treated animals were compared with the values obtained for untreated control rats run simultaneously. Significance of the difference between the values was calculated by Student's $t$ test. The data are expressed as percent reduction from control levels. Plasma cholesterol concentrations for typical control groups were 58 mg/100 ml by this method.

**7-Dehydrocholesterol Determination.** Nonsaponifiable lipids were extracted into petroleum ether (bp 40–60°) by the method of Abell et al.[27] Tentative identification of 3β-cholesta-5,7-dien-3-ol was indicated by immediate color development in the Lieberman–Burchard test. A silylated sample was gas chromatographed on 5% ECNSS-S on Chromosorb W DMCS at 203°. A peak at 14.8-min retention time was identical with one obtained with an authentic sample of 3β-cholesta-5,7-dien-3-ol. The retention time for cholesterol was 10.8 min on this column.

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# Catechol *O*-Methyltransferase. 9. Mechanism of Inactivation by 6-Hydroxydopamine

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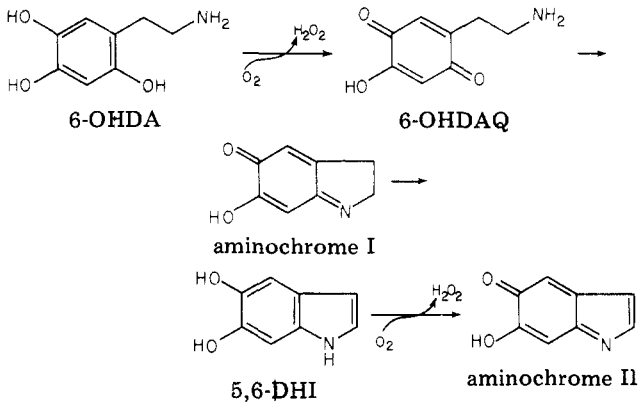
A series of methylated analogues of 6-hydroxydopamine (6-OHDA) has been synthesized and evaluated as irreversible inhibitors of catechol *O*-methyltransferase (COMT). These analogues have been prepared in an effort to elucidate the mechanism involved in the inactivation of this enzyme by 6-OHDA. The analogues prepared had methyl groups incorporated in the 2 and/or 5 positions of 6-OHDA so as to block nucleophilic attack at these positions in the corresponding oxidation products [6-hydroxydopamine-*p*-quinone (6-OHDAQ), aminochromes I and II]. Such 2- and/or 5-methylated 6-OHDA analogues were found to be inhibitors of COMT with the inactivation apparently resulting from modification of an essential amino acid residue at the active site of the enzyme. The activity of these analogues as inhibitors of COMT argues against a mechanism involving a 1,4 Michael addition reaction by a protein nucleophile at the 2 or 5 positions on 6-OHDAQ or on the corresponding aminochromes. Instead, an alternative mechanism is proposed to explain these data, which involves attack of a protein nucleophile at the carbonyl group in the 6 position of 6-OHDAQ or at the imine functionality on aminochromes I and II. The results of the present experiments have provided insight into the mechanism involved in inactivation of COMT by 6-OHDA. In addition, this study has provided considerable insight into the chemical reactivity of the electrophilic species generated after oxidation of 6-OHDA.

6-Hydroxydopamine (6-OHDA, 1)[2] has become a widely utilized pharmacological tool, because of its ability to produce selective destruction of norepinephrine- or dopamine-containing nerve terminals.[3,4] The mechanism by which 6-OHDA produces its degenerative effects remains a matter of speculation; however, in part its specificity

Scheme I. Air Oxidation Pathway of 6-Hydroxydopamine



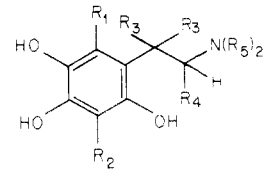

results from the fact that it is selectively transported by the neuronal membrane pump. This transport into the neuron appears to be a prerequisite to the degenerative effects.[3,4] The series of chemical events which subsequently occur within the neuron and eventually result in destruction of the nerve terminal has yet to be elucidated. Two general theories[3,4] have been proposed to explain these events: (a) the quinoid-like compounds produced upon oxidation of 6-OHDA may act as the alkylating agents of macromolecules; or (b) the hydrogen peroxide or superoxide anion also generated upon oxidation of 6-OHDA may function as oxidizing agents to produce alternations in the structures of important proteins and lipids.

Recently[5] in our laboratory it was observed that in vitro 6-OHDA and 6-aminodopamine rapidly and irreversibly inactivated the enzyme catechol *O*-methyltransferase (COMT, E.C. 2.1.1.6). From studies of the kinetics of inactivation, substrate protection studies, and incorporation studies, it was shown that this inactivation of COMT proceeds by an alkylation mechanism involving the quinoid oxidation products of 6-OHDA.[5] Therefore, we have utilized this system as a model to study the mechanism of interaction between such quinoid compounds and a protein nucleophile. In addition, the specificity involved in this inactivation process has provided a convenient method of affinity labeling COMT, thereby providing further knowledge concerning the active site of this enzyme and the mechanism of transmethylation.

From our earlier studies with 6-OHDA,[5a,b] we were able to show that the most reactive alkylating species were the aminochromes I and II (Scheme I) which arise from intramolecular cyclization of 6-hydroxydopamine-*p*-quinone (6-OHDAQ). The 6-OHDAQ, itself, appears to be substantially less reactive toward the enzyme.[5a,b] The question that still remains unanswered is the nature of the alkylation reaction itself. Saner and Thoenen[6] have previously proposed that the attack of a protein nucleophile on 6-OHDAQ (or its further oxidation products) proceeds by a 1,4 Michael addition reaction; however, no firm experimental evidence is available to support this mechanism.

In an effort to further elucidate the mechanism of this protein–ligand interaction, we have prepared a series of 6-OHDA analogues (Chart I) designed so as to block nucleophilic attack at specific locations on the electrophile (6-OHDAQ, aminochromes I and II). In this way we hope to characterize the nature and the location of the addition reaction. For example, compounds **2–5** are analogues of 6-OHDA in which the 2 and 5 positions are either independently or simultaneously blocked with methyl substituents. The 2 and 5 positions on 6-OHDAQ (or similar

Chart I. 6-Hydroxydopamine Analogues



| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| 6-OHDA (1) | H | H | H | H | H |
| 2,5-$Me_2$-6-OHDA (2) | $CH_3$ | $CH_3$ | H | H | H |
| *N*,*N*-$Me_2$-2,5-$Me_2$-6-OHDA (3) | $CH_3$ | $CH_3$ | H | H | $CH_3$ |
| 2-Me-6-OHDA (4) | $CH_3$ | H | H | H | H |
| 5-Me-6-OHDA (5) | H | $CH_3$ | H | H | H |
| β,β-$Me_2$-6-OHDA (6) | H | H | $CH_3$ | H | H |
| α-Me-6-OHDA (7) | H | H | H | $CH_3$ | H |

positions on aminochromes I and II) would be the potential sites of attack in a 1,4 Michael addition reaction. Evaluating these analogues for their ability to inactivate COMT has provided some useful information concerning the mechanism of nucleophilic attack in this protein–ligand interaction.

## Experimental Section

Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were conducted on a Hewlett-Packard Model 185B C, H, N analyzer, the University of Kansas, Lawrence, Kan. Unless otherwise stated, the ir, NMR, and uv data are consistent with the assigned structures. Ir data were recorded on a Perkin-Elmer Model 727 spectrophotometer and NMR data on a Varian Associates Model T-60 spectrophotometer ($Me_4Si$). Scintillation counting was done on a Beckman LS-150 scintillation counter.

