dropwise a $NaNO_2$ solution (280 mg, 4 mmol in 2.0 mL of H_2O). This diazonium mixture was then filtered and added to the cold (0–5 °C) CuCN solution prepared above. The reaction mixture was heated in a 50 °C bath with stirring for 1 h and then stirred at room temperature overnight. The mixture was concentrated in vacuo and mixed with EtOH. The residue was washed thoroughly with EtOH, the EtOH was acidified with dilute H_2SO_4, and the resulting solids were extracted again with EtOH. The total combined EtOH extracts were evaporated to give 220 mg (76%) of a crude solid: mp 192–195 °C. The crude solid was purified by preparative TLC (1000 μm, Woehlm silica gel, 90% $CHCl_3$ saturated with HCO_2H, 10% EtOH) and then sublimed (120–140 °C, 0.3 mm) to give the product: mp 225–227 °C (~50% total recovery from purification steps); NMR (acetone-d_6) δ 7.90–7.80 (2 d, 2, Ar), 4.10 (s, 3, OCH_3), no other absorptions observed; IR (KBr) 3250 (OH), 3200–2400 (COOH), 2235 (C≡N), 1740 (C=O), 1690, 1580, 1500, 1430 (Ar) cm^{-1}. Anal. ($C_9H_7NO_4$) C, H, N.

Acknowledgment. This work was supported by a grant from the National Institute of Neurological and Communicative Disorders and Stroke (NS-10918). The authors gratefully acknowledge the support provided by the Center for Biomedical Research, University of Kansas.

Catechol *O*-Methyltransferase. 12. Affinity Labeling the Active Site with the Oxidation Products of 5,6-Dihydroxyindole

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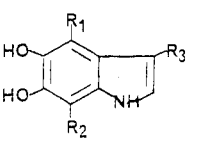
5,6-Dihydroxyindole (5,6-DHI) and a series of 4- and/or 7-methylated analogues of 5,6-DHI have been synthesized and evaluated for their ability to inactivate purified rat liver catechol *O*-methyltransferase (COMT). The inactivation of COMT by these agents could be prevented by excluding oxygen from the incubation mixtures, indicating the necessity for their oxidation to the corresponding aminochromes. Substrate protection studies and kinetic studies suggested that the loss of enzyme activity resulted from the modification of a crucial amino acid residue at the active site of COMT through reaction with the quinoid oxidation products. The COMT inhibitory activity of the 4- and/or 7-methylated analogues of 5,6-DHI argue against a mechanism involving a 1,4 Michael addition reaction at positions 4 or 7 on the aminochrome. Considering the number of potential electrophilic centers on the basic aminochrome structure, the site of the reaction might change depending on the aromatic substitution pattern. The preferred pathway of reaction may be determined in part by the juxtaposition of the protein nucleophile to the possible sites of attack on the electrophilic ligand but also in part on the reactivity of the electrophilic site which might change with substitution on the aromatic ring.

The extraneuronal inactivation of catecholamines and the detoxification of many xenobiotic catechols are dependent upon the enzyme catechol *O*-methyltransferase (COMT, EC 2.1.1.6). COMT is a soluble, magnesium-requiring enzyme which catalyzes the transfer of a methyl group from *S*-adenosylmethionine (SAM) to a catechol substrate, resulting in the formation of the meta- and para-O-methylated products.[1]

Until recently, limited information has been available concerning the nature of the amino acid residues present at the active site of COMT. Through the use of functional-group reagents[2,3] and affinity-labeling reagents,[2,4-9] our laboratory has been able to show that COMT has two nucleophilic residues at its active site; both groups are essential for enzymatic activity. The mechanisms by which these amino acid residues react with the functional-group reagent *N*-ethylmaleimide[3] and the affinity-labeling reagents, *N*-haloacetyl derivatives of 3,5-dimethoxy-4-hydroxyphenylethylamine and 3,4-dimethoxy-5-hydroxyphenylethylamine,[4-6] appears fairly straightforward because of the single electrophilic center present in each of these ligands. In contrast, the reactivity of the protein nucleophiles with the oxidation products of 6-hydroxydopamine[7,9] and 6-aminodopamine[8] is less clearly defined because of the multitude of electrophilic species generated upon air-oxidation of these hydroquinones and the various reactive centers present in each electrophilic species.

Chart I



	R_1	R_2	R_3
1	CH_3	CH_3	H
2	CH_3	H	H
3	H	CH_3	H
4	H	H	H
5	H	H	$CH_2CH_2NH_2$

Our earlier studies[7] suggested that one of the oxidation products of 6-hydroxydopamine which produced inactivation of COMT was aminochrome II which is generated by air-oxidation of 5,6-dihydroxyindole (DHI). In an attempt to better define the involvement of aminochrome II in the COMT inactivation process and to elucidate the nature of the chemical reaction that occurs between this electrophile and the protein nucleophile, a series of 4- and/or 7-methylated analogues of DHI have been prepared and their ability to inactivate COMT has been determined. The compounds of particular interest were 4,7-Me_2-5,6-

(1) R. T. Borchardt, "Enzymatic Basis of Detoxification", Vol. II, W. B. Jakoby, Ed., Academic Press, New York, 1980, p 43.
(2) R. T. Borchardt, "Biochemistry and Function of Monoamine Enzymes", E. Usdin and N. Weiner, Eds., Marcel Dekker, New York, 1977, p 707.
(3) R. T. Borchardt and D. R. Thakker, *Biochim. Biophys. Acta*, **445**, 598 (1976).
(4) R. T. Borchardt and D. R. Thakker, *Biochem. Biophys. Res. Commun.*, **54**, 1233 (1973).
(5) R. T. Borchardt and D. R. Thakker, *J. Med. Chem.*, 18, 152 (1975).
(6) R. T. Borchardt and D. R. Thakker, *Biochemistry*, 14, 4543 (1975).
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(8) R. T. Borchardt, E. E. Smissman, D. Nerland, and J. R. Reid, *J. Med. Chem.*, **19**, 30 (1976).
(9) R. T. Borchardt, J. R. Reid, D. R. Thakker, Y. O. Liang, R. W. Wightman, and R. N. Adams, *J. Med. Chem.*, **19**, 1201 (1976).

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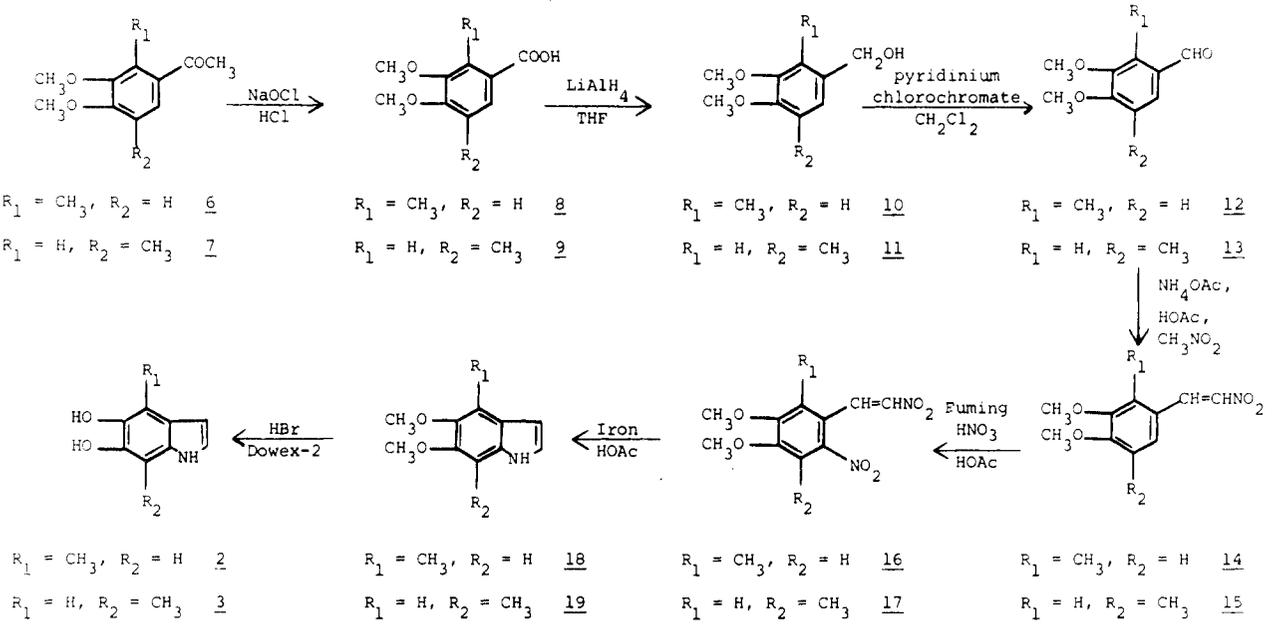
Scheme I



DHI (1), 4-Me-5,6-DHI (2), and 7-Me-5,6-DHI (3) (Chart I). These methylated analogues were chosen so that it would be possible to block either simultaneously or independently with methyl groups the 4 or 7 position of aminochrome II from nucleophilic attack. This substitution pattern should allow for clarification of the importance of these electrophilic sites in the COMT inactivation process. The results of these studies are described below.

