(2) G. Shtacker, M. Erez, and S. Cohen, J. Med. Chem., 16, 516 (1973).
(3) J. Augstein, D. A. Cox, A. L. Ham, P. R. Leeming, and M. Snavey, J. Med. Chem., 16, 1245 (1973).
(4) A. F. Crowther, D. J. Gilman, B. J. McLaughlin, L. H. Smith, R. W. Turner, and T. M. Wood, J. Med. Chem., 12, 638 (1969).
(5) C. B. Gairaud and G. R. Lappin, J. Org. Chem., 18, 1 (1953).
(6) F. A. Ramirez and A. Burger, J. Amer. Chem. Soc., 72, 2781 (1950).
(7) R. F. Meyer, C. D. Stratton, S. G. Hastings, and R. M. Corey, J. Med. Chem., 16, 1113 (1973).
(8) R. F. Furchgott, Ann. N. Y. Acad. Sci., 139, 553 (1967).
(9) A. F. Crowther and L. H. Smith, J. Med. Chem., 11, 1009 (1968).
(10) Chem. Abstr., 66, 46214p (1967).
(11) O. Stephenson, J. Chem. Soc., 1571 (1954).

Catechol O-Methyltransferase. 6. Affinity Labeling with N-Haloacetyl-3,5-dimethoxy-4-hydroxyphenylalkylamines

Ronald T. Borchardt*,† and Dhiren R. Thakker

Department of Biochemistry, McCollum Laboratories, University of Kansas, Lawrence, Kansas 66044. *Received September 23, 1974*

Several N-acyl-3,5-dimethoxy-4-hydroxyphenylalkylamines have been synthesized and evaluated for their ability to inactivate catechol O-methyltransferase (COMT). N-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine was found to rapidly and irreversibly inactivate this enzyme. The corresponding N-bromoacetyl derivative also produced inactivation of COMT but at a slower rate than the N-iodoacetyl derivative. The N-acetyl and N-fumaryl derivatives were completely inactive. 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 proximity of the amino acid residue being modified relative to the site which binds the aromatic portion of these inhibitors was determined using N-iodoacetylphenylaklylamines of varying chain length. The number of methylene carbons separating the aromatic ring and the iodoacetamide moiety in these inhibitors did not greatly influence the binding to COMT nor did it affect how rapidly the enzyme was inactivated. From these observations it was concluded that the amino acid moiety being modified by this class of affinity labeling reagents must be relatively close to or part of the site which binds the aromatic region of these inhibitors.

As part of our continuing studies of the enzyme catechol O-methyltransferase (COMT, E.C. 2.1.1.6), we have attempted to develop affinity labeling reagents which could be used to elucidate the relationship between the chemical structure and enzymatic function of this enzyme. Nikodejevic, *et al.*,[1] have previously observed that 3,5-dimethoxy-4-hydroxyphenylethylamine has affinity for the active site of COMT and is a reversible dead-end inhibitor of this enzyme. Therefore, one of our approaches to affinity labeling this enzyme has involved the preparation of various chemically reactive derivatives of 3,5-dimethoxy-4-hydroxyphenylalkylamines. These derivatives should have an affinity for COMT and, in addition, they should be capable of reacting to form a covalent bond with any nucleophiles present at or near the binding site, thereby producing modified amino acids readily amenable to isolation and identification. Since iodoacetamides are capable of alkylating histidine, cysteine, methionine, and lysine residues of proteins,[2,3] we initially prepared N-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (4) and found that it rapidly inactivates COMT. Our preliminary studies[4] suggested that this analog indeed serves as an affinity labeling reagent for COMT.

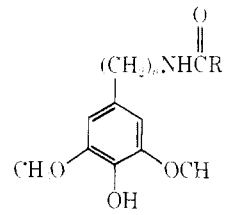
In an effort to further explore the mechanism and specificity of this interaction, we have synthesized a series of N-acyl-3,5-dimethoxy-4-hydroxyphenylalkylamines (Chart I) and studied their interaction with COMT. The objectives of this study were (1) to determine the reactivity of the amino acid residue being modified, and (2) to determine the proximity of this residue to the binding site. The present paper reports the results of this investigation.

