vitro uptake blockade. However, there was a correlation between the K_i for [^{3}H]NE uptake blockade and the EC_{50} for synaptosomal release of preloaded [^{3}H]NE for the eight analogs (R^2 = 0.96; for log:log plot, R^2 = 0.54), indicating that the results for these two in vitro tests both reflect interaction with the same NE neuronal membrane transport site. A similar correlation between K_i and EC_{50} was shown for all eight analogs using [^{3}H]DA (R^2 = 0.92; for log:log plot, R^2 = 0.52), indicating interaction with the same DA neuronal membrane transport site. These findings demonstrate that there is no single property that can account for selectivity of action and/or potency of catecholamine neurotoxins related to 6-hydroxydopamine.

Introduction

6-Hydroxydopamine (**1**),[1–4] originally reported as a product of dopamine found in urine,[5] was shortly thereafter found to produce long-term depletion of catecholamines[6] and actual degeneration of catecholaminergic neuronal pathways.[7] There are a number of striking similarities between the actions of **1** and the pathology observed in Idiopathic Parkinson's Disease, as recently summarized by Zigmond et al.[4] One would hope that a more complete understanding of the mode of action of **1** would be capable of being employed in the alleviation and/or delay of onset of such neurodegenerative disorders; for example, knowledge of the mode of action of another dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine,[8] was partly responsible for the recent finding that L-deprenyl, a monoamine oxidase blocking agent, was effective in delaying the onset of Idiopathic Parkinson's Disease.[9]

Not being able to pass the blood–brain barrier in adults, **1** is normally administered intraventricularly or into the parenchyma in a specific brain region when central nervous system (CNS) effects are desired.[10] And, while being a quite selective agent for noradrenergic or dopaminergic pathways under appropriate conditions,[1] it is neither complete nor specific with regard to its neurodegenerative action on either of these systems in the CNS. The mode of action of **1** in eliciting degeneration has been shown to clearly involve uptake of this agent into the targeted neurons via the transport system of the endogenous neurotransmitter located on the cell membrane, since prior administration of uptake inhibitors completely eliminates the subsequent degeneration observed.[11] Presumably a minimal intraneuronal concentration of **1** must be achieved before it can elicit its degenerative effects; Jonsson and Sachs have estimated that concentrations as high as 50 mM are required.[12] But, beyond the absolute requirement for transport, the subsequent means by which **1** leads to degeneration of neuronal processes is not well-known.[1] The ease of oxidation of **1** has frequently been invoked in the mode of action; indeed, **1** is much easier to oxidize than the endogenous transmitters norepinephrine and dopamine.[13,14] One of three major hypotheses concerning the mode of action of **1** implicates the toxic oxygen byproducts resulting from oxidation of **1** by molecular O_2 (including H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet}$) as being the primary cytotoxins; this is supported by the ability of catalase

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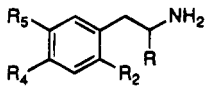


Cmpd	R_2	R_4	R_5	R
1	OH	OH	OH	H
2	NH_2	OH	OH	H
3	OH	NH_2	OH	H
4	OH	OH	NH_2	H

Cmpd	R_2	R_4	R_5	R
5	OH	OH	OH	CH_3
6	NH_2	OH	OH	CH_3
7	OH	NH_2	OH	CH_3
8	OH	OH	NH_2	CH_3

Figure 1. The eight 6-hydroxydopamine related neurotoxins.

and various radical trapping agents to block the action of **1**.[15] On the other hand, the originally proposed mode of action,[7] involving interaction of the oxidized, quinone form of **1** with nucleophilic components of functionally important cellular proteins or other nucleophiles, is equally viable and strongly supported by the correspondence between irreversible binding of ^{3}H-labeled **1** to endogenous proteins and the observed degeneration.[16] Studies by Liang et al.[17,18] have clearly demonstrated the nucleophilic attack of glutathione at the unsubstituted ring carbon atom ortho to the side chain in **1**; the isolation of the glutathionyl adduct of **1** following its injection into hypothalamus established that such reactions are possible in vivo. A third hypothesis concerning the mode of action of **1** focuses on the ability of this compound to interfere with the oxidative phosphorylation process; in fact, **1** has been claimed to be equipotent to 2,4-dinitrophenol, a classic uncoupler of this process.[19] Additionally, it is possible that the mode of action of this neurotoxin is different in noradrenergic neurons than it is in dopaminergic neurons and, perhaps, even different in subpopulations of individual neuronal types.[1] Studies by Borchardt et al.[20] aimed primarily at measuring the relative import of the first two hypotheses in the mode of action of **1** and reported on the properties of the two ring monomethylated and the one ring dimethylated derivatives of this agent. However, both of the monomethylated derivatives appeared to be capable of eliciting destructive actions; thus, one might reasonably conclude that both of the first two hypothesized actions and/or the third are involved, although to unknown degrees for each, in the ultimate degeneration process.

In an attempt to assess the relative importance of these different factors in the mode of action of **1**, we have synthesized various congeners of this toxin and examined some of their properties. Additionally, we thought it possible that such agents might provide more complete and/or more selective degeneration for noradrenergic and/or dopaminergic pathways than the original **1**; such agents could advantageously be employed in subsequent studies in place of **1**. In fact, many of the analogs of **1** that we have selected have already been shown to be neurotoxic by Tranzer and Thoenen in peripheral tissues using electron microscopy.[21] From this previous report, neurotoxic activity toward catecholaminergic neuronal systems is easily projected for all the 2,4,5-trisubstituted-phenethylamines or -α-methylphenethylamines in which the ring substitution is of the trihydroxy or aminodihydroxy variety. As seen in Figure 1, this group of compounds encompasses eight possibilities, including **1**.

Synthesis. Synthesis of analogs **2**, **5**, and **6** followed previously reported procedures.[22-24] The syntheses of analogs **3** and **4** are outlined in Schemes 1 and 2, respectively, while the syntheses of both **7** and **8** are sufficiently similar to allow incorporation into the single Scheme 3. Compounds **3**, **4**, and **8** have previously been reported. However, in the two previous reports for **3**, one[21] provided no synthetic information, no physical/spectral data, and no statement as to the salt form obtained, while the second[25] reported a different form of the final product (**3**·HCl instead of **3**·2HBr) and employed a different pathway. In the only previous report of **4**,[21] no synthetic information, no physical/spectral data, and no statement as to the salt form obtained were provided. In the only previous report of **8**,[26] no synthetic information and no physical/spectral data were provided. The standard **1** is commercially available.

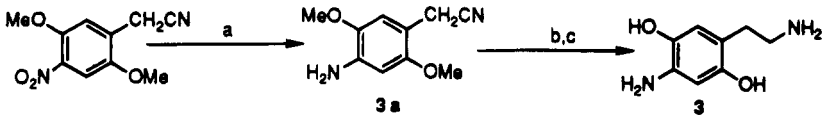
Preparation of **3**, as shown in Scheme 1, began with reduction of the nitro group of the previously reported[27] 2,5-dimethoxy-4-nitrobenzyl cyanide, yielding the desired **3a**. Subsequent conversion of the acetonitrile side chain to the ethylamine was achieved by dissolution in an EtOH/concentrated HCl solvent and hydrogenation under more forcing conditions, yielding **3b**. Demethylation of the ring methoxy groups employed concentrated HBr, providing the desired **3**·2HBr. The overall yield for the reaction sequence shown was 35%, with the lowest yield, 52%, occurring in the formation of **3b**.

In the synthesis of **4**, as shown in Scheme 2, the 2,4-dimethoxy-5-nitrobenzyl cyanide starting material was prepared from 2,4-dichloronitrobenzene as described previously.[28,29] Conversion to the amino derivative **4a** was accomplished with hydrogenation under moderate conditions using Pd/C catalyst. The cyanide group of **4a** was converted to the acetamide with concentrated HCl, and the resulting acetamide of **4b** was reduced to the saturated ethylamine with BH_3. The two ring methoxy groups were demethylated by treatment with concentrated HBr to yield **4**·2HBr·H_2O. The overall yield for the reaction sequence shown was 29%.