Results

Chemistry. The derivatives of 5,6-DHI that were synthesized for this study are listed in Chart I. These potential affinity-labeling reagents for COMT and their synthetic intermediates were characterized by several methods, including their spectral and chromatographic properties and elemental analyses.

The synthesis of 4,7-Me_2-5,6-DHI (1) was modeled after the procedure of Cromartie and Harley-Mason[10] with few modifications. Reaction of 2,5-dimethyl-3,4,6-trimethoxyphenylethylamine[9] with BBr_3 afforded 2,5-dimethyl-3,4,6-trihydroxyphenylethylamine in quantitative yields. The trihydroxyphenylethylamine was cyclized using K_3-[$Fe(CN)_6$] in $NaHCO_3$ to yield the corresponding 4,7-Me_2-5,6-DHI (1). The syntheses of 4-Me-5,6-DHI (2) and 7-Me-5,6-DHI (3) were achieved by initially preparing the intermediate acetophenone derivatives 6 and 7 (Scheme I). For the synthesis of 2-methyl-3,4-dimethoxyacetophenone (6), 3-methylcatechol was methylated[10] using dimethyl sulfate and K_2CO_3 to give 2,3-dimethoxytoluene. Acetylation of 2,3-dimethoxytoluene with polyphosphoric acid and acetic anhydride afforded a good yield of 2-methyl-3,4-dimethoxyacetophenone (6).[11] The position of the ketone functionality was confirmed from the ^1H NMR spectrum of 6, which exhibited ortho coupling of the two aromatic protons (J = 8 Hz).

For the synthesis of 3,4-dimethoxy-5-methylacetophenone (7), 3-methylcatechol was acetylated using NaOAc and acetic anhydride, affording in quantitative yield 2,3-diacetoxytoluene. 2,3-Diacetoxytoluene in the presence of chlorobenzene and anhydrous $AlCl_3$ underwent a Fries rearrangement[12] to yield 3,4-dihydroxy-5-methylacetophenone. The structure of 3,4-dihydroxy-5-methylacetophenone was confirmed from the ^1H NMR spectrum by the meta coupling of the two aromatic protons (J = 2 Hz). This acetophenone was converted to 3,4-dimethoxy-5-methylacetophenone (7) in quantitative yield using dimethyl sulfate and acetone. The structure of 7 was confirmed from the ^1H NMR spectrum by the meta coupling of the two aromatic protons (J = 2 Hz).

The general pathway used for the preparation of 4-Me-5,6-DHI (2) and 7-Me-5,6-DHI (3) from the acetophenones 6 and 7, respectively, is outlined in Scheme I. The acetophenones 6 and 7 were oxidized using NaOCl to their corresponding acids 8 and 9. The acids 8 and 9 were then reduced to their corresponding alcohols 10 and 11 using $LiAlH_4$ and THF. Oxidation of alcohols 10 and 11 to their respective aldehydes 12 and 13 was achieved by using pyridinium chlorochromate in dichloromethane.[13] The aldehydes 12 and 13 were converted to their corresponding β-nitrostyrene derivative 14 and 15 using nitromethane and ammonium acetate in glacial acetic acid.[14] Aromatic nitration of 14 and 15 using fuming nitric acid and glacial acetic acid afforded good yields of nitrostyrenes 16 and 17. Reduction[14] of nitrostyrenes 16 and 17, followed by cyclization using iron and glacial acetic acid, yielded indoles 18 and 19.

The assigned structures of 18 and 19 were confirmed from the ^1H NMR spectra by comparing the positions of the aromatic and vinylic protons with related indoles previously reported in the literature.[15] Demethylation of 18 and 19 using 48% HBr, followed by neutralization with ion-exchange resin (Dowex-2), afforded the desired 5,6-DHI derivatives 2 and 3. Cromartie and Harley-Mason[10] previously reported the synthesis of 2 and 3 by oxidation of 2- or 5-methyl-3,4-dihydroxyphenylalanine. The physical characteristics, e.g., melting points, of 2 and 3 synthesized by Scheme I were identical with those reported

(10) R. J. T. Cromartie and J. Harley-Mason, *J. Chem. Soc.*, 3525 (1953).
(11) L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis", Vol. 1, Wiley, New York, 1967, p 898.
(12) J. D. Moed, J. Vandijk, and H. Niewind, *Recl. Trav. Chim. Pays-Bas*, 77, 273 (1958).
(13) E. J. Corey and J. W. Sugge, *Tetrahedron Lett.*, 2547 (1975).
(14) J. D. Benigni and R. L. Minnis, *J. Heterochem.*, 2, 387 (1965).
(15) "Sadtler's Standard NMR Spectra", Sadtler Research Laboratories, Philadelphia, spectra 19523N, 20534M, 20580M, 21343M, 21626M, and 22754M.

Table I. Inactivation of COMT by Analogues of 5,6-DHI

no.[a]	residual act., cpm	
	before dialysis[b]	after dialysis[c]
	3684	3448
1	322	212
2	396	351
3	277	262
4	282	228
5	242	232

[a] The standard preincubation mixture consisted of inhibitor (0.5 mM), $MgCl_2$ (1.20 mM), phosphate buffer (pH 7.60), enzyme preparation, and water to a final volume of 0.8 mL. The incubation was carried out for 60 min. Residual activity was determined as described under 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. [c] Residual activity was from controls (as shown in footnote *b*), which were dialyzed for 24 h.

Table II. Effect of Aerobic and Anaerobic Conditions on the Inactivation of COMT by Analogues of 5,6-DHI

inhibitor[a] (0.5 mM)	% residual act. after 60 min at 37 °C	
	aerobic	anaerobic
1	4.5	96
2	0	81
3	0	84
4	0	96
5	0	89

[a] The standard preincubation mixture consisted of inhibitor (0.5 mM), magnesium chloride (1.20 mM), phosphate buffer (pH 7.60), enzyme preparation, and water to a final volume of 0.8 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 under 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.

earlier by Cromartie and Harley-Mason.[10]

5,6-DHI (**4**) was prepared from 5,6-bis(benzyloxy)indole by catalytic hydrogenation.

Biology. When 5,6-DHI (**4**), 5,6-DHT (**5**), and the methylated analogues of 5,6-DHI (**1–3**) were preincubated with rat liver COMT, complete loss of enzymatic activity was observed. The inactivation of COMT produced by these compounds could not be reversed by dialysis (Table I) or gel filtration chromatography (data not shown). In earlier studies,[7] we have shown that the air-oxidation of 5,6-DHI (**4**) was required for it to produce inactivation of COMT. As shown in Table II, air-oxidation of the 5,6-DHI analogues **1–3** and 5,6-DHT (**5**) was required for these compounds to produce inactivation of COMT. In these experiments the preincubations were carried out under either aerobic or anaerobic conditions. If oxygen was excluded completely from the incubation mixture, inactivation of COMT by compounds **1–3** and **5** was not observed. If oxygen was included, total inactivation of the enzyme was observed. These observations strongly support the premise that initial air-oxidation of the 5,6-DHI analogues **1–3** and 5,6-DHT (**5**) to quinoid oxidation products (aminochrome II) are necessary in this enzyme-inactivation process.

In order to explore in more detail the mechanism of this protein–ligand interaction, we determined the time course of enzymatic inactivation produced by 4,7-Me_2-5,6-DHI

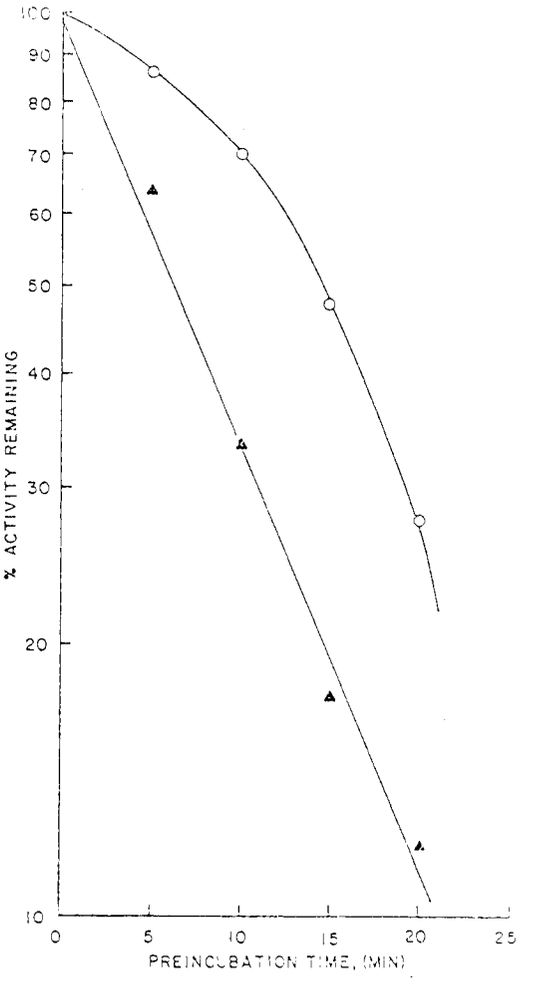
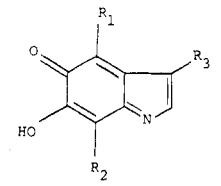