**Materials.** *S*-Adenosyl-L-methionine-*methyl*-$^{14}C$ (SAM-$^{14}CH_3$) (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored frozen. *S*-Adenosylmethionine chloride (Sigma) was stored as a 0.01 M aqueous stock solution. 6-Hydroxydopamine (6-OHDA, 1) and related structural analogues **2–7** were prepared fresh daily as stock solutions of 10 μmol/ml under nitrogen.

**2-(2′,5′-Dimethyl-3′,4′,6′-trimethoxyphenyl)ethylamine Hydrochloride (8).** To a cooled solution (−20°) of $AlCl_3$ (2.00 g, 8.5 mmol) in 100 ml of anhydrous $Et_2O$ was added portionwise $LiAlH_4$ (0.57 g, 15 mmol). This solution was then stirred at ambient temperature for 5 min after which a solution of 2,5-dimethyl-3,4,6-trimethoxybenzyl cyanide[7] (2.00 g, 15 mmol) in 50 ml of anhydrous $Et_2O$ was added dropwise. After addition was completed the reaction mixture was stirred for 1 h at ambient temperature and then cooled (0°) and the excess $LiAlH_4$ decomposed by the dropwise addition of 50 ml of 4 N NaOH. The suspension was filtered and washed with $Et_2O$ to remove occluded product. The combined etheral fractions were dried ($Na_2SO_4$) and filtered and the solvent was removed under reduced pressure. The residue was dissolved in EtOH (30 ml) and $Et_2O$ (150 ml), and HCl (gas) was bubbled into the solution for 30 s. The resulting hydrochloride salt was removed by filtration to yield 1.62 g (62%): mp 264–266° (lit.[7] mp 266–267°).

**2-(2′,5′-Dimethyl-3′,4′,6′-trihydroxyphenyl)ethylamine Hydrobromide (2,2,5-$Me_2$-6-OHDA).** To a suspension of amine **8** (300 mg, 1.1 mmol) in 40 ml of dry $CH_2Cl_2$ under an argon atmosphere was added 5 ml of a solution of 1 M $BBr_3$ in $CH_2Cl_2$. The reaction mixture was stirred for 12 h at ambient temperature. The mixture was cooled (0°) and $CH_3OH$ (50 ml) was added dropwise. The resulting precipitate was collected by filtration to yield 208 mg (68%): mp 230° dec (lit.[7] mp 227–228°). Anal. ($C_{10}H_{16}NO_3Br$) C, H, N.

***N*,*N*-Dimethyl-2-(2′,5′-dimethyl-3′,4′,6′-trimethoxyphenyl)ethylamine Hydrochloride (9).** The amine hydrochloride **8** (1.15 g, 4.17 mmol) was dissolved in 50 ml of $H_2O$ and the solution made basic with 20 ml of 4 N NaOH. The basic solution was saturated with NaCl and extracted twice (100 ml) with $Et_2O$. The combined $Et_2O$ fractions were dried ($Na_2SO_4$)

and filtered and the $Et_2O$ was removed under reduced pressure. The resulting oil was dissolved in 20 ml of 98% formic acid and 6 ml of 37% formaldehyde. The reaction mixture was heated at 100° for 48 h. The yellow reaction mixture was poured into 0.25 N HCl (70 ml). The acidic solution was concentrated in vacuo and the resulting solid collected by filtration to yield 750 mg (71%): mp 177–180° dec (very hygroscopic). Anal. ($C_{15}H_{26}NO_3Cl \cdot 0.25H_2O$) C, H, N.

**N,N-Dimethyl-2-(2′,5′-dimethyl-3′,4′,6′-trihydroxyphenyl)ethylamine Hydrobromide (3, N,N-Me$_2$-2,5-Me$_2$-6-OHDA).** The N,N-dimethylamine **9** (100 mg, 0.32 mmol) was cleaved under argon using $BBr_3$ as described above for the preparation of **2**. The reaction mixture was diluted with $CH_3OH$ (50 ml) and the resulting precipitate was collected by filtration. The sample was recrystallized (EtOAc) to yield 62 mg (64%): mp 188–190°. Anal. ($C_{12}H_{20}NO_3Br$) C, H, N.

**2-Methyl-3,4,6-trimethoxybenzaldehyde (12a).** The aldehyde **12a** was prepared from 2,3,5-trimethoxytoluene[8,9] using a modification of the procedure previously described by Cromartie and Harley-Mason.[10] To a cooled solution (0°) of 2,3,5-trimethoxytoluene[8,9] (**10**, 5.00 g, 27.4 mmol) in 25 ml of $C_6H_6$ was added $AlCl_3$ (6.0 g, 45 mmol) portionwise. To this solution was added liquid HCN (17.5 g, 650 mmol) (*hazard*: HCN is extremely toxic) and the reaction mixture stirred for 5 min. The solution was stirred under an atmosphere of HCl (gas) for 8 h, after which the suspension was poured into a mixture of 100 ml of concentrated HCl and 200 ml of crushed ice. The suspension was boiled for 2 min, then cooled, and extracted with ether (four times; total 450 ml). The ether solution was dried ($Na_2SO_4$) and filtered, the solvent removed in vacuo, and the product crystallized (hexane) to yield 3.30 g (57%): mp 103–104.5° (lit.[11] mp 103–104°); NMR ($CDCl_3$) δ 10.38 (s, 1 H, CHO), 6.21 (s, 1 H, $C_5$-H), 3.90, 3.80, 3.60 (3 s, 9 H, $OCH_3$), 2.28 (s, 3 H, $CH_3$). Anal. ($C_{11}H_{14}O_4$) C, H.

**1-(2′-Methyl-3′,4′,6′-trimethoxyphenyl)-2-nitroethene (13a).** A solution of 2-methyl-3,4,6-trimethoxybenzaldehyde (**12a**, 1.0 g, 4.76 mmol), $NH_4OAc$ (0.25 g, 3.17 mmol), and nitromethane (0.55 g, 9 mmol) in 15 ml of glacial HOAc was refluxed for 2.5 h and then cooled. The material was diluted with 50 ml of absolute EtOH and concentrated to dryness in vacuo at room temperature. The residue was crystallized (EtOH) to yield 690 mg (58%): mp 130–131.5°. Anal. ($C_{12}H_{15}NO_5$) C, H, N.

**2-(2′-Methyl-3′,4′,6′-trimethoxyphenyl)ethylamine Hydrochloride (14a).** A solution of $LiAlH_4$ (0.60 g, 15.8 mmol) in 100 ml of $Et_2O$ in a round-bottom flask, to which was attached a Soxhlet extractor containing 1-(2′-methyl-3′,4′,6′-trimethoxyphenyl)-2-nitroethene (**13a**, 1.0 g, 3.95 mmol), was refluxed continuously for 10 h. The excess $LiAlH_4$ was decomposed by dropwise addition of 1 ml of $H_2O$, followed by addition of 3 ml of 4 N NaOH. The inorganic salts were removed by filtration, the etheral solution was dried ($CaSO_4$) and filtered, and the $Et_2O$ was removed under vacuum. The colorless residue was dissolved in 150 ml of $Et_2O$ and the solution saturated with HCl (gas). The resulting solid was crystallized from *i*-PrOH to yield 550 mg (53%) of the desired product: mp 218.5–219.5°. Anal. ($C_{12}H_{20}NO_3Cl$) C, H, N.

**2-(2′-Methyl-3′,4′,6′-trihydroxyphenyl)ethylamine Hydrobromide (4,2-Me-6-OHDA).** A solution of 2-(2′-methyl-3′,4′,6′-trimethoxyphenyl)ethylamine hydrochloride (**14a**, 0.50 g, 1.91 mmol) in 8 ml of 48% HBr under an argon atmosphere was refluxed for 30 min, after which it was diluted with 10 ml of EtOH and 30 ml of $H_2O$. The mixture was concentrated in vacuo and the residue crystallized from *i*-PrOH–$Et_2O$. The unstable product **4** was rapidly isolated by filtration and dried in vacuo over KOH to yield 483 mg (96%): mp 205–210° dec. Anal. ($C_6H_{14}NO_3Br \cdot 0.25H_2O$) C, H, N.