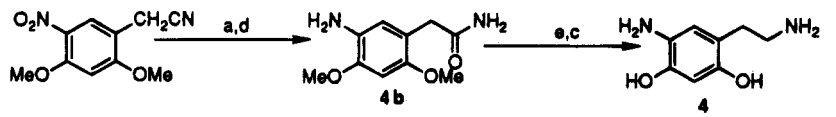
Compounds **7** and **8** were synthesized as their dihydrobromide salts according to Scheme 3. The starting materials, 2,5-dimethoxyphenyl- and 2,4-dimethoxyphenyl-2-nitropropene for **7** and **8**, respectively, were obtained through condensation of the corresponding benzaldehydes with nitroethane, as described previously.[30,31] Ring nitration was position specific in both cases, giving an 85% yield for **8a** and a 72% yield for **7a**. In both cases, simultaneous reduction of the ring nitro group to an amine and the side chain to an α-methylethylamine employed Raney nickel, while demethylation of the methoxy groups utilized concentrated HBr. The overall yield for the sequence shown was 44% for **7**·2HBr and 50% for **8**·2HBr.

Long-Term Depletion of Endogenous Neurotransmitters. Absolute proof of degeneration by a neurotoxin like **1** requires verification of biochemical measurements by electron microscopy[7] and/or histochemical fluorescence techniques.[32] But, the use of either of these approaches to routine quantitative measures of degeneration is impractical. Thus, once degeneration has been demonstrated by these techniques, more rapid and readily available biochemical measurements are generally accepted to provide the desired quantitation.[32] In the case of the compounds investigated, six (**1**–**6**) have been previously shown to produce degeneration of sympathetic innervation to the heart and/or vas defer-

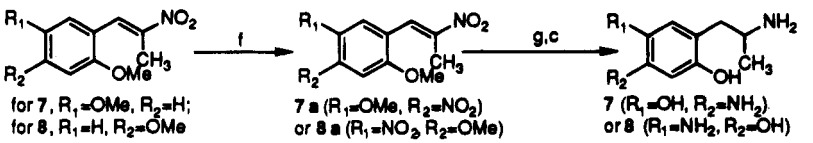
Scheme 1



Scheme 2



Scheme 3



Reagents: (a) H_2/Pd–C, (b) HCl, H_2/Pd–C, (c) concentrated HBr, (d) concentrated HCl, (e) BH_3/THF, and (f) HNO_3/HOAc, (g) H_2/Raney Ni.

ens 7 days following iv administration[21] or in other preparations,[10,33–36] and it would appear reasonable to expect a priori that the same catecholaminergic destructive capability exists for the remaining two compounds (**7**–**8**). The three most commonly employed biochemical measures for catecholaminergic degeneration following treatment with **1** and related compounds include (1) remaining levels of endogenous transmitter, (2) remaining activity levels of tyrosine hydroxylase, and (3) remaining transporter activity levels. Each of these measures has both advantages and disadvantages. For example, levels of neurotransmitters may be very rapidly depleted shortly after treatment due to releasing effects of the agent. Enzyme activities may exhibit short-term and/or long-term enhancements in response to neurotransmitter depletion and/or degeneration. Transporter density responses, if any, are unknown. A further complication arises in that neuronal pathways subjected to neurotoxic degeneration generally exhibit regeneration and/or sprouting shortly after the degenerative event. Degeneration following administration of **1** tends to predominate during the first 24–48 h following treatment, while regeneration slowly begins to predominate thereafter.[32] Endogenous transmitter levels tend to decline more rapidly and recover more slowly than transporter activity levels, but both seem to offer comparable results at ca. 7 days following treatment.[32] Given these factors, we decided to employ the simple measurement of remaining levels of pertinent neurotransmitters at 7 days following treatment as our quantitative determination of degeneration. The choice of 7 days provides some assurance, particularly for agents **7** and **8**, that the measurements were of degeneration rather than simple depletion. This approach also allows simultaneous assessment of the effects of the agents on three different transmitter systems, i.e., the noradrenergic, dopaminergic, and serotonergic systems; thus, an assessment of selectivity is additionally provided by the approach.

Results and Discussion

Table 1 shows the long-term depletion of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) achieved in whole mouse brains following a low dose of the eight agents, while Table 2 shows the same for a higher dose.

Table 1. Depletion of Whole Mouse Brain Neurotransmitters 7 Days after a Low Dose (60 nmol) of Various 6-Hydroxydopamine Related Neurotoxins[a]

		levels, % control ×b1 SEM		
compd	*n*	NE	DA	5-HT
control	5	100 ± 8	100 ± 2	100 ± 4
1	5	40 ± 8***	86 ± 1***	97 ± 2
2	5	40 ± 1***	90 ± 3*	78 ± 1***
3	6	37 ± 2***	100 ± 5	73 ± 9**
4	6	40 ± 2***	96 ± 2	91 ± 4
5	5	25 ± 2***	87 ± 0***	93 ± 8
6	6	32 ± 4***	96 ± 5	72 ± 4***
7	6	33 ± 1***	100 ± 7	79 ± 6**
8	5	26 ± 3***	100 ± 4	103 ± 5

[a] Significant differences compared to control values (*t* test): *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. Depletion of Whole Mouse Brain Neurotransmitters 7 Days after a High Dose (300 nmol) of Various 6-Hydroxydopamine Related Neurotoxins[a]

		levels, % controls ± SEM		
compd	*n*	NE	DA	5-HT
control	6	100 ± 5	100 ± 6	100 ± 4
1	6	35 ± 6***	71 ± 6**	88 ± 3*
2	5	37 ± 3***	79 ± 7	74 ± 6***
3	9	39 ± 2***	85 ± 2*	72 ± 3***
4	6	36 ± 1***	84 ± 3	96 ± 4
5		ND[b]	ND	ND
6	4	26 ± 3***	77 ± 3*	59 ± 2***
7	8	40 ± 11***	88 ± 5	83 ± 7
8	6	22 ± 1***	74 ± 9*	83 ± 4*

[a] Significant differences compared to control values (*t* test):*P < 0.05; **P < 0.01; ***P < 0.001. [b] ND = not determined.

Due to the apparently superior and more selective NE depletion afforded by **8**, we measured the long-term depletion of a high dose in eight mouse brain regions for this compound and compared it to the same for the standard **1**, as shown in Table 3. We also measured the NE, DA, and 5-HT depletions as a function of the dose administered for compounds **1** and **8**, as shown respectively in Figures 2–4. Each of these figures was fitted to an equation of the form[37]

$$\%\ \text{controls} = 100\left[1 - \frac{10^{n \log d}}{10^{n \log d_{50}} + 10^{n \log d}}\right]$$

where d is the dose (nmol), n is a slope factor, and d_{50}

Table 3. Depletion of Mouse Brain Region Neurotransmitter Levels 7 Days after a High Dose (300 nmol) of **1** and **8**[a]

region	compd	n	levels, % Control ± SEM NE	DA	5-HT
cerebellum	control	6	100 ± 4	100 ± 9	100 ± 4
	1	6	3.8 ± 0.8***	72 ± 9*	79 ± 3
	8	6	3.4 ± 1.4***	63 ± 9*	77 ± 4
medulla-pons	control	6	100 ± 9	100 ± 8	100 ± 6
	1	6	47 ± 8***	158 ± 10	109 ± 8
	8	6	59 ± 11*	110 ± 11	101 ± 3
midbrain	control	6	100 ± 8	100 ± 14	100 ± 2
	1	6	70 ± 7*	88 ± 14	89 ± 6
	8	6	74 ± 11*	116 ± 24	75 ± 6*
hypothalamus	control	6	100 ± 2	100 ± 14	100 ± 8
	1	6	6.9 ± 0.3***	116 ± 24	89 ± 6
	8	6	1.3 ± 1.4***	120 ± 15	75 ± 6*
thalamus	control	6	100 ± 8	100 ± 10	100 ± 3
	1	6	18 ± 6***	151 ± 23	90 ± 5
	8	6	14 ± 7***	154 ± 5**	93 ± 5
hippocampus	control	6	100 ± 10	100 ± 21	100 ± 9
	1	6	5.3 ± 3.2***	25 ± 6**	71 ± 7*
	8	6	6.4 ± 4.9***	42 ± 9	63 ± 5*
striatum	control	6	100 ± 28	100 ± 6	100 ± 4
	1	6	23 ± 5***	76 ± 7	103 ± 8
	8	6	7 ± 3***	71 ± 4*	104 ± 5
cortex	control	6	100 ± 6	100 ± 8	100 ± 5
	1	6	9 ± 2***	53 ± 6***	82 ± 2**
	8	6	8 ± 1***	57 ± 8**	78 ± 2*

[a] Significant differences compared to control values (t test): *P < 0.05; **P < 0.01; ***P < 0.001.