Experimental Section

Melting points were obtained on a calibrated Thomas-Hoover Uni-melt and were corrected. Microanalyses were conducted on a F & M Model 185 C, H, N analyzer. The University of Kansas, Lawrence, Kan., and the Microanalytical Laboratory, National Institutes of Health, Bethesda, Md. 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 Varian Associates Model T-60 spectrophotometer (TMS), and uv data on a Cary Model 14 spectrophotometer. Scintillation counting was done on a Beckman LS-150 scintillation counter. Tlc were run on Analtech silica gel GF (250 μ). Spots were detected by visual examination under uv light and/or ninhydrin for compounds containing amino moieties.

† Established Investigator of the American Heart Association.

Chart I. Derivatives of 3,5-Dimethoxy-4-hydroxyphenylalkylamines Synthesized as Potential Affinity Labeling Reagents for COMT



Compd	n	R
1	2	$-CH_3$
2	2	$-CH{=}CHCO_2Et$ (trans)
3	2	$-CH_2Br$
4	2	$-CH_2I$
5	3	$-CH_2I$
6	1	$-CH_2I$

Materials. SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored at −20°F. SAM iodide (Sigma) was stored as a 0.01 *M* aqueous stock solution. Phosphate buffers were prepared as 0.5 *M* stock solutions.

COMT Isolation and Assay. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) according to the methods previously described.[4,5] The enzyme was purified through the calcium phosphate gel step resulting in a preparation which contained 1.34 mg of protein per milliliter with a specific activity of 47.8 nmol of product/mg of protein/min with 3,4-dihydroxyben-

zoate as a substrate. The enzyme activity was determined using *S*-adenosylmethionine-*methyl*-^{14}C and 3,4-dihydroxybenzoate as substrates according to a previously described radiochemical procedure.[5] Processing of enzyme kinetic data was accomplished as previously described.[5]

3,5-Dimethoxy-4-benzyloxybenzaldehyde (8). To 3,5-dimethoxy-4-hydroxybenzaldehyde[6] (7, 5.0 g, 27.5 mmol) in 350 ml of MeOH were added anhydrous K_2CO_3 (4.93 g, 35.8 mmol) and benzyl chloride (4.5 g, 34.8 mmol). The reaction mixture was refluxed for 72 hr, after which time the solvent was removed under reduced pressure. The residue was dissolved in benzene and the benzene solution was washed with H_2O, 5% NaOH solution, and saturated NaCl solution, and dried ($MgSO_4$). The solvent was removed under reduced pressure to yield a yellow oil, which was crystallized (EtOH) to yield 4.94 g (66%), mp 61–62°. *Anal.* ($C_{16}H_{16}O_4$) C, H.

3,5-Dimethoxy-4-benzyloxy-β-nitrostyrene (9). Aldehyde 8 (4.08 g, 15.0 mmol) was dissolved in 150 ml of MeOH to which was added nitromethane (1.0 g, 16.5 mmol). The reaction mixture was cooled to 10–15° and NaOH (0.66 g, 16.5 mmol) dissolved in 4 ml of H_2O was added dropwise at such a rate that the temperature did not rise above 15°. When the addition was complete, the reaction mixture was poured slowly into 15 ml of 6 *N* HCl. The resulting product was collected by filtration, washed with cold MeOH, and crystallized (EtOAc–MeOH) to afford 4.14 g (88%) of pure product, mp 131–132°. *Anal.* ($C_{17}H_{17}NO_5$) C, H, N.