Figure 1. Effects of 4,7-Me_2-5,6-DHI (**1**) on COMT activity. 4,7-Me_2-5,6-DHI (0.17 mM) was added directly to the preliminary incubation mixture containing purified COMT, and enzyme activities were determined at appropriate times by removing aliquots (O–O). A solution of 4,7-Me_2-5,6-DHI (5.0 mM) in phosphate buffer, pH 7.60, was incubated at 37 °C for 20 min, during which time it was air-oxidized to the corresponding aminochrome **20** (▲–▲). An aliquot of this aminochrome solution was added to an incubation mixture containing purified enzyme, and incubation was carried out at 37 °C. At appropriate times, aliquots were removed and residual enzyme activities were determined. Points represent averages of duplicate determinations.

(**1**). As shown in Figure 1, when 4,7-Me_2-5,6-DHI (**1**) was added directly to the preincubation mixture containing the enzyme, a nonlinear relationship was observed between the log percentage of activity remaining and the preincubation time. Results similar to those shown in Figure 1 were also obtained with 4-Me-5,6-DHI (**2**), 7-Me-5,6-DHI (**3**), and 5,6-DHI (**4**). All of the analogues studied, except for 5,6-DHT (**5**), exhibited these nonlinear inactivation kinetics. This lag time in inactivation apparently results from the fact that the rates of oxidation of **1–4** are slow and that the reactive species (aminochromes **20–23**, Chart II) are generated only with prolonged incubation. This assumption is further substantiated by the fact that this lag time for inactivation can be eliminated as shown in Figure 1 by an initial preincubation (20 min, 37 °C, pH 7.60) of 4,7-Me_2-5,6-DHI (**1**), allowing it to air-oxidize to the corresponding aminochrome **20** prior to exposure to the enzyme. When an aliquot of air-oxidized 4,7-Me_2-5,6-DHI (**1**) was then added to the enzyme, a linear relationship between log percentage activity remaining and preincubation time was observed (Figure 1). Data similar to those shown in Figure 1 for 4,7-Me_2-5,6-DHI have also been observed for

Chart II



	R_1	R_2	R_3
20	CH_3	CH_3	H
21	CH_3	H	H
22	H	CH_3	H
23	H	H	H
24	H	H	$CH_2CH_2NH_2$

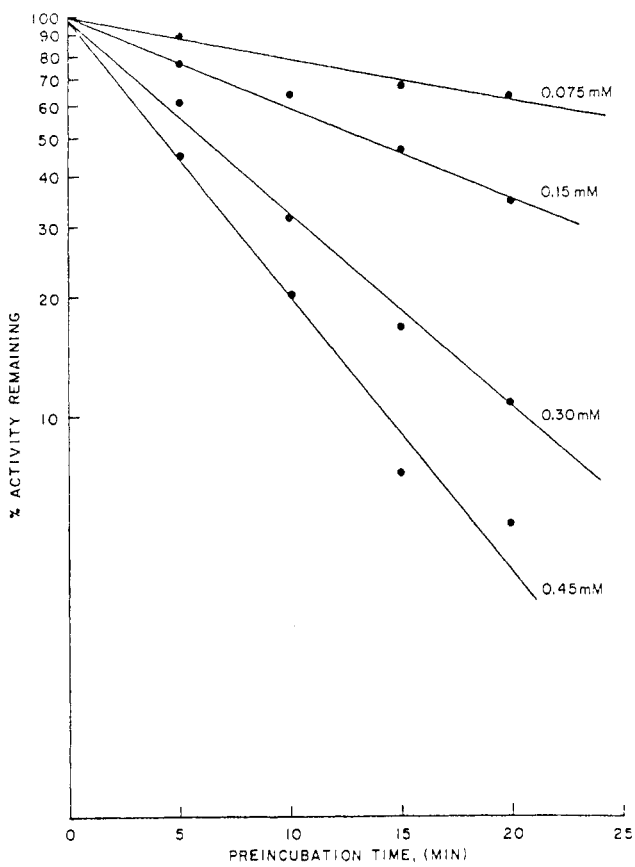


Figure 2. Effect of aminochrome **20** on COMT activity. Preliminary incubation mixtures containing purified enzyme and varying concentrations of aminochrome **20** were prepared by air-oxidation of 4,7-Me_2-5,6-DHI (Experimental Section). Enzyme activities were determined after the appropriate preliminary incubation time. The k_{app} were calculated from the slopes for each concentration of inhibitor.

4-Me-5,6-DHI (**2**), 7-Me-5,6-DHI (**3**), and 5,6-DHI (**4**). These data suggest that 5,6-DHI (**4**) and its analogues **1–3** are producing inactivation of COMT through the intermediate formation of the corresponding aminochrome II's shown in Chart II. In an effort to explore in more detail the kinetics of reaction between COMT and the aminochrome compounds **20–24** (Chart II), the time courses for enzyme inactivation were determined. As shown in Figure 2, when 4,7-Me_2-5,6-DHI (**1**) was allowed to air-oxidize to **20** and then was added to the enzyme, the inactivation plots were linear, indicating that the reactions were first-order with respect to active enzyme remaining. Plots similar to that shown in Figure 2 for 4,7-Me_2-5,6-DHI (**1**) have also been observed for 4-Me-5,6-DHI (**2**), 7-Me-5,6-DHI (**3**), 5,6-DHI (**4**), and 5,6-DHT (**5**), where the reactive species were **21**, **22**, **23**, and **24**, respectively.

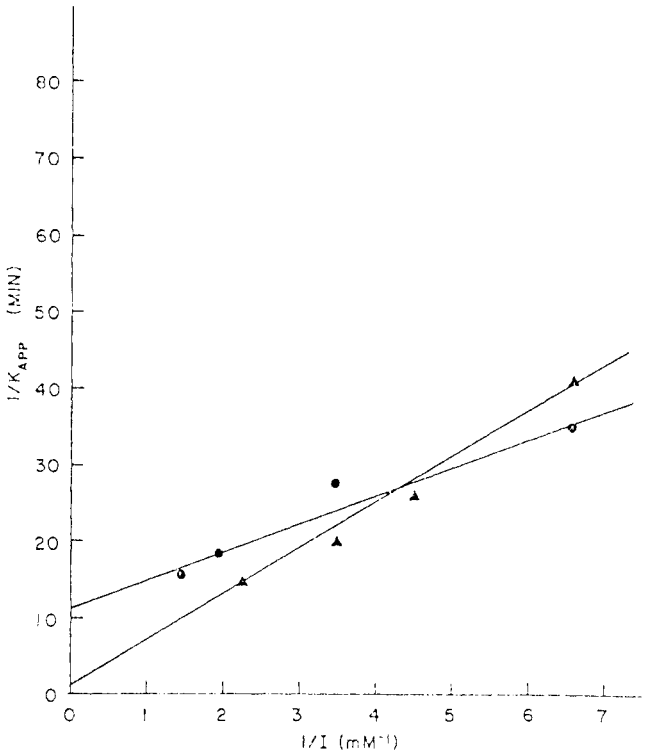


Figure 3. Double-reciprocal plots of pseudo-first-order rate constants of inactivation, k_{app}, vs. inhibitor concentration: (●–●) 4,7-Me_2-5,6-DHI (**1**), (▲–▲) 5,6-DHT (**5**). Kinetic constants k_2 and K_I were calculated from the Y intercept and the slope, respectively, using the least-squares method.