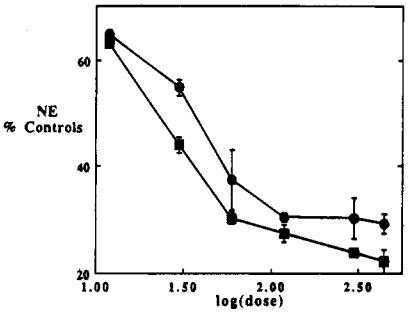


Figure 2. Whole mouse brain norepinephrine depletion dose–response effects for **1** (●) and **8** (■). Data points represent mean ± SD for n = 6. Dose in nanomoles.

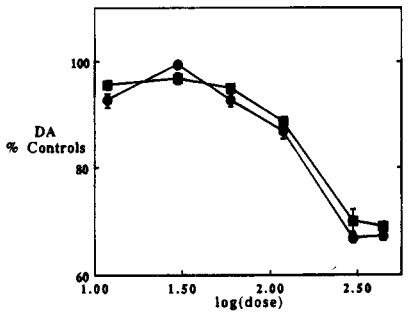


Figure 3. Whole mouse brain dopamine depletion dose–response effects for **1** (●) and **8** (■). Data points represent mean ± SD for n = 6. Dose in nanomoles.

is the dose (nmol) which provides 50% depletion by the tested agent. The results of these fittings for compounds **1** and **8**, shown in Table 4, are considered reasonably reliable for NE and DA effects, but less so for the 5-HT effects, since the amount of observed 5-HT depletions observed over the range of doses tested was relatively minimal. From the similar slope factors observed for **1** and **8** with NE depletion and, separately, with DA depletion, it would appear that the same general mechanism of toxicity is involved in the effects of both of these

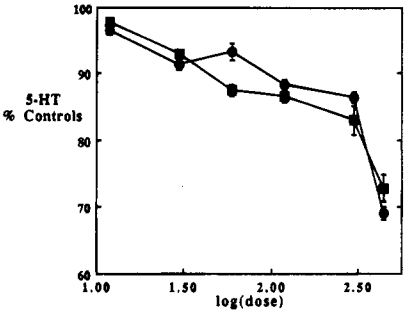


Figure 4. Whole mouse brain serotonin depletion dose-response effects for **1** (●) and **8** (■). Data points represent mean ± SD for n = 6. Dose in nmol.

Table 4. Dose–Response Depletion Effects for **1** and **8**[a]

$$\% \text{ controls} = 100\left[1 - \frac{10^{n \log d}}{10^{n \log d_{50}} + 10^{n \log d}}\right]$$

	parameter	neurotoxin **1**	**8**
NE	slope factor (n)	0.446 ± 0.086	0.511 ± 0.091
	log d_{50}, nmol	1.53 ± 0.11	1.33 ± 0.11
	d_{50}, nmol	34.3	21.5
DA	slope factor (n)	0.916 ± 0.204	0.943 ± 0.158
	log d_{50}, nmol	2.92 ± 0.12	2.96 ± 0.09
	d_{50}, nmol	831	918
5-HT	slope factor (n)	0.719 ± 0.232	0.558 ± 0.109
	log d_{50}, nmol	3.28 ± 0.30	3.51 ± 0.24
	d_{50}, nmol	1929	3218

[a] Results for slope factor and log d_{50} given as mean ± SD.

agents on each of these transmitter systems. Likewise, from the d_{50} values, **8** is seen to be 1.6 times more potent in its NE depletion than **1**, while it is only 0.90 times as potent for DA effects and only 0.60 times as potent for 5-HT effects. To quantitatively assess the selectivity of **1** and **8** with respect to the NE and DA effects, we compared the difference between the log d_{50}, DA and the log d_{50}, NE for each. For **1**, this difference was 1.39 ± 0.16, while for **8** it was 1.63 ± 0.14; these values are significantly different (P < 0.05). Thus, **8** is both a more potent and more selective noradrenergic agent in whole mouse brain than the commonly employed **1**.

Direct comparisons of depleting effects of **1** and **8** in mouse brain regions, as presented in Table 4, show that the noradrenergic effects of **8** are generally comparable to or more extensive than those of **1**. The noradrenergic effects of **8** are considerably greater than those of **1** in both the hypothalamus and striatum. This result is consistent to the enhanced noradrenergic effects previously reported for **8** in the rat hypothalamus.[26] Whether the differential effects of **1** and **8** are due to regional differences in the nature of noradrenergic projections or the character of the agents is unknown. The mouse brain regional analyses further indicate that both **1** and **8** elicit roughly equivalent depletions of both dopaminergic and serotonergic systems; the only exception to this occurs in the hippocampus, where **1** appears to produce greater DA depletion than does **8**.

The overall greater potency and selectivity of **8** compared to **1** for noradrenergic neurotoxicity is only meaningful, however, if at least comparable doses of these two agents can be tolerated. To examine this, we determined the maximum tolerable dose (MTD), defined as that which caused expiration of 50% of the mice at 1 h following injection. Using the Dixon up-and-down

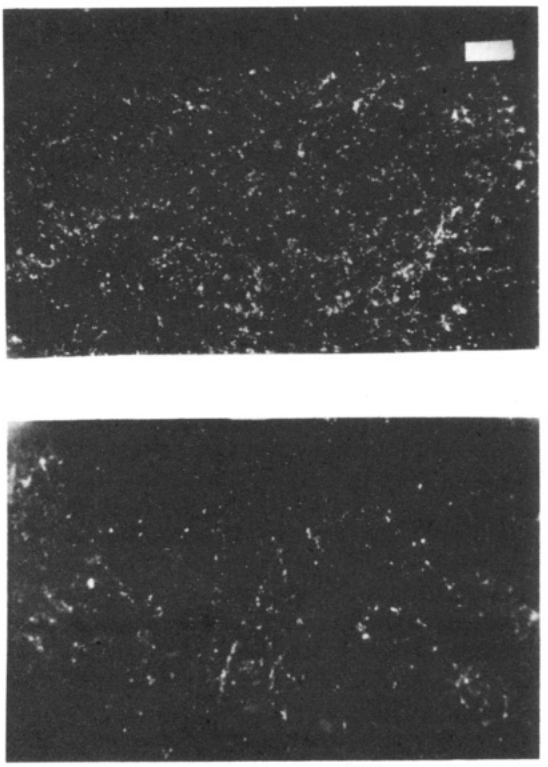


Figure 5. Histofluorescent fiber density in hypothalamic nucleus interstitialis stria terminalis of control (top) mice and mice treated with **8** (89 nmol) at 24 h following treatment (bottom). Bar in upper right region of control sample indicates 20 μm.

approach,[38] the MTD for **1** and **8**, respectively, were found to be 2.90 ± 0.02 and 3.25 ± 0.02, expressed as mean ± SD on a log(dose, nmol) scale. On a linear scale, this corresponds to approximately 793 ± 43 and 1784 ± 66 nmol, respectively. The ratio of MTD values (**8**/**1**) is thus 2.25 ± 0.15, with higher doses of **8** being tolerated. The combination of this ratio with the previously found 1.6 factor yields an overall potency advantage of 3.6 for **8** compared to **1** for noradrenergic effects. Thus, **8** is, overall, approximately 3−4 times more potent than **1** in its mouse whole brain noradrenergic neurotoxicity while being simultaneously more selective.