3,5-Dimethoxy-4-hydroxyphenylethylamine Hydrochloride (10). A general method[7] for reduction of β-nitrostyrenes to the corresponding phenylethylamines with $LiAlH_4$ was used in the preparation of 10. To $LiAlH_4$ (0.938 g, 25 mmol) in 100 ml of dry THF was added dropwise a solution of 3,5-dimethoxy-4-benzyloxy-β-nitrostyrene (9, 1.5 g, 4.76 mmol) dissolved in 100 ml of dry THF. The addition took approximately 1 hr, after which time the reaction mixture was refluxed for 3 hr and then cooled in ice bath, and the excess $LiAlH_4$ was decomposed by adding "wet" Et_2O followed by H_2O. The precipitates were filtered and extracted with $CHCl_3$–EtOAc to recover any occluded product. The filtrate and the $CHCl_3$–EtOAc washes were combined and concentrated under reduced pressure to yield a yellow oil. The oil was dissolved in Et_2O; the Et_2O solution was dried ($MgSO_4$) and filtered. Dry HCl gas was bubbled into the cooled Et_2O solution and the resulting precipitate collected by filtration to yield 0.70 g (45%) of 3,5-dimethoxy-4-benzyloxyphenylethylamine hydrochloride: mp 154–155°; nmr ($CDCl_3$) δ 7.40 (m, 5 H, aromatic), 6.40 (s, 2 H, aromatic), 4.90 (s, 2 H, $-OCH_2Ph$), 3.75 (s, 6 H, $-OCH_3$), 3.05 (m, 4 H, $-CH_2CH_2NH_3^+$).

The 3,5-dimethoxy-4-benzyloxyphenylethylamine hydrochloride (0.5 g, 1.55 mmol) was dissolved in 50 ml of MeOH to which was added 0.05 g of 5% palladium on carbon. The reaction was hydrogenated at 25° under 2 atm of pressure for 2 hr. The catalyst was removed by filtration and the MeOH removed under reduced pressure. The residue was crystallized (MeOH–Et_2O) to yield 0.34 g (95%) of 3,5-dimethoxy-4-hydroxyphenylethylamine hydrochloride (10), mp 254–255° [lit.[8] mp 258–259° (prepared by a different route)]. *Anal.* ($C_{10}H_{16}ClNO_3$) C, H, N.

***N*-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (4).** To a solution of 3,5-dimethoxy-4-hydroxyphenylethylamine (10, 0.245 g, 1.24 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC) (0.256 g, 1.24 mmol) in 150 ml of dry acetonitrile was added iodoacetic acid (0.231 g, 1.24 mmol). The solution was stirred for 72 hr at ambient temperature, after which time the reaction mixture was filtered and the solvent removed under reduced pressure. The crude product was purified using thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 1:9). The product with an R_f of 0.44 was extracted from the silica gel with EtOAc and the solvent was removed under reduced pressure. The residual oil was crystallized ($CHCl_3$–hexane) to yield 0.19 g (42%) of 4: mp 122–123.5°; nmr ($CDCl_3$) δ 6.40 (s, 2 H, aromatic), 6.20 (m, 1 H, $-NHCO-$), 5.4 (s, 1 H, phenol), 3.90 (s, 6 H, $-OCH_3$), 3.65 (s, 2 H, $-COCH_2I$), 3.45 (apparent q, 2 H, $-CH_2NH-$, J = 7 Hz), 2.75 (t, 2 H, $ArCH_2-$, J = 7 Hz). *Anal.* ($C_{12}H_{16}INO_4$) C, H, N.

***N*-Bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (3).** 3,5-Dimethoxy-4-hydroxyphenylethylamine (10, 0.29 g, 1.46 mmol) was treated with bromoacetic acid (0.203 g, 1.46 mmol) and DCC (0.206 g, 1.46 mmol) in acetonitrile under conditions similar to those described for the preparation of 4. The crude product was purified using thick-layer chromatography on silica gel (hexane–2-propanol–$CHCl_3$, 1:7.5:91.5) and crystallized ($CHCl_3$–hexane–EtOAc) to afford 0.185 g (40%) of product, mp 104–105°. *Anal.* ($C_{12}H_{16}BrNO_4$) C, H, N.