In order to provide evidence that the inactivation of COMT by these aminochrome II analogues proceeds via a unimolecular reaction within a dissociable complex rather than a nonspecific bimolecular reaction, the rates of enzyme inactivation as a function of inhibitor concentration were investigated. Kinetic evidence for the existence of such a rate-limiting step in the irreversible inhibition of cholinesterase,[16] trypsin,[17] and carboxypeptidase[18] by substrate-like reagents has been demonstrated previously. The model[18] for this type of inactivation is shown in eq 1 and 2 where E·I is the reversible complex, E–I is the inactive enzyme, K_I is the steady-state constant of inactivation, and k_2 is the first-order rate constant.

$$E + I \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} E{\cdot}I \xrightarrow{k_2} EI \quad (1)$$

$$K_I = \frac{[E][I]}{[E{\cdot}I]} \quad (2)$$

The variation of the rate of COMT inactivation as a function of the concentration of aminochrome **20** is shown in Figure 2. As predicted for each concentration of aminochrome **20**, pseudo-first-order kinetics were observed and apparent pseudo-first-order rate constants (K_{app}) were calculated.[18]

$$\frac{1}{k_{app}} = \frac{K_I}{k_2}\frac{1}{[I]} + \frac{1}{K_2} \quad (3)$$

By plotting the reciprocal of the pseudo-first-order rate constants ($1/k_{app}$) vs. the reciprocal of the inhibitor con-

(16) E. Shaw and G. Glover, *Arch. Biochem. Biophys.*, **139**, 298 (1970).
(17) G. M. Haas and H. Neurath, *Biochemistry*, **10**, 3535 (1971).
(18) P. H. Petra, *Biochemistry*, **10**, 3163 (1971).

Table III. Kinetic Constants for the Inactivation of COMT by Analogues of 5,6-DHI

	kinetic constants[a]	
no.	k_2, min^{-1}	K_I, mM
1	1.12	7.26
2	0.47	15.2
3	0.16	1.14
4	0.55	3.46
5	0.086	0.328

[a] Kinetic constants, k_2 and K_I, were calculated from the Y intercept and the slope, respectively, of double-reciprocal plots of the pseudo-first-order rate constants of inactivation, k_{app}, vs. inhibitor concentration, using the least-squares method according to eq 3.

Table IV. Substrate Protection Studies

			% residual act. after 60 min, 37 °C[c]		
reaction mixture	concn, mM DHB[b]	concn, mM SAM[b]	4-Me-5,6-DHI[a]	4,7-Me$_2$-5,6-DHI[a]	5,6-DHT[a]
1			1	4	3
2	5.0		9	12	6
3		2.5	96	45	76

[a] Concentration of inhibitor used was 0.5 mM. [b] When SAM and DHB were included in the preincubation mixture, they were not added in the assay mixture. [c] Residual activity after 60 min was calculated relative to the activity of control samples preincubated for 0 min.

centrations (1/[I]) according to eq 3 (Figure 3), a steady-state constant of inactivation, K_I, and a first-order rate constant at saturation, k_2, were calculated (Table III). The linearity of this plot and the positive Y intercept observed in this reciprocal plot provide evidence for the formation of a dissociable enzyme–inhibitor complex. Plots similar to that shown in Figure 3 for aminochrome **20** have also been observed for aminochromes **21–24**, which were derived from 4-Me-5,6-DHI (**2**), 7-Me-5,6-DHI (**3**), 5,6-DHI (**4**), and 5,6-DHT (**5**), respectively (Figures 3 and 4). From these replots, the first-order rate constants (k_2) and steady-state constant of inactivation (K_I) were calculated, and the data are listed in Table III.

In an attempt to further elucidate the nature of the interaction between these affinity-labeling agents and the active site of COMT, substrate protection studies were carried out. Since these affinity-labeling reagents would be expected to bind at the catechol binding site on COMT, the catechol substrate should protect the enzyme from inactivation by these agents. Therefore, the preincubations of COMT with 4,7-Me$_2$-5,6-DHI (**4**) were performed in the presence of catechol substrate [e.g., 3,4-dihydroxybenzoic acid (DHB)] or the methyl donor (SAM), and the inactivation of the enzyme was determined, with the results shown in Table IV. DHB alone in high concentrations produces only partial protection of the enzyme, whereas SAM alone produces nearly complete protection of the enzyme from inactivation. Also shown in Table IV are protection data obtained with 4-Me-5,6-DHI (**2**) and 5,6-DHT (**5**). Protection data similar to those shown in Table IV have also been obtained with 7-Me-5,6-DHI (**3**) and 5,6-DHI (**4**).

Discussion

The generally accepted experimental criteria[19,20] for an affinity-labeling reagent includes (1) a rate saturation effect on the rate of inactivation of the enzyme by the affinity-labeling reagent, (2) protection against inactivation by substrate or competitive inhibitor, and (3) stoichiometric incorporation of one molecule per binding site. From the evidence presented in this study, it is clear that 5,6-DHI, 5,6-DHT, and the methylated analogues of 5,6-DHI (**1–3**) satisfy many of the criteria for affinity-labeling reagents for COMT.

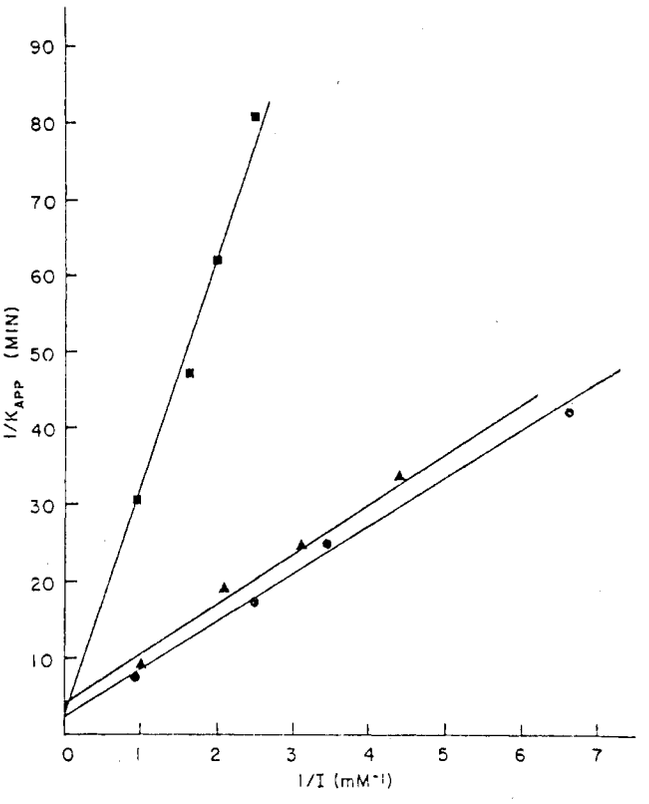


Figure 4. Double-reciprocal plots of pseudo-first-order rate constants of inactivation, k_{app}, vs. inhibitor concentration: (■–■) 4-Me-5,6-DHI (**2**), (▲–▲) 7-Me-5,6-DHI (**3**), (●–●) 5,6-DHI (**4**). Kinetic constants k_2 and K_I were calculated from the Y intercept and the slope, respectively, using the least-squares method.

The inactivation of COMT by these reagents appears to proceed by a unimolecular reaction within a dissociable complex rather than by a nonspecific bimolecular reaction. The inactivation of COMT by these affinity-labeling reagents appears to result from alkylation of a crucial amino acid residue at the active site of the enzyme. The results of the substrate protection studies with catechol substrate and the methyl donor, SAM, provided evidence that an active-site amino acid residue is being modified by these affinity-labeling reagents. The protection studies reported here showed that a catechol substrate only partially protected COMT from inactivation by the 5,6-DHI analogues **1–4** and 5,6-DHT (**5**). Nearly complete protection of COMT could be obtained when the methyl donor SAM was present in the preincubation mixture. The protection of COMT by SAM or the catechol substrate suggests that when these ligands bind to the enzyme there is either a physical protection of the nucleophilic residue or a conformational change of the enzyme which makes the nucleophilic group less accessible to the affinity-labeling reagent. Similar results have been observed by Borchardt et al. for protection of COMT from inactivation by 6-OHDA,[9] adrenochrome,[7] 6-aminodopamine,[8] and *N*-(iodoacetyl)-3,4-dimethoxy-5-hydroxyphenylethylamine.[6] However, these results are in sharp contrast to the rate enhancement effects that SAM or SAH have on the inactivation of COMT produced by *N*-(bromo-

(19) H. P. Maloche, *Biochemistry*, **6**, 2273 (1967).
(20) F. Wold, *Methods Enzymol.*, **46**, 3 (1977).