Histologically, compounds **1**−**5** had all previously been shown to elicit degeneration of the peripheral sympathetic nervous system neuronal projections by electron microscopy.[7,21] Electron microscopy and histochemical fluorescence had also been employed to demonstrate degeneration of noradrenergic and dopaminergic terminals in the central nervous system by **1**,[10,33,34] **2**,[35] and **6**.[36] Due to its relatively superior noradrenergic depletion effects, we were thus prompted to examine **8** from a histological perspective. Results of a histochemical fluorescence examination[39,40] of mouse hypothalamic nucleus interstitialis stria terminalis 24 h following a single intraventricular injection of 89 nmol of **8** are shown in Figure 5. A decrease of approximately 20−25% in the noradrenergic innervation was observed after treatment with 10−25 nmol. Increasing doses of **8** exhibited a dose-related decrease in the number of visible fibers; a dose of 370 nmol elicited ca. 95% decrease in such fibers. Similar results were obtained at 7 days following treatment. Changes in the number of fluorescent dopaminergic fibers in the nucleus accumbens were not observed at any but the highest doses tested, for which these fibers appeared to simply have somewhat less intense fluorescence than controls. Overall, these histochemical changes parallel the depletion results presented above. To further demonstrate degeneration, we examined rat hypothalamic tissue slices 3 or 14 days following intraventricular injection of 740 nmol of **8** using the Fink−Heimer silver-staining procedure.[41] In the treated animals, silver deposition was clearly evident, compared to controls, in and around the hypothalamus. Thus, the observed NE depletion and loss of NE fluorescence following **8** are clearly due to the loss of NE projections rather than simple depletion of the neurotransmitter.

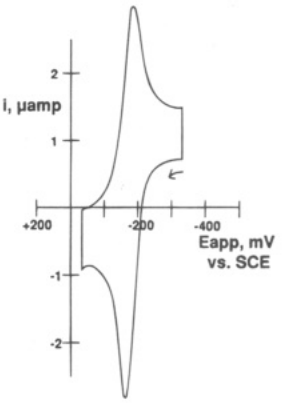


Figure 6. Cyclic voltammogram of 0.106 mM **8**. Recorded at hanging mercury drop electrode in 0.10 M phosphate buffer, pH 7.40; scan rate 92 mV/s; Ag/AgCl reference.

The ease of oxidation is frequently mentioned as an important characteristic of **1**, and this property is universally invoked in discussions of its mode of neurotoxic action.[1] The in vivo existence of the oxidized quinone form of **1** is strongly supported by the isolation of the glutathionyl adduct of this species.[18] A general correlation between the ease of oxidation and the degree of neurodegeneration afforded by analogs of **1** was provided in the study by Cheng and Castognoli.[25] Fundamentally, they showed that, for a series of 4-substituted 2,5-dihydroxyphenethylamines, only substitution with electron-releasing groups provided neurotoxicity; substitution with electron-withdrawing groups provided analogs with no detectable neurodegeneration. Among the successful substitutions, the order of strength as a neurotoxin (NH_2, OH, OCH_3, and CH_3) corresponded to the order for ease of oxidation, measured as the $E^{\circ\prime}$. Similar investigations attempting to examine the importance of oxidation examined the rate of autoxidation of various isomers of **1** and compared these to the long-term effects of these isomers on the accumulation of [^{3}H]NE by mouse heart.[42] These latter studies did not demonstrate any clearly discernible relationship between the two phenomena. Thus, we decided to examine the possible relationship between ease of oxidation and degree of neurodegeneration for the current group of compounds, since these represent a relatively homogeneous group. The ease of oxidation was determined as the $E^{\circ\prime}$ value, measured by standard electrochemical techniques employing a hanging mercury drop electrode. A typical cyclic voltammogram is shown for compound **8** in Figure 6. The $E^{\circ\prime}$ values, taken as the average of the anodic and cathodic peak potentials, for the eight neurotoxins are shown in Table

Table 5. Cyclic Voltammetry Derived Ease of Oxidation[a]

compd	concn, μM	potentials (mV vs SCE) $E_{p,c}$	$E_{p,a}$	$E^{o\prime}$
1	164	−227	−197	−212
2	92	−114	−147	−130
3	194	−167	−203	−185
4	112	−154	−192	−173
5	152	−198	−225	−212
6	92	−106	−141	−123
7	158	−167	−210	−188
8	106	−156	−186	−171

[a] $E^{o\prime}$ is the average of the anodic and cathodic peak potentials. All measurements obtained at hanging mercury drop electrode in 0.1 M phosphate buffer at pH 7.4.

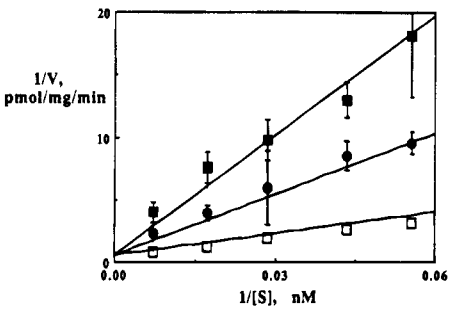


Figure 7. Kinetic analysis of NE uptake blockade by compound **2**. The concentrations of **2** for the three lines shown were, respectively, 0 (□), 10 (●), and 20 (■) μM.

5. There is no discernible relationship which could be established between the derived $E^{o\prime}$ values and the observed depletions or ratio of depletions found in Tables 1 and 2. Thus, it would appear that the simple, thermodynamic ease of oxidation is not directly correlated with the degree of degeneration afforded by these agents. Nonetheless, since all are known to be neurotoxic (**1**−**6**, **8**) or strongly suspected to be neurotoxic (**7**), one can at least infer that the range of formal potentials pertinent to eliciting toxicity for neurotoxins such as these at least encompasses those shown in Table 5. This statement, of course, presumes that oxidation is an obligatory component of the intraneuronal neurotoxic mode of action.

The interaction between each of the eight neurotoxins and the NE, DA, and 5-HT cell membrane transport sites were examined using radiolabeled transmitters and synaptosomal preparations. Preliminary determinations of IC_{50}, the concentration of toxin which blocked 50% of the accumulation in controls, indicated K_i values greater than 0.1 mM for each of the toxins with respect to the blockade of 5-HT; thus, no further investigations of this system were undertaken. Kinetic analyses, however, were performed for each of the eight toxins for both NE and DA systems. A typical such kinetic analysis is shown for the NE uptake blockade by **2** in Figure 7. The results of these analyses for both NE and DA uptake blockade for all eight toxins are presented in Table 6. Previously reported such values for uptake blockade by **1** are comparable. For example, Iversen[43,44] found K_i(NE) = 21.9 ± 2.5 μM and K_i (DA) = 36.7 ± 3.0 μM, while Borchardt et al.[20] reported approximately equal affinities of **1** toward the noradrenergic and dopaminergic uptake sites. However, more than a cursory comparison is probably unwarranted, since both of these other studies used tissue blocks or slices from differing brain regions as opposed to the synaptosomal preparations of the current study, and the DA results

Table 6. Inhibition of Norepinephrine and Dopamine Uptake[a]

compd	inhibition constants (K_i, μM) NE	DA
1	51 ± 1	12 ± 0
2	11 ± 2	17 ± 2
3	5 ± 0	9 ± 2
4	13 ± 2	47 ± 5
5	26 ± 1	16 ± 0
6	8 ± 2	9 ± 2
7	1 ± 0	3 ± 0[a]
8	46 ± 5	112 ± 33

[a] All data fitted to competitive model except that for the NE uptake blockade by **7**, which was fitted with a noncompetitive model.

in the former case were obtained using radiolabeled NE as the substrate. In addition, the incubation times for each of the studies was considerably different, ranging from the 2 min for the current investigation to 5 min[43] to 20 min.[20] And, perhaps most important of all, the actual fraction of the toxin which is in the reduced, hydroquinone, form as opposed to the oxidized, *p*-quinone or *p*-quinonimine, form during the course of these in vitro studies is unknown; it is presumed that the reduced form is actively transported by the uptake site(s), while the oxidized form is not.

In comparing the uptake results to the previously presented depletion results, it is obvious that there is no apparent relationship between the uptake blockade potency and the neurotoxic degeneration potency for the eight neurotoxins examined. Strikingly, the preferred noradrenergic agent, **8**, appears to be one of the very least potent of the neurotoxins in its ability to block uptake of either NE or DA. Attempted relationships between ratios of depletion and ratios of uptake blockade abilities similarly yielded negative results. Multiple previous studies have clearly shown that transport into the targeted neuron is essential to subsequent degeneration by such agents.[3] But, the present results would indicate, assuming that the portion of the neurotoxin in the reduced form is roughly comparable for all eight compounds investigated in these in vitro preparations, that there is no discernible relationship between the interaction of the agent with the transport site and either the degree of degeneration obtained or the selectivity of degeneration obtained.