**2,3,6-Trimethoxytoluene (11).** 3-Bromo-2,6-dimethoxytoluene was prepared in 56% yield from 2,6-dimethoxytoluene by bromination with dioxane dibromide according to the procedure of Doyle et al.[12] 2,3,6-Trimethoxytoluene (**11**) was prepared from 3-bromo-2,6-dimethoxytoluene using a general procedure for conversion of aryl halides via the intermediate Grignard derivative to the corresponding phenols.[13] The intermediate Grignard reagent was prepared by refluxing a mixture of magnesium (2.92 g, 0.12 mol) and 3-bromo-2,6-dimethoxytoluene (23.11 g, 0.10 mol) in $Et_2O$ for 18 h. The solution of the Grignard reagent was added dropwise to a solution of $B(OCH_3)_3$[13] in 150 ml of $Et_2O$. The reaction mixture was warmed to ambient temperature with vigorous stirring, after which 100 ml of 10% HCl was added. The acidic solution was extracted with $Et_2O$ (twice; total 150 ml) and the etheral solution was washed with $H_2O$. To the washed $Et_2O$ was added 100 ml of 10% $H_2O_2$ dropwise and the mixture stirred and heated at reflux for 1 h. The etheral layer was extracted with 300 ml of 4 N NaOH. The basic solution was acidified with concentrated HCl and the acidic solution extracted with $Et_2O$ (five times; total 500 ml). The $Et_2O$ was removed under vacuum to yield 10.2 g of 2,6-dimethoxy-3-hydroxytoluene as an oil. The intermediate phenol was methylated in 75 ml of 4 N NaOH and dimethyl sulfate (30 g). The reaction mixture was stirred at ambient temperature for 48 h, after which it was extracted with $Et_2O$. The combined etheral solution was dried ($CaSO_4$) and filtered and the $Et_2O$ removed under reduced pressure. The residual oil was distilled to yield 5.15 g (28%) of the desired **11**: bp 70–72° (0.5 mm). Anal. ($C_{10}H_{14}O_3$) C, H.

**3-Methyl-2,4,5-trimethoxybenzaldehyde (12b).** Benzaldehyde **12b** was prepared from 2,3,6-trimethoxytoluene (**11**, 5.0 g, 27.4 mmol), $AlCl_3$ (6.0 g, 45 mmol), and liquid HCN (18.1 g, 670 mmol) as described above for the preparation of **12a**. Recrystallization (Skelly B) afforded 4.10 g (71%) of the desired product: bp 102–104° (0.5 mm); NMR ($CDCl_3$) δ 10.10 (s, 1 H, CHO), 7.19 (s, 1 H, $C_6$-H), 3.92, 3.90, 3.88 (3 s, 9 H, $OCH_3$), 2.15 (s, 3 H, $CH_3$). Anal. ($C_{11}H_{14}O_4$) C, H.

**1-(3′-Methyl-2′,4′,5′-trimethoxyphenyl)-2-nitroethene (13b).** The β-nitrostyrene **13b** was prepared from 3-methyl-2,4,5-trimethoxybenzaldehyde (**12b**, 1.0 g, 4.76 mmol), $NH_4OAc$ (0.6 g, 7.8 mmol), and nitromethane (1.0 g, 16 mmol) in HOAc as described above for the preparation of **13a**. Recrystallization (EtOH) afforded 0.82 g (68%) of the desired product **13b**: mp 113–116°. Anal. ($C_{12}H_{15}NO_5$) C, H, N.

**2-(3′-Methyl-2′,4′,5′-trimethoxyphenyl)ethylamine Hydrochloride (14b).** The β-nitrostyrene **13b** (2.4 g, 9.48 mmol) was reduced with $LiAlH_4$ (1.08 g, 28.4 mmol) using a procedure identical with that described above for the preparation of **14a**. Formation of the hydrochloride salt and crystallization (*i*-PrOH–$Et_2O$) afforded 1.10 g (44%) of the desired product **14b**: mp 181–183° dec. Anal. ($C_{12}H_{20}NO_3Cl$) C, H, N.

**2-(3′-Methyl-2′,4′,5′-trihydroxyphenyl)ethylamine Hydrobromide (5, 5-Me-6-OHDA).** The ethylamine **14b** (0.30 g, 1.15 mmol) was cleaved under argon using $BBr_3$ as described above for the preparation of **2**. The reaction mixture was diluted with $CH_3OH$ and the resulting precipitate was collected by filtration and recrystallized (MeOH–EtOAc–$CH_2Cl_2$) to yield 254 mg (84%) of the desired **5**: mp 207–210° dec. Anal. ($C_9H_{14}NO_3Br$) C, H, N.

**1-(2′,4′,5′-Trimethoxyphenyl)-2-nitropropene (13c).** The 2-nitropropene derivative **13c** was prepared from 2,4,5-trimethoxybenzaldehyde[14,15] (**12c**, 19.2 g, 0.1 mol), $NH_4OAc$ (4 g), and nitroethane (12 g, 0.16 mol) in HOAc as described above for the preparation of **13a**. Recrystallization (EtOH–$H_2O$) afforded 11.5 g (45%): mp 92–94°. Anal. ($C_{12}H_{15}NO_5$) C, H, N.

**1-(2′,4′,5′-Trimethoxyphenyl)-2-aminopropane (14c).** The 2-nitropropene **13c** (7.00 g, 27.6 mmol) was reduced with $LiAlH_4$ (5.00 g, 142 mmol) using a procedure identical with that described above for the preparation of **14a**. Formation of the HCl salt and crystallization (*i*-PrOH–$Et_2O$) afforded 5.92 g (82%). Anal. ($C_{12}H_{20}NO_3Cl$) C, H, N.

**1-(2′,4′,5′-Trihydroxyphenyl)-2-aminopropane (7, α-Me-6-OHDA).** The 2-aminopropane derivative **14c** (1.0 g, 3.8 mmol) was cleaved under argon using $BBr_3$ as described above for the preparation of **2**. The reaction mixture was diluted with $CH_3OH$ and the resulting precipitate was collected by filtration and recrystallized ($Et_2O$) to yield 197 mg (75%): mp 195–199° dec. Anal. ($C_9H_{14}NO_3Br$) C, H, N. (The pharmacological effects of the title compound have been previously reported in the literature;[16–18] however, to our knowledge a detailed synthesis of **7** has not appeared in the literature.)

**2-Methyl-2-(2′,4′,5′-trimethoxyphenyl)propionitrile (16).** A mixture of 2,4,5-trimethoxyphenylacetonitrile[14,15] (**15**, 5.35 g, 26 mmol) and MeI (20 g, 140 mmol) in 50 ml of $Me_2SO$ was stirred vigorously as 15 ml of 50% NaOH (aqueous) was added dropwise over a period of 1 h, while maintaining the temperature of the reaction mixture at 40° (±2°) with an ice bath. The reaction

mixture was stirred at 40° for 3 h after addition was complete and then the reaction mixture heated to 65° for 15 min. The reaction mixture was diluted with 250 ml of $H_2O$ and extracted with 250 ml of $CHCl_3$. The $CHCl_3$ solution was washed with cold $H_2O$ (four times; total 1200 ml), dried ($MgSO_4$), and filtered, and the $CHCl_3$ was removed in vacuo to yield 4.92 g of impure product. Distillation of the product afforded 3.70 g (61%): bp 127–129° (0.4 mm). Anal. ($C_{13}H_{17}NO_3$) C, H, N.