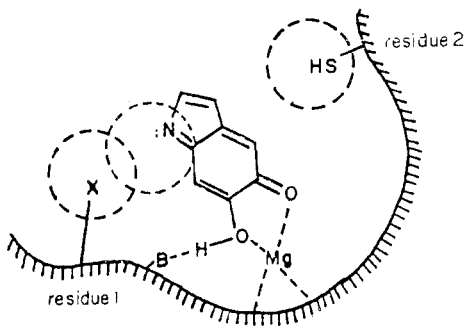


Figure 5. Proposed binding mode for aminochrome at the active site of COMT.

acetyl)-3,5-dimethoxy-4-hydroxyphenylethylamine and *N*-(iodoacetyl)-3,5-dimethoxy-4-hydroxyphenylethylamine.[6]

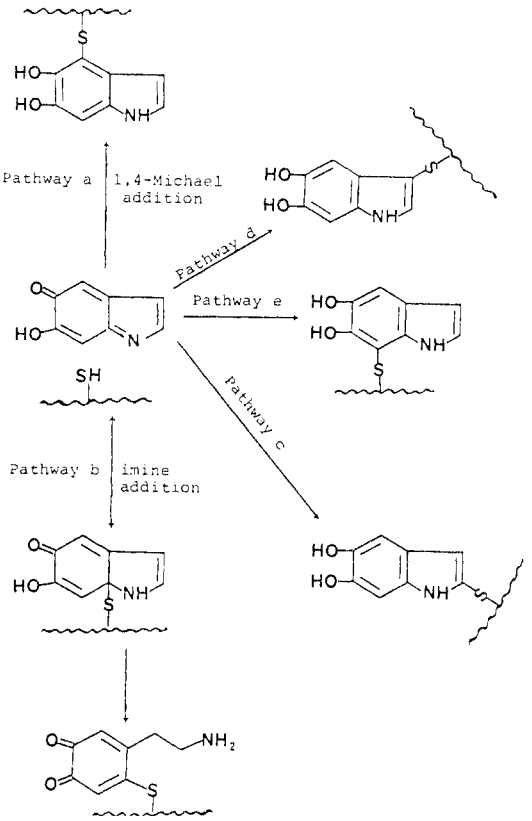
These basic differences in the properties of affinity-labeling reagents for COMT provide further support for two different nucleophilic residues at the active site of this enzyme[3] and the fact that these residues can be modified selectively using chemical reagents. Thus, from the evidence presented in this study and earlier studies,[7-9] it can be concluded that the inactivation of COMT by the 5,6-DHI analogues 1–4 and 5,6-DHT (**5**) results from alkylation of the nucleophilic residue (as depicted in Figure 5) at the active site of this enzyme. The alkylating species appears to be the quinoidlike compounds (aminochrome **20–24**) generated from the air-oxidation of the 5,6-dihydroxyindoles. The evidence suggests a mechanism involving attack by a protein nucleophile on the enzymatically bound electrophile (aminochrome **20–24**).

Depicted in Scheme II are five possible modes of nucleophilic addition to aminochrome II. Pathway a depicts a 1,4 Michael addition reaction similar to that suggested by Saner and Thoenen.[21] Pathways c, d, and e also depict similar 1,4 Michael additions at positions 2, 3, and 7 of aminochrome II, respectively. Another 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.[22] Therefore, the first reaction in pathway b should be chemically reversible. However, such a modification of a protein could result in changes in the tertiary structure of a protein, thereby changing the ability of the protein to carry out its biological function. The initial adduct in pathway b could undergo further reaction to form the *o*-quinone, thereby making this essentially an irreversible process.

The inhibitory activities of 4,7-Me_2-5,6-DHI (**1**), 4-Me-5,6-DHI (**2**) and 7-Me-5,6-DHI (**3**) toward COMT reported in this study would argue against a mechanism involving a 1,4 Michael addition reaction at positions 4 or 7 on the aminochrome (pathways a or e, Scheme II). The presence of methyl substituents in the 4 or 7 positions of 5,6-DHI should block such addition reactions at those sites.

The strong inhibitory activity of 5,6-DHT (**5**) toward COMT was of particular interest. The presence of the ethylamine side chain on 5,6-DHT (**5**) seems to enhance enzymatic binding relative to 5,6-DHI (**4**). This increasing affinity is evident by comparing the K_I for 5,6-DHT (**5**) with that of 5,6-DHI (**4**). 5,6-DHT also appears to produce

Scheme II. Possible Pathways for the Addition of a Protein Nucleophile to the Aminochrome Intermediate Generated upon Air-Oxidation of 5,6-DHI



an enhanced rate of COMT inactivation as compared to 5,6-DHI (**4**) or its methylated analogues **1–3**.

All these observations suggest that 5,6-DHI analogues (**1–5**) are active inhibitors of COMT. The general mechanism for all five analogues may be the formation of the same dissociable complex, followed by modification of an active-site amino acid residue. This modification may occur by attack at any one of a number of electrophilic sites on the aminochrome (Scheme II). However, inclusion of an alkyl substituent might, in fact, redirect the position of the attack and change the relative rates at which any of these pathways proceeds. The preferred pathway may be determined in part by the position of the protein nucleophile to the possible sites of attack on the electrophilic ligand but also in part on the availability of the electrophile site. Therefore, the interaction of the 5,6-DHI analogues with COMT may each represent a special or different case.

Experimental Section

Melting points were obtained on a calibrated Thomas-Hoover Uni-Melt apparatus and were uncorrected. Microanalyses were conducted on a Hewlett-Packard Model 185B C,H,N analyzer, University of Kansas, Lawrence, KS. Unless otherwise stated, the IR, NMR, and UV data were consistent with the assigned structures. IR data were recorded on a Beckman IR-33 spectrophotometer, NMR data on a Hitachi Perkin-Elmer high-resolution Model R-248 spectrophotometer (Me_4Si), and UV data on a Cary Model 118 spectrophotometer. Scintillation counting was done on a Beckman 3133-T scintillation counter. TLC were run on Analtech silica gel GF (250 μm). Spots were detected by visual examination under UV light and/or iodine vapors for compounds containing hydroxyl moieties.

Materials. SAM-$^{14}CH_3$ (New England Nuclear Corp., 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/mL and stored at −20 °F. SAM chloride (Sigma Chemical Co.) was stored as 0.01 M stock solution. Phosphate buffer was stored as 0.5 M solution. The following compounds were commercially available

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(22) C. A. Bishop, R. F. Porter, and L. K. J. Tong, *J. Am. Chem. Soc.*, **85**, 3991 (1963).
(23) Y. O. Liang, R. M. Wightman, and R. N. Adams, *Eur. J. Pharmacol.*, **36**, 455 (1976).

from the indicated sources: 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 5,6-bis(benzyloxy)-indole, and SAM chloride, Sigma Chemical Co.; 3,4-dihydroxybenzoic acid (DHB), Aldrich Chemical Co.; 3-methylcatechol, Pfaltz & Bauer. 2,5-Dimethyl-3,4,6-trimethoxyphenylethylamine and 5,6-dihydroxytryptamine were generously provided by Drs. J. R. Reid and C. R. Creveling, respectively. COMT was purified from rat liver according to methods previously described.[6,24] Because of stability problems, analogues of 5,6-DHI (**1–5**) were prepared in a stock solution of 2.5–5.0 μmol/mL in sealed ampules under nitrogen, and aliquots were removed as needed for inactivation experiments.

COMT Inactivation Experiments. The COMT inactivation experiments were carried out using procedures similar to those described earlier by Borchardt et al.[4-9] A preincubation mixture was prepared consisting of the following components (in micromoles): water so that the final volume was 3.20 mL; magnesium chloride (4.80); phosphate buffer, pH 7.60 (400); inhibitor (variable); and purified enzyme preparation. The preincubation step was started by the addition of enzyme, and incubation was carried out at 37 °C. After the appropriate preincubation time, an aliquot (0.20 mL) of the preincubation mixture was removed and assayed by the addition of 0.5 μCi of SAM-$^{14}CH_3$, SAM (0.25 μmol), and 3,4-dihydroxybenzoic acid (DHB; 0.40 μmol) to a final volume of 0.25 mL. The assay mixtures were incubated for 10 min at 37 °C, and reaction was stopped by the addition of 0.1 mL of 1.0 N HCl. The assay mixture was extracted with 10 mL of toluene–isoamyl alcohol (7:3), and after centrifugation, a 5-mL aliquot of the organic phase was measured for radioactivity. The results were corrected using appropriate blanks. The percent activity remaining at any given time was calculated relative to zero-time activity. The pseudo-first-order rate constants of inactivation, K_{app}, were calculated from the slopes of the plots of log of percent activity remaining vs. time.[18]

In the anaerobic experiments, the preincubation mixtures and inhibitor solution were prepared in a similar way to those described above, except that the anaerobic experiments were done in sealed ampules under nitrogen and samples were removed using a syringe. Enzyme assays were also carried out in ampules under nitrogen.

2,5-Dimethyl-3,4,6-trihydroxyphenylethylamine Hydrobromide. To a suspension of 2,5-dimethyl-3,4,6-trimethoxyphenylethylamine hydrochloride (0.300 g, 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 room temperature. The mixture was cooled to 0 °C, and CH_3OH (50 mL) was added dropwise. The CH_3OH, $(CH_3O)_3B$, and CH_2Cl_2 were removed under pressure, and the process of addition of 50 mL of CH_3OH and evaporation was repeated three times. Addition of benzene to the resulting oil produced precipitation of the product. The resulting precipitate was collected by filtration to yield 0.220 g (70%): mp 227–230 °C dec; NMR (D_2O) δ 3.0 (s, 4 H, CH_2CH_2N), 2.15 (s, 6 H, CH_3). Anal. ($C_{10}H_{16}NO_3Br$) C, H, N.