Since uptake transport studies conducted in the manner described above might be contaminated by substantial release effects of the neurotoxins,[45] we also examined the releasing effects of the eight neurotoxins on both NE and DA synaptosomal preparations. A typical result for these studies is shown for the release of DA by **3** in Figure 8. The results for both NE and DA release by each of the eight tested neurotoxins are presented in Table 7. Comparisons to the uptake blockade results in Table 6 yielded three conclusions. First, none of the neurotoxins exhibit release effects for either NE or DA which were significantly or substantially greater than the corresponding uptake blockade effects. Second, for NE, a plot of release EC_{50} vs uptake K_i values for the eight neurotoxins reveals a correlation between these two parameters. Thus, both the transport into the neuron and the release from the neuron examined in these experiments would appear to be mediated by the same NE transport site. Third, a similar correlation was observed for all eight neurotoxins for the EC_{50} and K_i results obtained with labeled

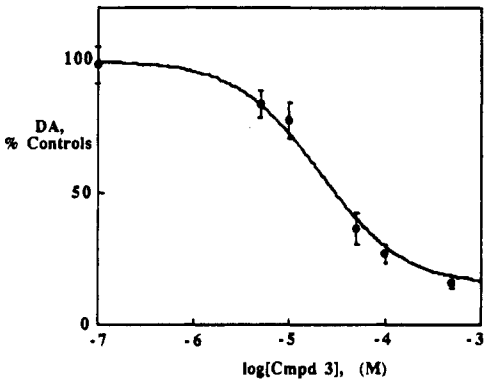


Figure 8. EC_{50} determination of DA release by compound **3**.

Table 7. Stimulation of Norepinephrine and Dopamine Synaptosomal Release

	release constants (EC_{50}, μM)	
compd	NE	DA
1	828 ± 470	60 ± 18
2	23 ± 12	3 ± 2
3	34 ± 8	21 ± 2
4	212 ± 56	105 ± 61
5	317 ± 106	101 ± 43
6	21 ± 3	7 ± 1
7	51 ± 13	8 ± 1
8	672 ± 234	380 ± 204

DA, indicating mediation by the same DA transport site. Correlation coefficients for these EC_{50}:K_i relationships were 0.96 (NE) and 0.92 (DA); log:log plots of the same data yielded correlation coefficients of 0.54 (NE) and 0.52 (DA).

The above reported results provide five specific and pertinent conclusions concerning these 6-hydroxydopamine related neurotoxins. First, the potency related to degeneration and the potency related to the interaction with the transport sites do not appear to be directly related. Second, the selectivity related to degeneration and the selectivity related to the interaction with the transport sites do not appear to be directly related. Third, the potency related to degeneration and the ease of oxidation of the agents do not appear to be directly related. Fourth, the uptake blockade effects and the neurotransmitter releasing effects of such neurotoxins for each of NE and DA, separately, are related and appear to be mediated by the corresponding transport protein in each case. Fifth, compound **8** appears to be a more potent and more selective noradrenergic neurotoxin than the standardly employed **1**.

Experimental Section

Chemical. The 60 MHz ^{1}H NMR spectra were recorded with a Varian model A60A spectrometer and the 300 MHz ^{1}H NMR spectra with a Varian XL300 unit. Chemical shifts are reported in ppm downfield from $SiMe_4$ ($CDCl_3$) and from DSS (D_2O). Mass spectra were recorded using a V.G. ZAB25 instrument. Melting points were determined in a open capillary with a Fisher melting point unit. Infrared analyses were taken as KBr pellets using a Beckman model Acculab 7 spectrometer. Elemental analyses, reported as only the elements analyzed when within 0.4% of theoretical, were performed by Desert Analytics, Tuscon, AZ. Reagents were generally used as received. However, the concentrated HBr employed for the demethylation reactions was always freshly distilled prior to use. For syntheses described below, only data for (1) final products or (2) novel intermediates and/or pathways are presented. Additional synthetic details are available as supporting information.

1-(2-Amino-4,5-dihydroxyphenyl)-2-ethylamine (2) Dihydrobromide. This compound was prepared from 1-(3,4-dimethoxyphenyl)-2-aminoethane by (a) nitration with HNO_3, (b) catalytic reduction with PtO_2, and (c) demethylation with BBr_3.[22,23,46-48] The final product was a fine whitish-gray powder. ^{1}H NMR (60 MHz, D_2O): δ 2.70–3.45 (m, 4H), 6.88 (s, 2H). Anal. ($C_8H_{12}N_2O_2$·2HBr): C, H, N.

4-Amino-2,5-dimethoxybenzyl Cyanide (3a). 2,5-Dimethoxy-4-nitrobenzyl cyanide was prepared by (a) replacement of the hydroxyl of the corresponding alcohol with chloride, (2) replacement of the chloride with cyanide, and (3) nitration.[27,49,50] This compound (2.90 g, 15.2 mmol) was suspended in 120 mL of EtOH along with 680 mg of 5% Pd/C catalyst, and the mixture was hydrogenated at 45 psi for 5 h. The light orange solution was filtered under N_2, and the catalyst was washed with 25 mL of deaerated EtOH. The solution, which rapidly darkened in air at room temperature, was cooled to 5°C, and the solvent was removed by evaporation. The resulting light gray solid was triturated with Et_2O and dried under vacuum for 5 h to give a nearly colorless product (2.40 g, 93% yield) which, after sublimation at ~95 °C, exhibited a mp of 123–125 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 3.25 (br, s, 2H), 3.60 (s, 2H), 3.73 (s, 3H), 3.80 (s, 3H), 6.27 (s, 1H), 6.70 (s, 1H).

1-(4-Amino-2,5-dimethoxyphenyl)-2-ethylamine (3b). Compound **3a** (2.8 g, 66.7 mmol) was dissolved in a mixture of 180 mL of EtOH and 12 mL of concentrated HCl. The solution was deaerated, 10% Pd/C (1.50 g) catalyst was added, and the mixture was hydrogenated at 50 psi for 36 h. The catalyst was removed by filtration, and the solvent was removed by evaporation. The semisolid remaining was treated with EtOH (50 mL), and the alcohol was removed by evaporation. The remaining dark gray solid was dissolved in 2 M HCl, impurities were extracted with $CHCl_3$ (2 × 50 mL), and the solution was raised to pH 6.5 with 40% NaOH and extracted again with $CHCl_3$ (2 × 50 mL) to remove unreacted cyanide and any intermediate amide. The pH was adjusted to 10.0 using solid NaOH, and the free base was extracted into $CHCl_3$ (3 × 150 mL). The combined $CHCl_3$ layers were dried ($MgSO_4$) and the solvent removed to give 2.20 g of a gray viscous material. Distillation at 120 °C and 2.5 mmHg for 18 h gave a colorless solid (1.49 g, 52% yield, mp 88–90 °C). The dihydrochloride salt, prepared by dissolving 100 mg of the free base in $CHCl_3$ (50 mL), passing HCl gas through the mixture, initiating precipitation by addition of Et_2O, and recrystallizing from MeOH/Et_2O, had a mp of 247–250 °C. ^{1}H NMR (free base, 300 MHz, $CDCl_3$): δ 1.55 (br, s, 2H), 2.65 (t, 2H, J = 6.9 Hz), 2.85 (t, 2H, J = 6.9 Hz), 3.72 (s, 3H), 3.79 (s, 3H), 6.32 (s, 1H), 6.60 (s, 1H). Anal. ($C_{10}H_{16}N_2O_2$·0.75H_2O): C, N; H: calcd, 8.41; found, 7.38).

1-(4-Amino-2,5-dihydroxyphenyl)-2-ethylamine (3) Dihydrobromide. Compound **3b** (1.40 g, 7.13 mmol) was dissolved in 18 mL of deaerated concentrated HBr (47.5%), and the mixture was sealed and heated at 90–95 °C for 12 h. After cooling, the solution was transferred to a smaller flask inside a glovebox and lyophilized to give 1.95 g of nearly colorless solid. The product was triturated with a deaerated solution containing CH_3CN (25 mL), MeOH (6 mL), and Et_2O (200 mL) for 1 h inside the glovebox, filtered, transferred to a vial, and dried overnight under vacuum to give 1.70 g (72% yield) of the colorless dihydrobromide salt with mp (sealed tube, dec) of 235–240 °C. Related compounds have been previously reported as **3** (salt unknown) by Tranzer and Thoenen[21] (no pathway or data given) and as **3**·HCl by Cheng and Castagnoli[25] (alternate pathway; different precursor). ^{1}H NMR (300 MHz, D_2O): δ 2.95 (t, 2H, J = 7.0 Hz), 3.26 (t, 2H, J = 7.0 Hz), 6.92 (s, 1H), 6.94 (s, 1H). Anal. ($C_8H_{12}N_2O_2$·2HBr): C, H, N.