**2-Methyl-2-(2′,4′,5′-trimethoxyphenyl)propylamine Hydrochloride (17).** To a solution of $AlCl_3$ (4.0 g, 30 mmol) in 100 ml of $Et_2O$, cooled in a dry ice–$CH_2Cl_2$ bath, was added portionwise $LiAlH_4$ (1.14 g, 30 mmol). The reaction mixture was stirred for 10 min followed by dropwise addition of 2-methyl-2-(2′,4′,5′-trimethoxyphenyl)propionitrile (**16**, 3.70 g, 16 mmol) which was dissolved in 75 ml of $Et_2O$. After addition was complete the reaction mixture was warmed to ambient temperature and stirred for 3 h. The reaction was stopped by dropwise addition of 30 ml of 4 N NaOH. The resulting inorganic precipitate was removed by suction filtration (Celite 545) and washed with $Et_2O$. The etheral solution was dried ($Na_2SO_4$) and the ether was removed in vacuo to yield 2.90 g of a yellow oil. The oil was dissolved in 175 ml of $Et_2O$ and the etheral solution saturated with HCl (gas). The resulting hydrochloride salt was recrystallized from *i*-PrOH–EtOH–$Et_2O$ to yield 2.20 g (50%) of the desired product **17**: mp 233–236°. Anal. ($C_{13}H_{22}NO_3Cl$).

**2-Methyl-2-(2′,4′,5′-trihydroxyphenyl)propylamine Hydrobromide (6, β,β-Me$_2$-6-OHDA).** A solution of propylamine **17** (1.0 g, 3.63 mmol) in 10 ml of 48% HBr (aqueous) under an argon atmosphere was heated at reflux for 1.5 h. The reaction mixture was cooled and diluted with 50 ml of cold $H_2O$. The solution was concentrated in vacuo and the residue crystallized (EtOH–$C_6H_6$) to yield 450 mg (45%) of the desired **6**: mp 185–188° dec. Anal. ($C_{10}H_{16}NO_3Br \cdot 1.3HBr$) C, H, N.

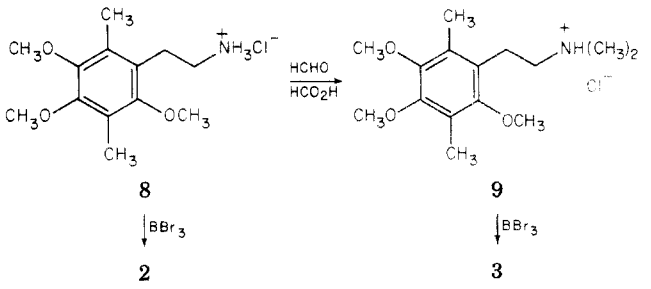
**COMT Isolation and Assay.** COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) according to the methods previously described.[19] Purification was carried through the affinity chromatography stage resulting in approximately a 500-fold purification of the COMT activity.[19] The enzyme activity was determined using *S*-adenosylmethionine-*methyl*-$^{14}C$ and 3,4-dihydroxybenzoate (DHB) or 3,4-dihydroxyacetophenone (DHA) as substrates according to a previously described radiochemical assay.[20,21]

**COMT Inactivation Experiments.** The COMT inactivation experiments were carried out using procedures similar to those described earlier from our laboratory.[5b,c,22–24] A typical preincubation mixture consisted of the following components: water, so that the final volume was 3.20 ml; magnesium chloride (4.80 μmol); phosphate buffer, pH 7.60 (400 μmol); inhibitor (variable); and purified enzyme preparation. The preincubation step was started by the addition of enzyme, and incubation was carried out at 37°. After the appropriate preincubation time an aliquot (0.20 ml) of the preincubation mixture was removed and assayed by addition of 0.05 μCi of *S*-adenosylmethionine-*methyl*-$^{14}C$, *S*-adenosylmethionine (0.25 μmol), and DHB (or DHA) (0.50 μmol) to a final volume of 0.25 ml. The assay mixtures were incubated for 5 min at 37°.[5b,c,22–24] In the anaerobic experiments the preincubation mixtures and inhibitor solutions were prepared in a similar way to those described above, except the anaerobic experiments were done in sealed ampules under nitrogen and samples were removed using a syringe. Enzyme assays in these anaerobic experiments were also carried out in ampules under nitrogen.

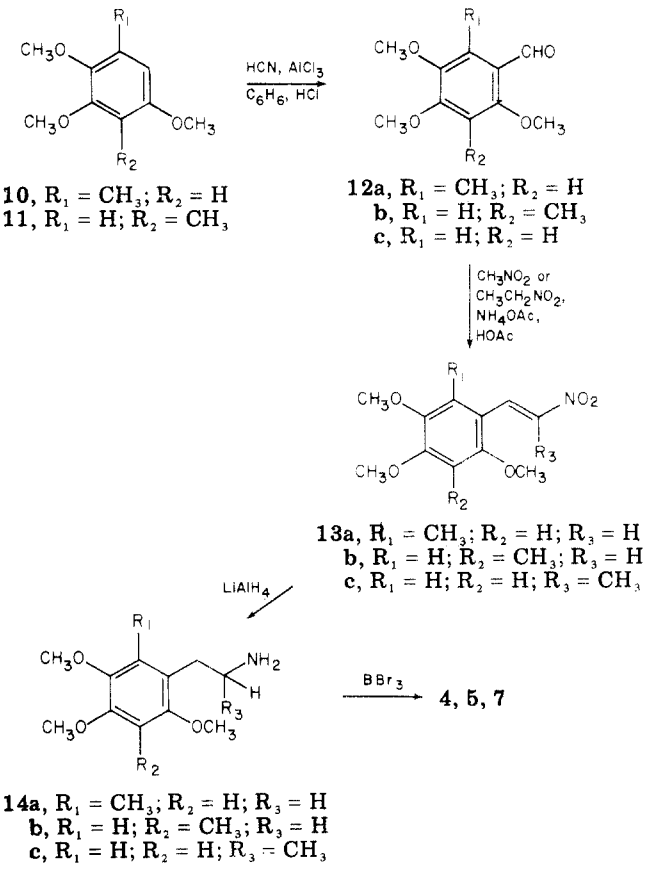
Because of stability problems, stock solutions of 6-OHDA and compounds **2–7** were prepared fresh for each set of inactivation experiments. Stock solutions of 5 μmol/ml in $H_2O$ were prepared in sealed ampules under nitrogen and aliquots were removed as needed for inactivation experiments. In general, no evidence of oxidation was observed in these stock solutions for up to 4 h.