4,7-Dimethyl-5,6-dihydroxyindole (1). To a solution of 2,5-dimethyl-3,4,6-trihydroxyphenylethylamine hydrobromide (0.200 g, 2.5 mmol) in 5 mL of H_2O was added a solution of potassium ferricyanide (0.160 g, 4 mmol) and $NaHCO_3$ (0.068 g, 8 mmol) in 4 mL of H_2O. The intensely violet-colored reaction mixture was kept under H_2 (Parr shaker) for 24 h. The color faded considerably during this time. After sodium dithionite (0.01 g, 0.04 mmol) was added, the aqueous solution was extracted with EtOAc. The EtOAc was removed under reduced pressure. The resulting product was dissolved in H_2O and lyophilized to yield 0.045 g (35%) of the desired product: mp 170–172 °C dec (lit.[10] mp 170 °C); NMR (Me_2SO-d_6) δ 7.5 (broad, 1 H, NH), 7.0 (m, 1 H, CH=CHN), 6.20 (m, 1 H, CH=CHN), 2.25 (s, 6 H, CH_3). Anal. ($C_{10}H_{11}NO_2$) C, H, N.

2,3-Dimethoxytoluene. To a refluxing mixture of 3-methylcatechol (8.0 g, 54 mmol) and K_2CO_3 (15.0 g, 108.0 mmol) in 150 mL of acetone was added dimethyl sulfate (10.2 g, 81.0 mmol) dropwise. The reaction mixture was heated under reflux for 3 h, after which it was filtered under suction. The solvent was removed under reduced pressure to yield an oil (8.4 g). The crude oil was distilled at 2 mm at 56–60 °C to yield 7.5 g (83%) of 2,3-dimethoxytoluene: NMR ($CDCl_3$) δ 6.80 (m, 3 H, aromatic), 3.80 (d, 6 H, OCH_3), 2.30 (s, 3 H, CH_3); IR (salt plate), 1590, 1480, 1270, 1220, 1170, 1080, 1000 cm^{-1}.

2-Methyl-3,4-dimethoxyacetophenone (6). To a mixture of 2,3-dimethoxytoluene (50 g, 33.0 mmol) and Ac_2O (3.4 g, 33.0 mmol) was added polyphosphoric acid (42.0 g, 124.0 mmol). The thick semisolid material was stirred mechanically while heating at 45–50 °C for 1.5 h. To the reaction mixture was then added 150 mL of ice–water. The aqueous layer was then extracted with 100 mL of Et_2O. The Et_2O layer was washed with 1 N NaOH and H_2O, dried ($MgSO_4$), and filtered. The Et_2O was then removed under reduced pressure. The residue was distilled at 2 mm at 106–108 °C. The distillate on cooling gave white crystals of 2-methyl-3,4-dimethoxyacetophenone (**6**) in 70% yield: mp 48–50 °C; NMR ($CDCl_3$) δ 7.65 (d, 1 H, aromatic, J = 8 Hz), 6.95 (d, 1 H, aromatic, J = 8 Hz), 3.78 (s, 3 H, OCH_3), 3.73 (s, 3 H, OCH_3), 2.42 (s, 3 H, $COCH_3$), 2.35 (s, 3 H, CH_3); IR (KBr) 1680, 1600, 1495, 1270 cm^{-1}. Anal. ($C_{11}H_{14}O_3$) C, H.

2-Methyl-3,4-dimethoxybenzoic Acid (8). Calcium hypochlorite (2.94 g, 20.0 mmol) was dissolved in 11.76 mL of warm H_2O. To this was added Na_2CO_3 (2.058 g, 19.0 mmol) and NaOH (0.60 g, 15.0 mmol) dissolved in 5.88 mL of H_2O. The mixture was stirred vigorously until the semisolid gel became quite fluid. The suspended solid was removed by suction. The filtrate (NaOCl) was warmed to 55 °C and 2-methyl-3,4-dimethoxyacetophenone (**6**; 1.0 g, 5.0 mmol) was added. The mixture was vigorously stirred, and the temperature of the reaction was maintained at 60–70 °C by occasional warming and cooling. The reaction was stirred for 40 min, after which the excess hypochlorite was destroyed by adding a solution of $NaHSO_3$ (0.60 g, 5.0 mmol) in 2.5 mL of H_2O. The reaction mixture was stirred for 15 min. After cooling to room temperature, the reaction mixture was acidified with 2.4 mL of concentrated HCl. The crude acid which precipitated out was collected by filtration. The crude acid was crystallized (95% EtOH) to yield 0.80 g (81.5%) of desired product **8**: mp 173–174 °C; NMR (Me_2SO-d_6) δ 7.65 (d, 1 H, aromatic, J = 8 Hz), 6.95 (d, 1 H, aromatic, J = 8 Hz), 3.78 (s, 3 H, OCH_3), 3.73 (s, 3 H, OCH_3), 2.38 (s, 3 H, CH_3); IR (KBr) 3300 (broad), 3010 (broad), 1680 cm^{-1}. Anal. ($C_{10}H_{12}O_4$) C, H.

2-Methyl-3,4-dimethoxybenzyl Alcohol (10). To $LiAlH_4$ (0.577 g, 15.2 mmol) in 100 mL of dry THF was added dropwise a solution of 2-methyl-3,4-dimethoxybenzoic acid (**8**; 1.4 g, 7.6 mmol) dissolved in 50 mL of dry THF. The addition took approximately 20 min, after which the reaction mixture was refluxed for 12 h and then cooled in an ice bath, and excess $LiAlH_4$ was decomposed by adding "wet" EtOAc followed by H_2O. The precipitates were then extracted with Et_2O, the Et_2O layer was dried with $MgSO_4$ and filtered, and the solvent was removed under reduced pressure to give an oil. The oil was recrystallized (benzene–hexane) to give the desired product: yield 1.15 g (74%); mp 63–64 °C; NMR (acetone-d_6) δ 7.65 (d, 1 H, aromatic, J = 8 Hz), 6.75 (d, 1 H, aromatic, J = 8 Hz), 4.5 (s, 2 H, OCH_2), 3.77 (s, 3 H, OCH_3), 3.73 (s, 3 H, OCH_3), 2.85 (s, 1 H, OH), 2.15 (s, 3 H, CH_3); IR (KBr) 3040 (broad), 2995 and 1600 cm^{-1}. Anal. ($C_{10}H_{14}O_3$) C, H.

2-Methyl-3,4-dimethoxybenzaldehyde (12). E. J. Corey's method[13] of restricted oxidation of an alcohol to the corresponding aldehyde with pyridinium chlorochromate was used in the preparation of **12**. To a suspension of pyridinium chlorochromate (0.647 g, 3.0 mmol) in 5 mL of CH_2Cl_2 was added a solution of 2-methyl-3,4-dimethoxybenzyl alcohol (**10**; 0.364 g, 2 mmol) dissolved in 4 mL of CH_2Cl_2. The dark brown reaction mixture was stirred for 1 h at room temperature. The reaction was then quenched with 35 mL of Et_2O and filtered through a Florosil pad; the solvent was removed under pressure to give an oil. The oil was recrystallized (benzene–petroleum ether) to yield 0.568 g (88%): mp 48–50 °C; NMR (acetone-d_6) δ 10.10 (s, 1 H, CHO), 7.55 (d, 1 H, aromatic, J = 8 Hz), 6.95 (d, 1 H, aromatic, J = 8 Hz), 3.80 (s, 3 H, OCH_3), 3.70 (s, 3 H, OCH_3), 2.45 (s, 3 H, CH_3); IR (salt plate) 2995 and 1695 cm^{-1}. Anal. ($C_{10}H_{12}O_3$) C, H.

2-Methyl-3,4-dimethoxy-β-nitrostyrene (14). 2-Methyl-3,4-dimethoxybenzaldehyde (**12**; 6.3 g, 35.0 mmol) was dissolved in 50 mL of glacial HOAc to which was added ammonium acetate (5.6 g, 70.0 mmol) and nitromethane (6.2 g, 105.0 mmol). The reaction mixture was stirred and heated under reflux for 2 h. The

(24) R. T. Borchardt, *Methods Enzymol.*, **77**, 267 (1981).

reaction mixture was cooled to room temperature, and the solvent was removed under reduced pressure to give a copious yellow solid. This was recrystallized (95% EtOH) to yield 4.7 g (64%) of yellow needles: mp 84–86 °C; NMR (acetone-d_6) δ 8.15 (d, 1 H, CH=C*H*NO_2, J = 14 Hz), 7.55 (d, 1 H, C*H*=CHNO_2, J = 14 Hz), 7.45 (d, 1 H, aromatic, J = 8 Hz), 6.85 (d, 1 H, aromatic, J = 8 Hz), 3.80 (s, 3 H, OCH_3), 3.73 (s, 3 H, OCH_3), 2.35 (s, 3 H, CH_3); IR (KBr) 1690, 1620, 1320, 1285 cm^{-1}. Anal. ($C_{11}H_{13}NO_4$) C, H, N.