5-Amino-2,4-dimethoxybenzyl Cyanide (4a). 2,4-Dimethoxy-5-nitrobenzyl cyanide was initially prepared from 2,4-dichloronitrobenzene by (a) displacement of the chloro groups with methoxy groups,[28] (b) chloroformylation with paraformaldehyde and HCl,[29] and (c) displacement of the benzyl chloro group with a cyano group.[29] This compound (4.5 g, 0.0203 mol) was suspended in 200 mL of deaerated EtOH. To this was added 1.1 g of 5% Pd/C under argon, and the mixture was

hydrogenated at 50 psi of H_2 for 6 h. The catalyst was removed by filtration, and the solvent was evaporated to yield 4.5 g of a gray-brown residue which, when dried overnight under vacuum, yielded 4.5 g crude product. Distillation yielded 2.77 g (71% yield) of colorless solid with mp 74–75 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 3.43 (br, s, 2H), 3.57 (s, 2H), 3.80 (s, 3H), 3.87 (s, 3H), 6.43 (s, 1H), 6.67 (s, 1H).

5-Amino-2,4-dimethoxyphenylacetamide (4b). Compound **4a** (1.45 g, 7.54 mmol) was dissolved in 125 mL of deaerated concentrated HCl, stirred for 3 h under argon, and cooled to 0 °C. Neutralization was effected by dropwise addition of NaOH (60 g in 120 mL H_2O) at <15 °C. The amide was extracted with 4 × 150 mL of $CHCl_3$, the $CHCl_3$ portions were combined, washed with 2 × 150 mL of H_2O, and dried with $MgSO_4$, and the solvent was evaporated. The colorless solid was dried overnight under vacuum (1.34 g, 85% yield). Mp: 125–126 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 3.47 (s, 2H), 3.83 (s, 3H), 3.90 (s, 3H), 5.30–6.30 (br, s, 2H), 6.50 (s, 1H), 6.60 (s, 1H).

1-(5-Amino-2,4-dimethoxyphenyl)-2-ethylamine (4c). Via the procedure of Brown et al.,[51] **4b** (2.8 g, 13.3 mmol) and a stirring bar were placed in a 250 mL, three-necked flask; the flask, maintained in a water bath at room temperature, was equipped with an argon inlet, a reflux condenser with a Drierite guard tube, and an addition flask. After the system was flushed with argon for 15 min, 130 mL of BH_3 in THF (1 M, 130 mmol of BH_3) was transferred into the addition flask under argon at 5 °C and, subsequently, added dropwise to **4b** over 90 min. The water bath was removed, and the mixture was slowly brought to reflux, where it was maintained for 16 h under argon. The contents were cooled to room temperature, and 10 mL of 6 M HCl was added dropwise with considerable H_2 evolution. A further 15 mL of 6 M HCl was added, the mixture was refluxed for 4 h, and cooled to room temperature. Incomplete hydrolysis was indicated by precipitation upon cooling; in such cases, additional reflux was required. The THF was removed by distillation over a steam bath, and the mixture was basified to pH 10 with 40% NaOH. The diamine was extracted with 6 × 50 mL portions of deaerated $CHCl_3$, the $CHCl_3$ portions were combined, washed with 50 mL H_2O, dried with $MgSO_4$, and filtered, and the solvent was removed by evaporation to give a brown semisolid. The residue was dissolved in a minimum amount of 2 M HCl, and impurities were extracted into 3 × 100 mL of deaerated $CHCl_3$. The acid solution was again basified to pH 10 with 40% NaOH, extracted with 3 × 100 mL of deaerated $CHCl_3$, the $CHCl_3$ solutions were combined and dried, and the solvent was removed. The light brown residue was dissolved in 10 mL of $CHCl_3$, enough cold petroleum ether was added to form slight turbidity, and $CHCl_3$ was added to just remove the cloudiness. The solution was refrigerated (4 °C) overnight, and the solvent was removed by decantation to yield, after trituration with petroleum ether and drying under vacuum for 4 h, 1.68 g (64% yield) of product with mp 87.5–89 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55 (br, s, 4H), 2.67 (t, 2H, J = 8.2 Hz), 2.87 (t, 2H, J = 8.2 Hz), 3.76 (s, 3H), 3.82 (s, 3H), 6.33 (s, 1H), 6.60 (s, 1H). Anal. ($C_{10}H_{16}N_2O_2$): C, H, N.

1-(5-Amino-2,4-dihydroxyphenyl)-2-ethylamine (4) Dihydrobromide Monohydrate. Compound **4c** (1.68 g, 8.56 mmol) was demethylated with 26.0 mL of deaerated concentrated HBr and deaerated with argon, by refluxing under argon for 12 h. The mixture was cooled, lyophilized, and triturated with a mixture of 100 mL of Et_2O, 5 mL of CH_3CN, and 5 mL of MeOH. The solid was dried overnight under vacuum to give 2.15 g (75% yield) of the dihydrobromide salt. The related **4** has been reported previously, although synthetic information and the nature of the salt were not provided.[21] ^{1}H NMR (300 MHz, D_2O): δ 2.97 (t, 2H, J = 9.0 Hz), 3.27 (t, 2H, J = 9.0 Hz), 6.63 (s, 1H), 7.19 (s, 1H). Anal. ($C_8H_{12}N_2O_2$·2HBr·H_2O): C, H, N.

1-(2,4,5-Trihydroxyphenyl)-2-aminopropane (5) Hydrobromide and Hydrochloride Monohydrate. These salts were prepared from 2,4,5-trimethoxybenzaldeyde by (a) condensation with nitroethane, (b) reduction with H_2/Raney Ni, and (c) demethylation with either BBr_3, for **5**·HBr, or concentrated HCl, for **5**·HCl.[21–24,30,46–48,52,53] The **5**·HBr was very hygroscopic and apparently contained some H_3BO_3, which could not be removed by repeated attempts at recrystallization; electrochemical examination of the product, yielded only 70 ± 1% purity, corresponding to ca. **5**·HBr·2H_3BO_3. The compound was used for the depletion studies, accounting for the impurity. For the HBr salt, ^{1}H NMR (300 MHz, D_2O): δ 1.23 (d, 3H, J = 6.5 Hz), 2.75 (m, 2H), 3.55 (m, 1H), 6.45 (s, 1H), 6.67 (s, 1H). The **5**·HCl·H_2O salt was also very hygroscopic and was transferred and weighed under argon into multiple tightly sealed vials; once opened, each vial was immediately used, and any remainder was discarded. The HCl salt was used in all except the depletion studies. For the HCl salt, the ^{1}H NMR (300 MHz, D_2O) was the same as for the HBr salt. Also, for the HCl salt, ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.07 (d, 3H, J = 6.4 Hz), 2.46 (dd, 1H, J_1 = 13.2 Hz, J_2 = 8.8 Hz), 2.74 (dd, 1H, J_1 = 13.2 Hz, J_2 = 6.4 Hz), 3.29 (m, 1H), 6.34 (s, 1H), 6.41 (s, 1H), 7.94 (s, b, ~3H, NH or OH), 8.75 (s, b, ~1H, NH or OH). Anal. ($C_9H_{13}N_1O_3$·HCl·H_2O): C, H; N: calcd, 5.89; found, 5.19.

1-(2-Amino-4,5-dihydroxyphenyl)-2-aminopropane (6) Dihydrobromide. This compound, **6**·2HBr, was prepared from 3,4-dimethoxybenzaldehyde by (a) condensation with nitromethane, (b) reduction with $LiAlH_4$, (c) nitration with HNO_3, (d) reduction with H_2/PtO_2, and (e) demethylation with concentrated HBr.[22,48] ^{1}H NMR (60 MHz, D_2O): δ 1.28 (d, 3H, J = 6.5 Hz), 2.80 (br, s, 1H), 2.93 (br, s, 1H), 3.33–3.87 (m, 1H), 6.87 (s, 1H), 6.90 (s, 1H). Anal. ($C_9H_{14}N_2O_2$·2HBr): C, H, N, Br.