**Electrochemistry.** The reduction potentials ($E^{0\prime}$) and rate constants for cyclization were determined in pH 7.4 phosphate–citrate buffer and were measured with a PAR 174 polarograph, equipped with a dropping mercury electrode and a standard calomel reference electrode.[25] The electrochemical cell was immersed in a water bath at 37° (or 25°). The quinones were formed by air oxidation of the corresponding hydroquinones. Following air oxidation the solution was deoxygenated with a stream of nitrogen to allow polarographic measurements without interference from oxygen polarographic waves. The rate constants for cyclization were calculated by plotting the logarithm of the quinone concentration measured polarographically vs. time, and all were in good accord with first-order kinetic behavior. The uv spectra of the *p*-quinones and their reaction products were recorded on a Cary 14 spectrophotometer. Kinetic rate data for cyclization obtained spectrophotometrically for 2,5-Me$_2$-6-OHDA (**2**) were identical with that obtained polarographically. Spectroscopy also confirmed that the *p*-quinone generated from oxidation of **3** did not undergo a cyclization reaction to the corresponding aminochrome. Electrochemical data for β,β-Me$_2$-6-OHDA (**6**) and α-Me-6-OHDA (**7**) were reported earlier.[26]

**Scheme II**



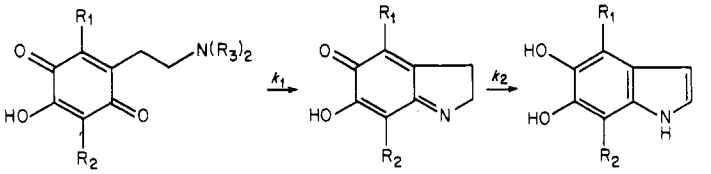

**Scheme III**




## Results and Discussion

**Synthesis.** In Scheme II are outlined the steps involved in the preparation of the 2,5-Me$_2$-6-OHDA derivatives **2** and **3**. The 2-(2′,5′-dimethyl-3′,4′,6′-trimethoxyphenyl)-ethylamine (**8**) was prepared by reduction of the corresponding nitrile according to the procedure of Cromartie and Harley-Mason.[7] Cleavage of amine **8** with $BBr_3$ afforded the desired 2,5-Me$_2$-6-OHDA (**2**). Alternatively, methylation of **8** using Eschweiler–Clark conditions afforded the corresponding *N*,*N*-dimethylamine **9**, which was

Table I. Electrochemical Rate Data for Analogues of 6-OHDA




*p*-quinone aminochrome indole

| Hydroquinone no. | $R_1$ | $R_2$ | $R_3$ | $E^{0\prime}$ (*p*-quinone), V vs. SCE | $E^{0\prime}$ (aminochrome), V vs. SCE | $k_1 \times 10^4$, $s^{-1}$ | $T_{1/2}$, min |
|---|---|---|---|---|---|---|---|
| 1 | H | H | H | −0.210 | Not obsd | 3.0[a,c] | 39[a,c] |
| 2 | $CH_3$ | $CH_3$ | H | −0.230 | −0.285 | 18.3 ± 0.5[b] | 6[b] |
| 3 | $CH_3$ | $CH_3$ | $CH_3$ | −0.235 | Not formed | No rxn[d] | |
| 4 | $CH_3$ | H | H | −0.220 | −0.305 | 6.8 ± 0.2[a] | 17[a] |
| 5 | H | $CH_3$ | H | −0.210 | Not obsd | 4.9 ± 0.2[a] | 24[a] |

[a] Measured at 37°. [b] Measured at 25°. [c] Reference 26. [d] No detectable cyclization of this *p*-quinone to the aminochrome was observed.

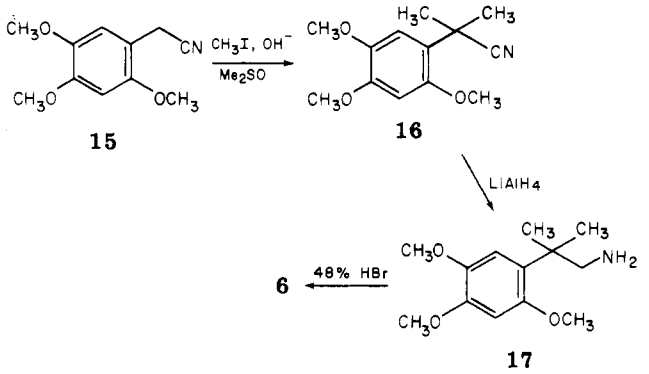
subsequently cleaved using $BBr_3$ to *N,N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**).

The general pathway used to prepare the monomethyl 6-OHDA derivatives **4**, **5**, and **7** is outlined in Scheme III. In the preparation of 2-Me-6-OHDA (**4**), the intermediate 2-methyl-3,4,6-trimethoxybenzaldehyde (**12a**) was prepared from 2,3,5-trimethoxytoluene (**10**)[8,9] using $AlCl_3$ and HCN in a general Gatterman reaction.[10] The aldehyde **12a** was converted to the corresponding β-nitrostyrene derivative **13a** using nitromethane and ammonium acetate in glacial acetic acid. Reduction of the β-nitrostyrene **13a** with $LiAlH_4$ afforded the desired phenylethylamine **14a**. Cleavage of **14a** with $BBr_3$ afforded the 2-Me-6-OHDA (**4**). 5-Me-6-OHDA (**5**) was prepared by a route identical with that described above for **4**. The key intermediate 2,3,6-trimethoxytoluene (**11**) was prepared from 3-bromo-2,6-dimethoxytoluene[12] using a general procedure for conversion of aryl halides via the intermediate Grignard derivatives to the corresponding phenols.[13] 2,3,6-Trimethoxytoluene (**11**) was then converted to 5-Me-6-OHDA (**5**) using the pathway described in Scheme III. α-Me-6-OHDA (**7**) was prepared from 2,4,5-trimethoxybenzaldehyde[14,15] (**12c**) by reaction with nitroethane followed by reduction of the intermediate β-nitrostyrene **13c** to the propylamine derivative **14c**. Cleavage of **14c** with $BBr_3$ afforded the desired **7**.

The β,β-$Me_2$-6-OHDA (**6**) was prepared from 2,4,5-trimethoxyphenylacetonitrile[14,15] (**15**) by alkylating **15** with methyl iodide to form the intermediate dimethylnitrile **16**. Reduction of **16** with $LiAlH_4$ to the amine **17**, followed by cleavage with 48% HBr, afforded the desired β,β-$Me_2$-6-OHDA (**6**) (Scheme IV). All of the synthetic intermediates and final compounds described in this paper were characterized by their ir, NMR, and uv spectra, their chromatographic properties, and elemental analyses.

**Electrochemistry.** The $E^{0\prime}$ data for the reduction of the quinones and their rates of cyclization are summarized in Table I. The quinones reported in this table exhibit a negative shift in reduction potential with an increasing number of methyl groups on the ring as expected for electron-donating groups. The cyclization rates $k_1$ were measured for the compounds shown in Table I. In addition, the spectral and polarographic data allowed us to draw certain conclusions concerning the magnitude of $k_2$ (the rate constant for the subsequent rearrangement of the aminochrome to the indole) relative to $k_1$. For 2,5-$Me_2$-6-OHDA (**2**), the aminochrome I intermediate was spectrally (and polarographically) observed as a stable intermediate, so in this case $k_1 \gg k_2$. Spectral and polarographic observations of 2-Me-6-OHDA (**4**) indicate that the aminochrome I intermediate is a more transient intermediate than was observed for compound **2**, but still $k_1 > k_2$. For 6-OHDA (**1**) and 5-Me-6-OHDA (**5**), the aminochrome intermediates were not observed spectrally or polarographically, which indicates that $k_1 \ll k_2$ for these two compounds. The *N,N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**) was observed not to cyclize to the aminochrome I intermediate as would be predicted.