2-Methyl-3,4-dimethoxy-6-nitro-β-nitrostyrene (16). To a stirred suspension of 2-methyl-3,4-dimethoxy-β-nitrostyrene (**14**; 4.5 g, 20.0 mmol) in 100 mL of glacial HOAc was added 25 mL of fuming HNO_3. The reaction temperature was maintained at 25 °C during the addition. The mixture was then stirred for 3 h at room temperature, after which it was poured into ice–water and a yellow solid precipitated. The solid was collected by filtration and air-dried. The crude product was recrystallized (95% EtOH) to yield 4.6 g (85%) of yellow needles: mp 112–114 °C; NMR (acetone-d_6) δ 8.25 (d, 1 H, CH=C*H*NO_2, J = 14 Hz), 7.65 (s, 1 H, aromatic), 7.35 (d, 1 H, C*H*=CHNO_2, J = 14 Hz), 3.95 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 2.30 (s, 3 H, CH_3); IR (KBr) 1690, 1330, 1270 cm^{-1}. Anal. ($C_{11}H_{12}N_2O_6$) C, H, N.

4-Methyl-5,6-dimethoxyindole (18). To a refluxing mixture of iron powder (21.4 g, 380.0 mmol) in 70 mL of glacial HOAc was added a hot solution of 2-methyl-3,4-dimethoxy-6-nitro-β-nitrostyrene (**16**; 4.0 g, 18.0 mmol) dissolved in 70 mL of glacial HOAc. The addition was controlled so as to maintain a uniform vigorous reflux. The reaction mixture was refluxed for an additional 2 h and then filtered while hot into a solution of sodium metabisulfite in 300 mL of H_2O. The filtrate and inorganic materials were extracted with $CHCl_3$. The combined extracts were dried (Na_2SO_4) and filtered, and the solvent was removed under reduced pressure to give an oil. The resulting dark oil was filtered through a 25-g silica gel column using 70:30 benzene–chloroform. The eluate was evaporated under reduced pressure to give a solid, which was recrystallized (benzene–hexane) to yield 1.7 g (37%) of desired product **18**: mp 112–114 °C; NMR ($CDCl_3$) δ 8.25 (broad, 1 H, NH), 7.05 (m, 1 H, CH=C*H*NH), 6.7 (s, 1 H, aromatic), 6.5 (m, 1 H, C*H*=CHNH), 3.80 (s, 6 H, OCH_3), 2.5 (s, 3 H, CH_3); IR (KBr) 3370, 2980, 1630, 1310 cm^{-1}. Anal. ($C_{11}H_{13}NO_2$) C, H, N.

4-Methyl-5,6-dihydroxyindole (2). To 4-methyl-5,6-dimethoxyindole (**18**; 0.2 g, 1.2 mmol) was added 3 mL of 48% HBr under an atmosphere of argon. The pale green solution was refluxed gently for 15 min. The reaction mixture was diluted with water, and the yellow hydrobromide salt of 4-methyl-5,6-dihydroxyindole precipitated. The crude HBr salt of 4-methyl-5,6-dihydroxyindole was converted to the free base by neutralization with Dowex-2 (OH^- form) in CH_3OH. The CH_3OH solution was filtered and evaporated under reduced pressure to give an oil. The oil was dissolved in H_2O and lyophilized to give 0.050 g (37%) of a yellow solid. The indole **2** was stored under argon: mp 146–147 °C dec (lit.[10] mp 146–149 °C); NMR (D_2O) δ 7.00 (m, 1 H, CH=C*H*N), 6.75 (s, 1 H, aromatic), 6.45 (m, 1 H, C*H*=CHN), 2.45 (s, 3 H, CH_3).

2,3-Diacetoxytoluene. To a mixture of NaOAc (5.0 g, 37.0 mmol) in 20 mL of Ac_2O was added 3-methylcatechol (5.0 g, 40.0 mmol). The reaction mixture was refluxed for 2 h. After cooling to room temperature, the reaction mixture was poured into ice–water. The resulting solid precipitate was recrystallized (HOAc–$CHCl_3$) to yield 7.25 g (87%) of the desired diacetate: mp 64–66 °C; NMR ($CDCl_3$) δ 7.30 (s, 3 H, aromatic), 2.45 (s, 3 H, $COCH_3$), 2.40 (s, 3 H, $COCH_3$), 2.35 (s, 3 H, CH_3); IR (KBr) 1760, 1460, 1370, 1210, 1080 cm^{-1}.

3-Methyl-4,5-dihydroxyacetophenone. A solution of 2,3-diacetoxytoluene (4.2 g, 20.0 mmol) in 8 mL of C_6H_5Cl was added to a well-stirred suspension of anhydrous $AlCl_3$ (8 g, 60.0 mmol) in 11 mL of C_6H_5Cl at such a rate that the evolution of HCl (gas) did not become too vigorous. The reaction mixture was then heated with stirring at 110 °C for 5 h and then cooled to room temperature and poured into a mixture of ice and 5.4 mL of concentrated HCl, resulting in a yellow precipitate. The precipitated product was collected by filtration and recrystallized (95% EtOH) to yield 2.98 g (71%) of the desired product: mp 139–140 °C; NMR (Me_2SO-d_6) δ 7.30 (s, 2 H, aromatic), 2.40 (s, 3 H, CH_3), 2.15 (s, 3 H, $COCH_3$); IR (KBr) 3010 (broad), 1770 cm^{-1}. Anal. ($C_9H_{10}O_3$) C, H.

3-Methyl-4,5-dimethoxyacetophenone (7). To a solution of 3-methyl-4,5-dihydroxyacetophenone (1.5 g, 9.0 mmol) in 100 mL of acetone was added K_2CO_3 (3.7 g, 27.0 mmol) and dimethyl sulfate (2.1 g, 16.2 mmol) dropwise. The synthesis of **7** was carried out under conditions similar to those described for the preparation of **6**. The crude product was distilled at 85–90 °C (2 mmHg) to yield 3.1 g (84%) of pure crystalline material: NMR ($CDCl_3$) δ 7.30 (s, 2 H, aromatic), 3.85 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), 2.45 (s, 3 H, CH_3), 2.20 (s, 3 H, $COCH_3$); IR (KBr) 1760, 1600, 1275 cm^{-1}.

3-Methyl-4,5-dimethoxybenzoic Acid (9). The preparation of 3-methyl-4,5-dimethoxybenzoic acid (**9**) from 3-methyl-4,5-dimethoxyacetophenone (**7**; 1.0 g, 5.0 mmol) was done exactly as described for the synthesis of **8**. The acid was crystallized (95% EtOH) to yield 0.74 g (74%) of pure product: mp 129–130 °C; NMR (Me_2SO-d_6) δ 7.30 (s, 2 H, aromatic), 3.84 (s, 3 H, OCH_3), 3.79 (s, 3 H, OCH_3), 2.20 (s, 3 H, CH_3); IR (KBr), 3030 (broad), 3010 (broad), 1680 cm^{-1}. Anal. ($C_{10}H_{12}O_4$) C, H.

3-Methyl-4,5-dimethoxybenzyl Alcohol (11). To a solution of $LiAlH_4$ (0.276 g, 7.6 mmol) in 100 mL of dry THF was added a solution of 3-methyl-4,5-dimethoxybenzoic acid (**9**; 0.70 g, 3.8 mmol) dissolved in 25 mL of dry THF. The reaction mixture was refluxed overnight. The product was isolated in a similar way as described for the preparation of **10**. The crude oil was crystallized (benzene-hexane) to yield 0.21 g (75%) of pure product: mp 52–54 °C; NMR ($CDCl_3$) δ 7.40 (s, 2 H, aromatic), 4.65 (s, 2 H, Ar CH_2O), 3.84 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), 2.30 (s, 3 H, CH_3); IR (KBr), 3030 (broad), 2990, 1660, 1270 cm^{-1}. Anal. ($C_{10}H_{14}O_3$) C, H.

3-Methyl-4,5-dimethoxybenzaldehyde (13). 3-Methyl-4,5-dimethoxybenzyl alcohol (**11**; 0.364 g, 2 mmol) was oxidized to the corresponding aldehyde **13** using pyridinium chlorochromate (0.647 g, 3 mmol). The reaction conditions and method of purification were similar to those described for the synthesis of **12**. The oil was recrystallized (benzene–petroleum ether) to yield 0.269 g (74%) of the desired product: mp 62–63 °C; NMR ($CDCl_3$) δ 9.9 (s, 1 H, CHO), 7.30 (s, 2 H, aromatic), 3.90 (s, 6 H, OCH_3), 2.33 (s, 3 H, CH_3); IR (salt plate) 2990, 1695, 1275 cm^{-1}. Anal. ($C_{10}H_{12}O_3$) C, H.