1-(2,5-Dimethoxy-4-nitrophenyl)-2-nitropropene (7a). 1-(2,5-Dimethoxyphenyl)-2-nitropropene (9.2 g, 41.2 mmol), prepared by condensation of 2,5-dimethoxybenzaldehyde with nitroethane,[30,54] was dissolved in 300 mL of $CHCl_3$, and the flask was equilibrated to 25 °C in a water bath; 18.0 mL of fuming nitric acid, cooled to 15 °C, was added dropwise, and the mixture was stirred for 2 h. The product was worked up as described below for **8a** to give a yellow-brown solid with mp 126–130 °C. Recrystallization from a 3:2 95% EtOH:ethyl acetate mixture gave yellow needles (7.95 g, 72% yield) with mp 134.5–135.6 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 2.40 (s, 3H), 3.91 (s, 3H), 4.00 (s, 3H), 7.05 (s, 1H), 7.47 (s, 1H), 8.12 (s, 1H).

1-(4-Amino-2,5-dimethoxyphenyl)-2-aminopropane (7b). Raney nickel (70 g wet wt.) was washed successively with 70, 60, 50, and 50 mL of EtOH and then placed in a hydrogenation bottle. To this was added **7a** (2.69 g, 10.0 mmol) suspended in 200 mL of EtOH and 250 mL of pyridine. The mixture was hydrogenated at 50 psi for 5 h, the catalyst was removed by filtration and washed with 100 mL EtOH, and the combined filtrate was reduced to ca. 200 mL by evaporation. Benzene (150 mL) was added and the solvent reduced to ca. 20 mL. EtOH (20 mL) and benzene (50 mL) were added, and the solvent was removed by evaporation. The remaining light brown solid was triturated with a mixture of 100 mL of Et_2O and 5 mL of CH_3CN and refrigerated overnight (4 °C), the solvents were decanted, and the solid was dried under vacuum for 8 h. The nearly colorless solid was distilled (135–140 °C, 2.5 mmHg) to give a white crystalline solid (1.34 g, 64% yield). The related **7b**·2HCl has been prepared previously by an alternate pathway.[30] ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (d, 3H, J = 6.6 Hz), 1.31 (br, s, 2H), 2.41 (dd, 1H, J_1 = 13.2 Hz, J_2 = 8.1 Hz), 2.64 (dd, 1H, J_1 = 13.2 Hz, J_2 = 5.1 Hz), 3.17–3.19 (m, 1H), 3.73 (s, 3H), 3.80 (s, 3H), 6.33 (s, 1H), 6.60 (s, 1H). Anal. ($C_{11}H_{18}N_2O_2$): C, N; H: calcd, 8.83; found, 7.99.

1-(4-Amino-2,5-dihydroxyphenyl)-2-aminopropane (7) Dihydrobromide. Compound **7b** (1.19 g, 5.66 mmol) was dissolved in 18 mL of concentrated HBr deaerated with argon, refluxed under argon for 12 h, cooled to room temperature, lyophilized, triturated with 100 mL of Et_2O, and dried under vacuum for 6 h. The solid was transferred to a vial and dried overnight under vacuum (2.5 mmHg) in a drying pistol to give the light gray dihydrobromide salt (1.65 g, 97% yield). ^{1}H NMR (300 MHz, D_2O): δ 1.39 (d, 3H, J = 7.0 Hz), 2.97 (d, 2H, J = 7.0 Hz), 3.50–3.90 (m, 1H), 6.61 (s, 1H), 7.14 (s, 1H). Anal. ($C_9H_{14}N_2O_2$·2HBr): C, H; N: calcd, 8.14; found, 7.36.

1-(2,4-Dimethoxy-5-nitrophenyl)-2-nitropropene (8a). 1-(2,4-Dimethoxyphenyl)-2-nitropropene (15.0 g, 67.2 mmol),

prepared by condensation of 2,4-dimethoxybenzaldehyde with nitroethane,[30,55] was dissolved in 250 mL of $CHCl_3$. To this was added dropwise, with stirring at 15–20 °C over 10 min, 50.0 mL of a 1:1 mixture of glacial acetic acid and fuming HNO_3 which was previously cooled to 5 °C; the mixture was brought to room temperature and stirred for 5 h. The $CHCl_3$ solution was washed with H_2O (2 × 100 mL), aqueous saturated $NaHCO_3$ (2.15 mL), and H_2O (100 mL); it was then dried ($MgSO_4$) and the solvent removed by evaporation. Recrystallization from 250 mL of a 3:2 mixture of 95% EtOH: ethyl acetate gave bright yellow crystals (15.3 g, 85% yield) with mp 178–180 °C. ^1H NMR (60 MHz, $CDCl_3$): δ 2.42 (s, 3H), 4.00 (s, 3H), 4.05 (s, 3H), 6.56 (s, 1H), 8.05 (s, 1H), 8.14 (br, s, 1H).

1-(5-Amino-2,4-dimethoxyphenyl)-2-aminopropane (8b). Compound **8a** (2.69 g, 10.0 mmol) was hydrogenated (45 psi, 5 h) in the presence of 150 mL of EtOH, 15 mL of pyridine, and 30 g (wet wt.) of Raney nickel which had previously been washed with EtOH (40, 30, and 30 mL). The catalyst was removed by filtration, and the solvent was removed after addition of 50 mL of EtOH and 200 mL of benzene to facilitate pyridine removal via the azeotrope. The crude product (1.93 g) was distilled (130–135 °C, 1.5–2.0 mmHg) to give a colorless solid (1.50 g, 71% yield) with mp 48–50 °C. ^1H NMR (300 MHz, $CDCl_3$): δ 1.06 (d, 3H, J = 6.6 Hz), 2.38 (dd, 1H, J_1 = 13.2 Hz, J_2 = 8.1 Hz), 2.59 (dd, 1H, J_1 = 13.2 Hz, J_2 = 5.3 Hz), 3.09 (m, 1H), 6.43 (s, 1H), 6.51 (s, 1H). Anal. ($C_{11}H_{18}N_2O_2$): C, H, N.

1-(5-Amino-2,4-dihydroxyphenyl)-2-aminopropane (8) Dihydrobromide. Compound **8b** (1.34 g, 6.37 mmol) was dissolved in previously deaerated (argon) concentrated HBr (26 mL), refluxed under argon for 12 h, cooled, and lyophilized. The crude product was triturated with a mixture containing 100 mL of Et_2O, 5 mL of CH_3CN, and 5 mL of MeOH. The solid was dried overnight under vacuum (2–3 mmHg) to give 1.90 g (83% yield) of the dihydrobromide salt. While **8**·HBr has been reported previously, no synthetic details or spectral/physical information was provided.[26] ^1H NMR (300 MHz, D_2O): δ 1.33 (d, 3H, J = 6.6 Hz), 2.90 (apparent d, 2H), 3.72 (m, 1H), 6.64 (s, 1H), 7.19 (s, 1H). Anal. ($C_9H_{14}N_2O_2$·2HBr): C, H; N: calcd, 8.14; found, 7.57.

Mouse Brain Neurotransmitter Levels. Male albino mice of the Hsd:ICR strain from Harlan Sprague-Dawley (Madison, WI) were employed. These were allowed access to food and water ad libitum; no animal was used in experiments until at least 1 week following receipt from the supplier. The mice typically weighed 30 g at the time of treatment. One-half of the specified dose of neurotoxin was administered in the vicinity of the left lateral ventricle[56,57] in 5 μL of isotonic saline containing 1 mg/mL ascorbic acid under light ether anesthesia; the remaining half of the dose was administered in the vicinity of the right lateral ventricle 24 h later. Control animals received injections of vehicle. The animals were sacrificed by microwave irradiation (7.5 kW, 200 ms) using an NJE model 2603-10kW (New Japan Radio, Tokyo, Japan) 7 days following the last injection. The brain was removed, dissected, if appropriate, into regions, and weighed for analysis.[58] Levels of NE, DA, and 5-HT were determined using liquid chromatography with electrochemical detection.[59] Typical control values for mouse whole brain were (nmol/g, mean ± SEM): NE, 3.74 ± 0.06; DA, 8.15 ± 0.52; and 5-HT, 3.95 ± 0.15.