Scheme IV



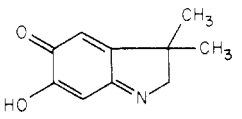

**COMT Inactivation Studies.** In our earlier studies[5b] on the inactivation of COMT by 6-OHDA, we were able to evaluate the reactivity of aminochrome II (Scheme I) by generating this species in situ from 5,6-dihydroxyindole. More recently,[5c] we have been able to test the reactivity of aminochrome I toward COMT by synthesizing various β,β-dialkyl-6-aminodopamine analogues which readily oxidize and undergo intramolecular cyclization to the corresponding aminochrome I derivatives, but because of the β,β-dialkyl substitutions these aminochromes do not rearrange to the corresponding 5,6-dihydroxyindoles. This provided us with a means of generating in situ aminochrome I analogues. Surprisingly, these aminochrome I analogues were found to be relatively weak inhibitors of COMT.[5c] In order to further confirm these findings, we have prepared in this study β,β-$Me_2$-6-OHDA (**6**), which provides a means of trapping an aminochrome I analogue. Blank et al.[26] have shown that the redox potential of **6** is identical with that of 6-OHDA (**1**), but that the cyclization rate to the aminochrome I species is approximately 230 times faster ($T_{1/2}$ = 10 s). Aminochrome **18** generated from β,β-$Me_2$-6-OHDA (**6**) is stable and does not rearrange

Table II. Effects of Aerobic and Anaerobic Conditions on Inactivation of Catechol O-Methyltransferase by Analogues of 6-OHDA

| Compd[a] | % residual act. after 60 min, 37°[b] Aerobic | Anaerobic |
|---|---|---|
| 6-OHDA (1) | 0 | 93 |
| 2,5-$Me_2$-6-OHDA (2) | 0 | 100 |
| N,N-$Me_2$-2,5-$Me_2$-6-OHDA (3) | 95 | 100 |
| 2-Me-6-OHDA (4) | 0 | 82 |
| 5-Me-6-OHDA (5) | 0 | 82 |
| β,β-$Me_2$-6-OHDA (6) | 35 | 90 |
| α-Me-6-OHDA (7) | 0 | 100 |

[a] The standard preincubation mixture consisted of inhibitor (0.60 mM), magnesium chloride (1.38 mM), phosphate buffer (pH 7.60), enzyme preparation, and water to a final volume of 1.32 ml. Anaerobic experiments were conducted in sealed ampules under nitrogen. Inhibitor stock solutions were prepared in ampules under nitrogen using deoxygenated water. Residual enzyme activity was determined as described in the Experimental Section. [b] Residual activity was calculated from controls which were not first incubated but were directly assayed after addition of the inhibitor. Values are the averages of duplicate determinations.

to the corresponding 5,6-dihydroxyindole.



18

When β,β-$Me_2$-6-OHDA (6) was incubated under aerobic conditions with purified COMT, enzyme inactivation resulted. This inactivation of COMT could be prevented by including antioxidants in the preincubation mixture or by carrying out the preincubation under anaerobic conditions (Table II). However, consistent with our earlier observations,[5c] it appears that 18 (generated in situ from 6) is substantially less reactive with COMT than aminochrome 2. This is clearly demonstrated in Figure 1 where the same concentrations of aminochrome II (generated in situ from 5,6-dihydroxyindole) and aminochrome 18 (generated in situ from 6) are compared for their abilities to inactivate COMT. It is highly unlikely that the weaker inhibitory activity of 18 results because of poor enzymatic binding, since alkyl substituents in comparable positions on COMT substrates produce little effect on enzymatic binding.[27] In addition, the potent COMT inactivating ability of α-Me-6-OHDA (7), which is comparable in reactivity to 6-OHDA, would suggest that alkyl groups on the side chain of 6-OHDA do not affect the compound's ability to bind to COMT. These results would then be consistent with the idea that aminochrome I is less chemically reactive toward COMT than aminochrome II.

In an effort to elucidate the nature of this protein–ligand reaction, we have evaluated various 2- and/or 5-substituted 6-OHDA analogues (2–5) for their ability to inactivate COMT. These compounds were prepared, since we felt that if the protein–ligand reaction involved a nucleophilic attack (via a 1,4 Michael addition reaction) at the 2 or 5 position of aminochrome I or II, methyl groups in these positions should block this type of addition reaction. Surprisingly, when 6-OHDA analogues 2, 4, and 5 were preincubated with COMT, complete loss of enzymatic activity was observed (Table II). This inactivation of COMT by compounds 2, 4, and 5 cannot be reversed by removal of the excess inhibitor by dialysis or gel filtration, indicating the protein–ligand reaction is irreversible. These

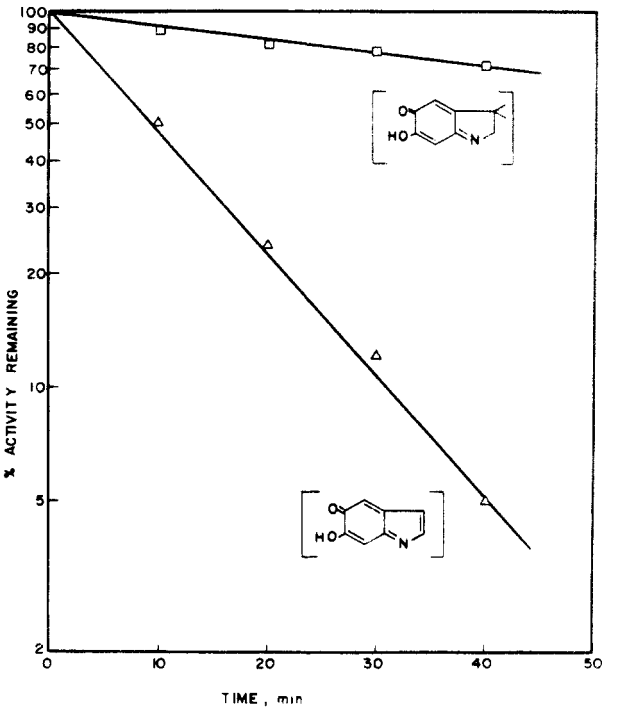



Figure 1. Effect of aminochromes II and 18 on COMT activity. (△—△) A solution of 5,6-dihydroxyindole (5.0 mM) in phosphate buffer, pH 7.60, was incubated at 37° for 20 min, during which time it was air oxidized to aminochrome II. An aliquot of this aminochrome II solution was added to a preliminary incubation mixture containing purified enzyme (final concentration of aminochrome II = 0.25 mM) and the incubation was carried out at 37°. At the appropriate times aliquots were removed and enzyme activities were determined by the methods described in the Experimental Section. (□—□) β,β-$Me_2$-6-OHDA (6) (0.25 mM) was added directly to a preliminary incubation mixture containing purified COMT and enzyme activities were determined at the appropriate times by removing aliquots. β,β-$Me_2$-6-OHDA (6) has been shown to instantaneously oxidize and cyclize to aminochrome 18 under these conditions.[26] Points represent averages of duplicate determinations.

Table III. Substrate Protection of COMT from Inactivation by 2,5-$Me_2$-6-OHDA (2)

| Rxn mixture | Additions,[a] mM DHB | SAM | SAH | % residual act. after 60 min, 37°[b] |
|---|---|---|---|---|
| 1 | | | | 0 |
| 2 | 6.0 | | | 16 |
| 3 | | 3.0 | | 37 |
| 4 | | | 0.5 | 41 |
| 5 | 6.0 | | 0.5 | 97 |

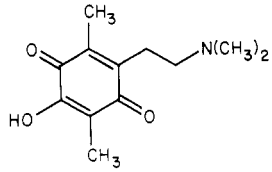
[a] The standard preincubation mixture described in Table II was used except the indicated additions were made. When DHB or SAM were incubated in the preincubation mixture, they were not included in the assay mixture. [b] Residual activity after 60 min was calculated relative to the activity of the control samples preincubated for 0 min.

results suggest the possibility that the reaction occurs by a mechanism other than a 1,4 Michael addition reaction (see Conclusion).

Evidence to suggest the alkylation of a specific amino acid residue at the active site of COMT in this inactivation process is shown in Table III. If the preincubation of COMT with 2,5-$Me_2$-6-OHDA (2) is performed in the

presence of catechol substrate (DHB), SAM, or S-adenosylhomocysteine (SAH), the inactivation of the enzyme is greatly reduced. If both a catechol substrate and SAH are included in the preincubation mixture, complete protection of the enzyme from inactivation results. The protection observed when both SAH and DHB were present in the preincubation mixture is substantially greater than the protection produced by SAH alone or than that expected from a simple combination of the two. We believe this enhanced protection results because SAH facilitates the binding of the catechol substrate. The SAH–COMT complex must have a higher affinity for DHB than does the enzyme alone, thereby allowing the catechol substrate to compete more effectively with the inhibitor for binding to the enzyme. Similar results have been observed in our laboratory for protection of COMT from inactivation by 6-OHDA,[5b] adrenochrome,[5b] 6-aminodopamine,[5c] and *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine.[24] Protection data similar to that shown in Table III have also been obtained with 2-Me-6-OHDA (**4**) and 5-Me-6-OHDA (**5**).