3-Methyl-4,5-dimethoxy-β-nitrostyrene (15). 3-Methyl-4,5-dimethoxybenzaldehyde (**13**; 3.1 g, 17.0 mmol) was converted to the corresponding β-nitrostyrene **15** using ammonium acetate (2.8 g, 35.0 mmol) and nitromethane (3.1 g, 52.0 mmol). The procedures were similar to those described for the preparation of **14**. The crude product was recrystallized (95% EtOH) to yield 2.35 g (63%) of a yellow crystalline material: mp 126–129 °C; NMR ($CDCl_3$) δ 7.95 (d, 1 H, CH=C*H*NO_2, J = 14 Hz), 7.45 (d, 1 H, C*H*=CHNO_2, J = 14 Hz), 6.95 (m, 2 H, aromatic), 3.90 (s, 3 H, OCH_3), 3.86 (s, 3 H, OCH_3), 2.25 (s, 3 H, CH_3); IR (KBR), 1690, 1320, 1275 cm^{-1}. Anal. ($C_{11}H_{13}NO_4$) C, H, N.

2-Nitro-3-methyl-4,5-dimethoxy-β-nitrostyrene (17). 3-Methyl-4,5-dimethoxy-β-nitrostyrene (**15**; 2.2 g, 10.0 mmol) was nitrated to give the β-nitrostyrene derivative **17** using fuming nitric acid. The preparation was in a manner similar to that described for the synthesis of **16**. The product was crystallized (95% ethanol) to yield 1.90 g (78%): mp 164–166 °C; NMR ($CDCl_3$) δ 7.95 (d, 1 H, CH=C*H*NO_2, J = 14 Hz), 7.45 (d, 1 H, C*H*=CHNO_2, J = 14 Hz), 6.90 (s, 1 H, aromatic), 3.90 (s, 3 H, OCH_3), 3.86 (s, 3 H, OCH_3), 2.25 (s, 3 H, CH_3); IR (KBr), 1690, 1330, 1270 cm^{-1}. Anal. ($C_{11}H_{12}N_2O_6$) C, H, N.

5,6-Dimethoxy-7-methylindole (19). To a refluxing mixture of iron powder (10.7 g, 190 mmol) and 35 mL of HOAc was added a hot solution of the β-nitrostyrene derivative **17** (2.0 g, 18.0 mmol) dissolved in 35 mL of HOAc. The reaction conditions and methods of purification were similar to those described for the synthesis of **18**. The crude product was recrystallized (benzene–hexane) to yield 0.60 g (32%) of the desired product: mp 76–80 °C; NMR ($CDCl_3$) δ 8.1 (broad, 1 H, NH), 7.05 (m, 1 H, CH=C*H*N), 6.95 (s, 1 H, aromatic), 6.40 (m, 1 H, C*H*=CHN), 3.80 (s, 3 H, OCH_3), 3.75 (s, 3 H, OCH_3), 2.30 (s, 3 H, CH_3); IR (KBr) 3365, 2985, 1620, 1320, 1280 cm^{-1}. Anal. ($C_{11}H_{13}NO_2$) C, H, N.

5,6-Dihydroxy-7-methylindole (3). To 5,6-dimethoxy-6-methylindole (**19**; 0.1 g, 0.6 mmol) was added 1.5 mL of 48% HBr under an atmosphere of argon. The reaction conditions and methods of purification were in a similar manner to that described

for the synthesis of compound **2**. The compound was isolated by lyophilization to give 0.0234 g (23%) of a yellow solid. The indole **3** was immediately stored under argon: mp 107–109 °C dec (lit.[10] mp 108–109 °C); NMR (D_2O) δ 7.05 (m, 1 H, CH=C*H*N), 6.90 (s, 1 H, aromatic), 6.45 (m, 1 H, C*H*=CHN), 2.35 (s, 3 H, CH_3).

5,6-Dihydroxyindole (4). 5,6-Bis(benzyloxy)indole was debenzylated in a manner similar to that described by Benigni and Minnis.[25]

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Novel Dimeric Derivatives of Leucomycins and Tylosin, Sixteen-Membered Macrolides

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The reductive amination of an aldehyde group on the aglycon moiety of leucomycins A_3 and A_5 and tylosin with sodium cyanoborohydride in the presence of $NH(CH_3)_2$ or NH_2CH_3 afforded the corresponding amine derivative. The use of NH_3 as an amine source in the reduction of leucomycin A_3 and tylosin afforded a novel dimeric derivative, 18,18′-dideoxo-18,18′-iminodileucomycin A_3 and 20,20′-dideoxo-20,20′-iminoditylosin, respectively. The structures of the dimers were elucidated by field desorption mass spectral analysis. The dimeric derivative of tylosin possesses considerable antibacterial activity. The binding activity of the dimer for *Escherchia coli* ribosome was approximately the same as for tylosin.

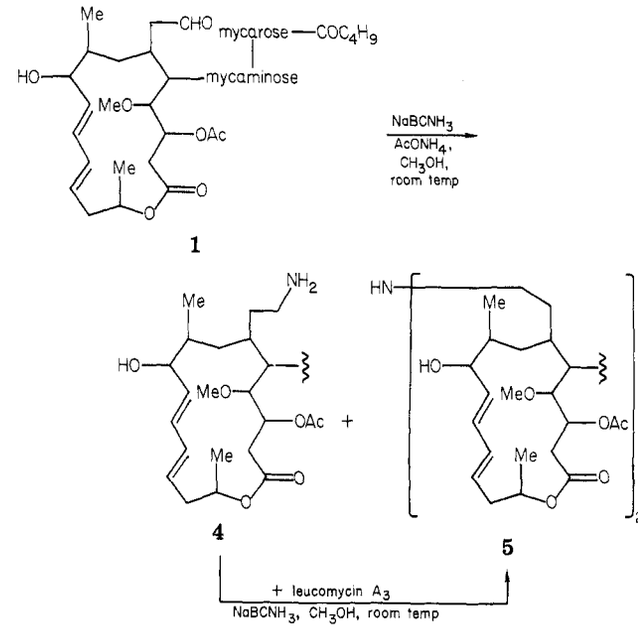
In previous structure–activity correlations of 16-membered macrolide antibiotics, leucomycins, carbomycins, tylosin, and chalcomycin, it was revealed that the presence of an aldehyde group on the lactone ring and a dimethylamino group on the mycaminose moiety may be important for antimicrobial activity.[1] We have also found that when a carbonyl group at the 9 position of the lactone ring is present, an aldehyde group at the 18 position is not necessary for antimicrobial activity. Therefore, we have focused on the modification of the aldehyde group on the aglycon moiety of leucomycins and tylosin. In this paper we describe the reductive amination of an aldehyde group and the structure of a dimer of the amine derivative coupled together through an amino function attached to the methylenic group at the 18 position of the aglycon moiety. In addition, antimicrobial activity (MIC) and binding to ribosomes were evaluated.

Results and Discussion

The reductive amination of leucomycins and tylosin was carried out according to the procedure of Jacquensy et al.[2] Reduction of leucomycin A_3 (**1**) with sodium cyanoborohydride and dimethylamine hydrochloride gave 18-deoxo-18-(dimethylamino)leucomycin A_3 (**2**).

The reductive amination of **1** with sodium cyanoborohydride and methylamine hydrochloride afforded 18-deoxo-18-(methylamino)leucomycin A_3. The use of ammonium acetate as an amine source gave three products differing from those obtained from the reactions described above. Three compounds, 18-amino-18-deoxoleucomycin A_3 (**4**), 18,18′-dideoxo-18,18′-iminodileucomycin A_3 (**5**), and 18-deoxoleucomycin A_3 (**6**), can also be obtained by using ammonium chloride instead of ammonium acetate. Their IR spectra showed the lack of an absorption band due to an aldehyde group (ν_{CH} 2920 and 2720 cm^{-1}). The structure

Scheme I



of **4** was easily assigned from the mass spectral data.

Elemental analysis of compound **5** gave a compositional formula, $C_{84}H_{141}N_3O_{28}\cdot H_2O$, suggesting the possibility of the dimerized structure of **1**. The ^{13}C chemical-shift values of **5** are approximate to those of **4**, except for that of each C-18 methylenic carbon at δ 38.7 and 47.1 in **4** and **5**, respectively. The field desorption (FD) mass spectrum of **5** showed a distinct molecular ion peak at m/z 1639 ($C_{84}H_{141}N_3O_{28}$) and a fragment peak, m/z 827, due to the aglycon moiety involving the di-*N*,*N*-ethylenic amino group, being the symmetric center in the molecule of **5**, clearly demonstrating that compound **5** has the structure of two molecules of leucomycin A_3 (**1**) condensed through an aminomethyl group introduced at the 18 position by amination. Compound **5** was also obtained by the con-

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