Maximum Tolerable Dose. The maximum tolerable dose (MTD) was defined as that dose, in nanomoles, which provided expiration of 50% of the mice when injected intraventricularly, as described above, and examined 1 h following administration. The MTD was assessed logarithmically using the standard up-and-down approach described by Dixon[38] for both compounds **1** and **8**.

Uptake of Radiolabeled Transmitters into Synaptosomes. For these investigations, male rats of the Hsd: Sprague-Dawley SD strain, weighing 225–250 g, were employed. The procedure was similar to that previously described.[60,61] Protein determinations were made on the synaptosomal preparation by the method of Smith et al.[62] Upon sacrifice by decapitation, the brain was quickly removed and the hindbrain (cerebellum, medulla, and pons) discarded. The remaining tissue was homogenized (8–12 up–down strokes in 10 mL Potter–Elvehjem unit using 3.5 mL of solution/g of tissue; homogenization solution, kept at 2–4 °C, contained 0.32 M sucrose, 20 mM Tris, adjusted to pH 7.40 with concentrated HCl, and 10 μM iproniazid). The homogenate was centrifuged at 1000g for 10 min, the pellet was discarded, and the supernate centrifuged again at 1000g for 10 min. The resulting supernate was centrifuged at 12000g for 20 min. The resulting pellet was resuspended in homogenization solution (ca. 1 mL/g of original tissue) to yield the synaptosomal preparation. In a typical incubation, 50 μL of synaptosomal preparation was preincubated for 5 min with 200 μL of Tris-Krebs buffer (140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 1 mM ascorbate, and 20 mM Tris buffered to pH 7.40 at room temperature with concentrated HCl; prepared fresh and kept at 2–4 °C). The tested neurotoxin (25 μL) was added, and the incubation, which proceeded for 2.0 min, was initiated by addition of 25 μL of the radiolabeled transmitter. The synaptosome concentration in the incubation mixture was 1.2–1.8 μg of protein/μL. The incubation was stopped by the addition of 2.5 mL of ice-cold Li-Tris-Krebs buffer (same as Tris-Krebs, except LiCl replaced NaCl) and rapidly filtered (Millipore, 0.45 μM, GN-6, 25 mm). The reaction vial was washed two successive times with 2.5 mL aliquots of the Li-Tris-Krebs buffer, with the washings being passed through the filter. The filter was placed in a liquid scintillation vial and air dried. 2-Methoxyethanol (1 mL) was added to "dissolve" the filter, 4 mL of Ecolite-(+) liquid scintillation fluid was added, and, after being placed in the dark for ≥24 h, the radioactivity was counted with a Beckman LS7000. Each data point represents the mean ± SD for at least four determinations. Incubation samples prepared with Li-Tris-Krebs in place of the Tris-Krebs buffer were used to determine nonspecific uptake, which was subtracted from the sample values.

The IC_{50} was determined using incubation concentrations of the agent which varied by powers of 10 (typically, 10^{-8}–10^{-4} M) and by 0.5 log units in the vicinity of the previously estimated IC_{50}. Subsequent analysis of the results was performed using a nonlinear least–squares fitting procedure, which yielded the IC_{50} ± SD. For NE uptake blockade, the IC_{50} values, μM, obtained for compounds **1**–**8**, respectively, were 93 ± 25, 12 ± 2, 6 ± 1, 15 ± 5, 56 ± 32, 32 ± 12, 2 ± 0, and 86 ± 14. For DA uptake blockade, the IC_{50} values, μM, obtained for compounds **1**–**8**, respectively, were 52 ± 12, 67 ± 14, 36 ± 2, 77 ± 22, 20 ± 3, 5 ± 1, 22 ± 2, and 201 ± 26.

Kinetic analysis of uptake inhibition by each of the agents for each of NE and DA were performed using four or five concentrations of the neurotransmitter in the vicinity of the K_m and two or three concentrations of the neurotoxin in the vicinity of the K_i. Curve fitting assumed a competitive model for all cases except the blockade of NE transport by **7**, which employed a noncompetitive model; the appropriate kinetic models were selected in accordance with preliminary observation of the $1/V$ vs $1/[S]$ plots. Kinetic analyses were not performed for 5-HT uptake, since the K_i value for all the tested neurotoxins with the 5-HT uptake system were determined in the IC_{50} studies to be greater than 1×10^{-4} M. Typical V_{max} values for the other two transmitters were (mean ± SD): NE, 2.4 ± 0.3 (pmol/min)/mg of protein; DA, 18.0 ± 2.4 (pmol/min)/mg of protein. Typical K_m values were (mean ± SD): NE, 115 ± 11 nM; DA, 311 ± 57 nM.

Release of Radiolabeled Transmitters from Synaptosomes.[45] For these investigations, the procedure and solutions described above for the uptake experiments were primarily employed to preload the synaptosomes with labeled neurotransmitter. However, the neurotoxin was not added prior to the uptake incubation, the total incubation volume was typically doubled, and the time of the uptake incubation was extended from 2 to 10 min. The preloaded synaptosomes were collected on a 0.45 μm Millipore filter and washed two times with 2.5 mL of Tris-Krebs buffer as described above. Subsequently, these synaptosomes were transferred, along with the filter, to a clean incubation vial containing 2.0 mL of the Tris-Krebs buffer. After a 5 min prerelease incubation with

shaking at 37 °C, a 25 μL aliquot of the tested agent was added and the mixture incubated for a further 20 min. Quenching of the process, achieved by addition of 6.0 mL of ice-cold Li-Tris-Krebs buffer, was followed by filtration, washing (2 × 6.0 mL Li-Tris-Krebs), air drying of the filters, addition of liquid scintillation reagents, and counting of the radioactivity as described above. The resultant graphs, constructed as percent controls vs log[neurotoxin], yielded sigmoidal shaped curves. The EC_{50}, determined as the concentration of the agent which produced 50% of the maximal observed release, was obtained by fitting with a least squares approach. Estimates of the SD were obtained from the fitting procedure.

Ease of Oxidation of Neurotoxins. The ease of oxidation of the agents was determined by assessing the $E^{0\prime}$ value for a 0.1 mM solution of the agent in 0.10 M phosphate buffer, pH 7.40 at 22 °C, with a hanging mercury drop working electrode and a Ag/AgCl reference electrode from Bioanalytical Systems (West Lafayette, IN). The $E^{0\prime}$ was taken as the average of the anodic and cathodic peak potentials. The scan rate was 92 mV/s in all cases.

Histological Examination. For the histochemical investigations of **8**, the mice were injected intraventricularly with 25–370 nmol of the tested compound. The animals were killed 1–7 days following treatment. The method of de la Torre and Surgeon,[39] as modified by de la Torre,[40] was employed. Cryostat sections were thawed on glass slides, which were immersed in a solution containing sucrose (200 mM), potassium phosphate (236 mM, pH 7.4), and glyoxylic acid (1%). After air drying for approximately 5 min, the slides were placed on a glass plate in an oven at 90 °C for 2.5 min. Light mineral oil was then applied to each section, and the slides were heated for another 90 s. Coverslips were placed on the slides, and the sections were viewed using an Olympus fluorescence microscope with a BG 12 excitation filter and a 505 barrier filter. The observers were unaware of the treatment at the time of observation.

To ascertain actual degeneration of neurons, rather than simple depletion of the neurotransmitter(s), we also treated adult Sprague-Dawley albino rats with an intraventricular injection of **8** (740 nmol in 5 μL) under pentobarbital anesthesia (40 mg/kg, ip). The animals were killed 3 or 14 days after treatment by intracardiac perfusion with a formalin (10%)–saline solution. The brains were removed, stored in 10% formalin for approximately 2 weeks, and then processed by the Fink–Heimer procedure I which allows direct observation of degeneration by silver staining.[41]

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Supporting Information Available: Synthetic details and associated spectral and physical information for **2**, **5**, **6**, and the precursors described above for **3**, **4**, **7**, and **8** (6 pages). Ordering information is given on any current masthead page.

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