Our earlier studies[5b] with 6-OHDA suggested that the species reacting with COMT were aminochromes I and II. A similar situation also appears to exist for inactivation of COMT by 2,5-$Me_2$-6-OHDA (**2**). This is demonstrated by the fact that the *N*,*N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**) is inactive as an inhibitor of COMT (Table II). As shown in Table I, *N*,*N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**) readily oxidizes to the *p*-quinone **19**; however, because of the substitution on the terminal nitrogen, cyclization of **19** to the corresponding aminochrome is prohibited. The weak inhibitory activity of **19** is consistent with our earlier observations on a structurally related quinone.[5b]




**19**

To explore in more detail the mechanism of COMT inactivation by 2,5-$Me_2$-6-OHDA (**2**), the time course of enzyme inactivation was studied. As shown in Figure 2, if 2,5-$Me_2$-6-OHDA was added directly to the enzyme, a nonlinear relationship was observed between the log percentage of activity remaining and preliminary incubation time. This apparent lag time in inactivation further suggests that the *p*-quinone generated from **2** is not the alkylating species, but rather with prolonged incubation times a more reactive species is being formed (the corresponding aminochromes I and II). This idea is further substantiated by the fact that this lag time can be eliminated as shown in Figure 2 by an initial prepreincubation (60 min, 37°, pH 7.60) of 2,5-$Me_2$-6-OHDA (**2**) allowing it to air oxidize and cyclize to the corresponding aminochromes. Also shown in Figure 2 for comparison is the inactivation of COMT by the *p*-quinone **19** generated in situ from the 6-OHDA analogue **3**, again demonstrating the poor reactivity of the *p*-quinone species. Data similar to that shown in Figure 2 for 2,5-$Me_2$-6-OHDA have also been observed for 2-Me-6-OHDA (**4**) and 5-Me-6-OHDA (**5**). These data suggest that 6-OHDA analogues **2**, **4**, and **5** are producing inactivation of COMT through the intermediate formation of the corresponding methylated aminochromes I and II. Since it would be expected that methylation at the 2 and/or 5 positions would block nucleophilic attack at these positions, the activity of compounds **2**, **4**, and **5** with COMT would argue against a mechanism involving a 1,4 Michael addition reaction.

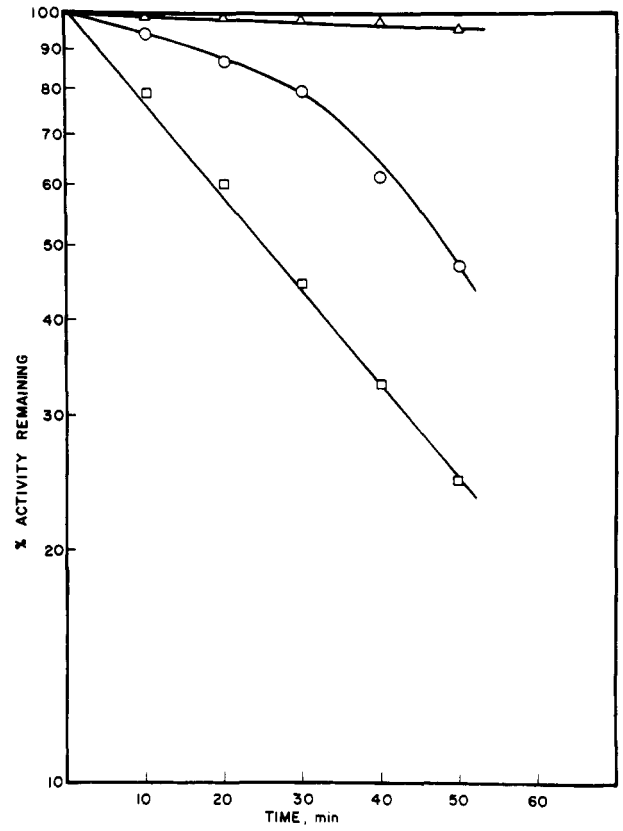
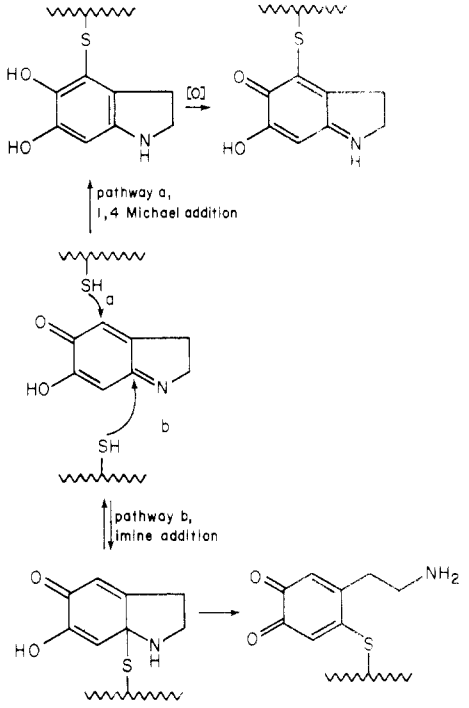



**Figure 2.** Effect of 2,5-$Me_2$-6-OHDA (**2**) and *N*,*N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**) on COMT activity. (○—○) 2,5-$Me_2$-6-OHDA (**2**) (0.125 mM) was added directly to a preliminary incubation mixture containing purified COMT and enzyme activities were determined at the appropriate times by removing aliquots (see Experimental Section). (△—△) *N*,*N*-$Me_2$-2,5-$Me_2$-6-OHDA (**3**) (0.75 mM) was added directly to a preliminary incubation mixture as described above. (□—□) A solution of 2,5-$Me_2$-6-OHDA (**2**) (5.0 mM) in phosphate buffer, pH 7.60, was incubated at 37° for 60 min, after which an aliquot of this solution (equivalent to a final concentration of 0.125 mM) was added to a preliminary incubation mixture containing purified enzyme and incubated at 37°. At the appropriate times aliquots were removed and enzyme activities were determined. Points represent averages of duplicate determinations.

## Conclusion

We have shown in this study and earlier studies[5b] that the in vitro inactivation of COMT by 6-OHDA results from the alkylation of a nucleophilic residue at the active site of this enzyme. The evidence suggests a mechanism involving attack by a protein nucleophile on an enzymatically bound electrophile (6-OHDAQ, aminochrome I or aminochrome II). The order of reactivity toward COMT appears to be aminochrome II ≥ aminochrome I ≫ 6-OHDAQ. Depicted in Scheme V are two possible modes of nucleophilic addition to aminochrome I. (Nucleophilic additions to 6-OHDAQ and aminochrome II could proceed by mechanisms similar to those described in Scheme V.) Pathway a depicts a 1,4 Michael addition reaction similar to that suggested earlier by Saner and Thoenen.[6] An alternative type of nucleophilic addition is described in pathway b, which involves addition across a carbon–nitrogen double bond (imine). Such carbonyl or imine type additions have been shown previously to be rapid and reversible reactions.[28] Therefore, the first reaction in pathway b should be chemically reversible; however, such a modification of a protein could result in changes in the

Scheme V. Possible Pathways for the Addition of a Protein Nucleophile to the Aminochrome Intermediate Generated from 6-OHDA Air Oxidation




tertiary structure of the protein, thereby changing the protein's ability to carry out its biological function. The initial adduct generated in pathway b could undergo further reaction to form an *o*-quinone, thereby making this an essentially irreversible process.

The inhibitory activity of 2,5-$Me_2$-6-OHDA (**2**), 2-Me-6-OHDA (**4**), and 5-Me-6-OHDA (**5**) toward COMT reported in this paper would argue against a mechanism involving a 1,4 Michael addition reaction (pathway a, Scheme V). The presence of the methyl substituents in the 2 and 5 positions of 6-OHDA should block such addition reactions at these positions. Evidence in support of this assumption comes from a study of glutathione addition to 6-OHDAQ. Adams et al.[29] have shown that glutathione addition to 6-OHDAQ proceeds by a 1,4 Michael addition with attack at the 2 position of the quinone. 2-Me-6-OHDAQ (**4**) and 2,5-$Me_2$-6-OHDAQ (**2**) do not react with glutathione, providing evidence that methyl substituents do indeed block such nucleophilic attack. However, it is of interest to note that in the absence of external nucleophiles 6-OHDAQ undergoes intramolecular cyclization (imine attack) to form aminochrome I,[26,30] rather than an intramolecular 1,4 Michael addition reaction. This observation suggests that an intermolecular nucleophilic attack such as that observed in the reaction with COMT may proceed by pathway b or pathway a. The preferred pathway may be determined by the position of the protein nucleophile to the possible sites of attack on the electrophilic ligand. The interaction of the oxidation products of 6-OHDA with COMT may represent a somewhat special case, since these ligands are bound at the active site of COMT in a reversible manner prior to covalent bond formation. Therefore, attack on the enzymatically bound ligand (6-OHDAQ, aminochrome I or II) would occur at the electrophilic site in the proper juxtaposition to a protein nucleophile. After enzymatic binding of aminochrome I or II (or 6-OHDAQ) to COMT, the protein nucleophile may be in closer proximity to the carbon–nitrogen double bond than to the 2 position on these molecules. It would be expected that the reaction of 6-OHDAQ with COMT, which is for all practical purposes a unimolecular reaction within a dissociable complex, may be much different than a bimolecular reaction such as that observed with glutathione. Until the addition product(s) formed from the reaction of COMT and the oxidation products of 6-OHDA are chemically characterized, the nature of the addition reaction will remain speculative. Attempts to characterize these addition products are presently under way in our laboratory.

The inactivation of COMT by 6-OHDA and 6-$NH_2$DA is probably of no significance in the in vivo degenerative effects of these compounds; therefore, the system described here should only be considered as a model system. However, we feel this system has provided new insight into the mechanisms by which these compounds can interact with proteins. It has also shown that because of certain structural features of the oxidation products of 6-OHDA[5b] and 6-aminodopamine[5c] (e.g., the conjugated $\alpha$-hydroxycarbonyl system), these molecules may have a built-in specificity for proteins which normally have an affinity for catecholamines.

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## References and Notes

(1) Established Investigator of the American Heart Association.
(2) Abbreviations used are COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosylhomocysteine; DHA, 3,4-dihydroxyacetophenone; DHB, 3,4-dihydroxybenzoic acid; 6-OHDA, 6-hydroxydopamine; 6-OHDAQ, 6-hydroxydopamine-*p*-quinone; 5,6-DHI, 5,6-dihydroxyindole.
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# Potential Antitumor Agents. 14. 4-Substituted 2-Formylpyridine Thiosemicarbazones[1]

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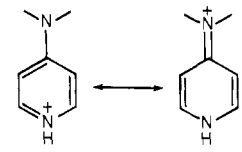
A series of 4-substituted 2-formylpyridine thiosemicarbazones has been synthesized which contain a tertiary N at the 4 position. These materials were obtained by reacting 4-nitro-2-picoline *N*-oxide, either directly or after conversion to the corresponding 4-chloro derivative, with a variety of secondary amines. Rearrangement of the 4-substituted 2-picoline *N*-oxides with $Ac_2O$ yielded respective methyl acetates, which upon acid hydrolysis, $MnO_2$ oxidation, and reaction with thiosemicarbazide resulted in the desired compounds. An alternate procedure which consisted of reacting 4-chloro-2-formylpyridine ethylene acetal with various amines, followed by hydrolysis and reaction with thiosemicarbazide, was also employed. Introduction of an alkyl group at the 3 position of the pyridine ring of 4-morpholino-2-formylpyridine thiosemicarbazone was achieved by utilizing 2,3-dimethyl-4-nitropyridine *N*-oxide; this material was converted to the corresponding 4-chloro derivative which was then subjected to nucleophilic substitution. 4-Morpholino-2-formylpyridine thiosemicarbazone was the most active antineoplastic agent of this series in mice bearing Sarcoma 180 ascites cells and was significantly superior to 5-hydroxy-2-formylpyridine thiosemicarbazone in this test system.

$\alpha$-(N)-Heterocyclic carboxaldehyde thiosemicarbazones have significant antineoplastic activity in a variety of experimental tumor systems.[2-10] The reductive conversion of ribonucleotides to deoxyribonucleotides, catalyzed by the enzyme ribonucleotide reductase, appears to be the major site of action of these agents; blockade of this enzyme results in inhibition of DNA synthesis.[11-15] Since these agents are extremely water insoluble, hydroxylated derivatives were synthesized to (a) solubilize these compounds as sodium salts[16] and (b) enhance therapeutic efficacy.[3,4] The potent antineoplastic activity of the most promising of the hydroxylated derivatives, 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP), in animal systems was not attained in man[17-19] for two reasons: (a) 5-HP has relatively low inhibitory potency for ribonucleotide reductase[14] and (b) rapid metabolism to an *O*-glucuronide and elimination of 5-HP occurred in man.[18] Amino-substituted derivatives were therefore synthesized to eliminate the problem of *O*-glucuronide formation and allow water solubility as acid salts. As a result, 5-amino-1-formylisoquinoline and 2-formyl-4-(*m*-amino)-phenylpyridine thiosemicarbazones have been synthesized;[20,21] these agents possessed broad-spectrum antineoplastic activity in murine neoplasms. Since these agents were designed to take advantage of a hypothetical hydrophobic zone of interaction between the inhibitor and ribonucleotide reductase, they had high affinity for the target enzyme.[20,21] The amino group of these compounds, however, is readily susceptible to certain metabolic transformations by N-substitution (e.g., acetylation, methylation, and glucuronidation) which may result in inactivation. Confirming this possibility was the finding that acetylation of 5-amino-1-formylisoquinoline resulted in a compound devoid of antineoplastic activity.[4] For this reason, 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone was synthesized with the methyl group placed adjacent to the 5-amino function to provide steric protection from metabolic substitution and potential inactivation.[22]

We now report the synthesis of a series of 4-substituted 2-formylpyridine thiosemicarbazones, in which a tertiary nitrogen, as part of different ring systems, was inserted to achieve the assets of $NH_2$-substituted derivatives while (a) minimizing conjugation of the N atom employed for solubilization and (b) utilizing the postulated hydrophobic bonding zone present in or adjacent to the inhibitor binding site on the enzyme by varying the size of the substituted ring. The latter feature was also enhanced by insertion of an additional $CH_3$ group at the 3 position of the pyridine nucleus in one of the most active members of this series; such substitution has been shown to increase the affinity of 2-formylpyridine thiosemicarbazone toward the target enzyme.[23] Introduction of an alkyl group at the 3 position would also result in steric hinderance which would be expected to decrease the base-strengthening effect of 4-substituted amines on the ring nitrogen; this would tend to reduce the cationic forms shown below.



Formation of these cations would be undesirable since N*N*S* tridentate chelate formation with transition