***N*-Fumaryl-3,5-dimethoxy-4-hydroxyphenylethylamine Ethyl Ester (2).** 3,5-Dimethoxy-4-hydroxyphenylethylamine (10, 0.266 g, 1.35 mmol) was treated with fumaric acid monoethyl ester (0.195 g, 1.35 mmol) and DCC (0.28 g, 1.35 mmol) in acetonitrile under conditions similar to those described for the preparation of 4. After removal of the solvent, the oil was purified by preparative thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 1:9) and the product was isolated from the band with R_f 0.44. Crystallization ($CHCl_3$–hexane) afforded 0.09 g (21%) of pure product, mp 124–125°. *Anal.* ($C_{16}H_{24}NO_6$) C, H, N.

***N*-Acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (1).** 3,5-Dimethoxy-4-hydroxyphenylethylamine (10, 0.06 g, 0.30 mmol) was dissolved in 5 ml of CH_3OH. Ac_2O (0.045 g, 0.44 mmol) was added and the reaction mixture was stirred at ambient temperature for 2 hr. The solvent and excess Ac_2O were removed under reduced pressure and the residual oil was crystallized ($CHCl_3$–EtOAc–hexane) to yield 0.053 g (73%), mp 97–98°. *Anal.* ($C_{12}H_{17}NO_4$) C, H, N.

3,5-Dimethoxy-4-benzyloxy-β-carboxycinnamonitrile (11). To a solution of 3,5-dimethoxy-4-benzyloxybenzaldelhyde (8, 2.1 g, 7.72 mmol) in 100 ml of toluene was added cyanoacetic acid (0.656 g, 7.72 mmol), ammonium acetate (0.022 g), and pyridine (0.8 ml, 10.1 mmol). The reaction mixture was refluxed for 48 hr using a Dean-Stark trap to collect the H_2O which formed. The solvent was removed under reduced pressure; the residue was dissolved in $CHCl_3$ and filtered. Crystallization ($CHCl_3$–hexane) yielded 2.0 g (76.4%): mp 140–141°; nmr ($CDCl_3$) δ 9.05 (s, 1 H, –COOH), 8.15 (s, 1 H, ArCH=C–), 7.3 (m, 7 H, aromatic), 5.1 (s, 2 H, $-OCH_2Ph$), 3.85 (s, 6 H, $-OCH_3$). Ir, nmr, and uv data were consistent with the proposed structure, but an accurate C, H, and N analysis could not be obtained.

3,5-Dimethoxy-4-hydroxyphenylpropylamine Hydrochloride (14). The carboxycinnamonitrile 11 (2.0 g, 5.9 mmol) was dissolved in 15 ml of pyridine and the reaction mixture was refluxed for 2 days after which time the pyridine was removed under reduced pressure. The residual oil was purified by column chromatography on silica gel (EtOAc–$CHCl_3$, 1:1) yielding 1.5 g (86%) of a light green oil, which appeared to be a mixture of the *cis*- and *trans*-3,5-dimethoxy-4-benzyloxycinnamonitrile (12): nmr ($CDCl_3$) δ 7.45 (m, 6 H, aromatic and ArCH=C<), 6.65 (s, 2 H, aromatic), 5.75 [d, 0.7 H, –C=CHCN (trans), J = 16 Hz], 5.35 [d, 0.3 H, –C=CHCN (cis), J = 12 Hz], 5.10 (s, 2 H, $-OCH_2Ph$), 3.85 (2 s, 6 H, $-OCH_3$). The material was pure enough to be utilized in subsequent steps.

The mixture of *cis*- and *trans*-cinnamonitrile 12 (1.4 g, 4.75 mmol) was dissolved in 75 ml of absolute EtOH to which was added 0.2 g of 10% palladium on carbon. The reaction mixture was hydrogenated at 25° under 1 atm of pressure for 2 hr. The catalyst was removed by filtration and the solvent removed under reduced pressure to yield 0.7 g (71%) of an oil: nmr ($CDCl_3$) δ 6.45 (s, 2 H, aromatic), 3.85 (s, 6 H, $-OCH_3$) 2.70 (m, 4 H, $ArCH_2CH_2$); ir (KBr) 3400 and 2200 cm^{-1}. The spectral evidence supported the structural assignment of 3,5-dimethoxy-4-hydroxyphenylethylnitrile (13). This product was used directly for the synthesis of the corresponding amine 14 without further purification.

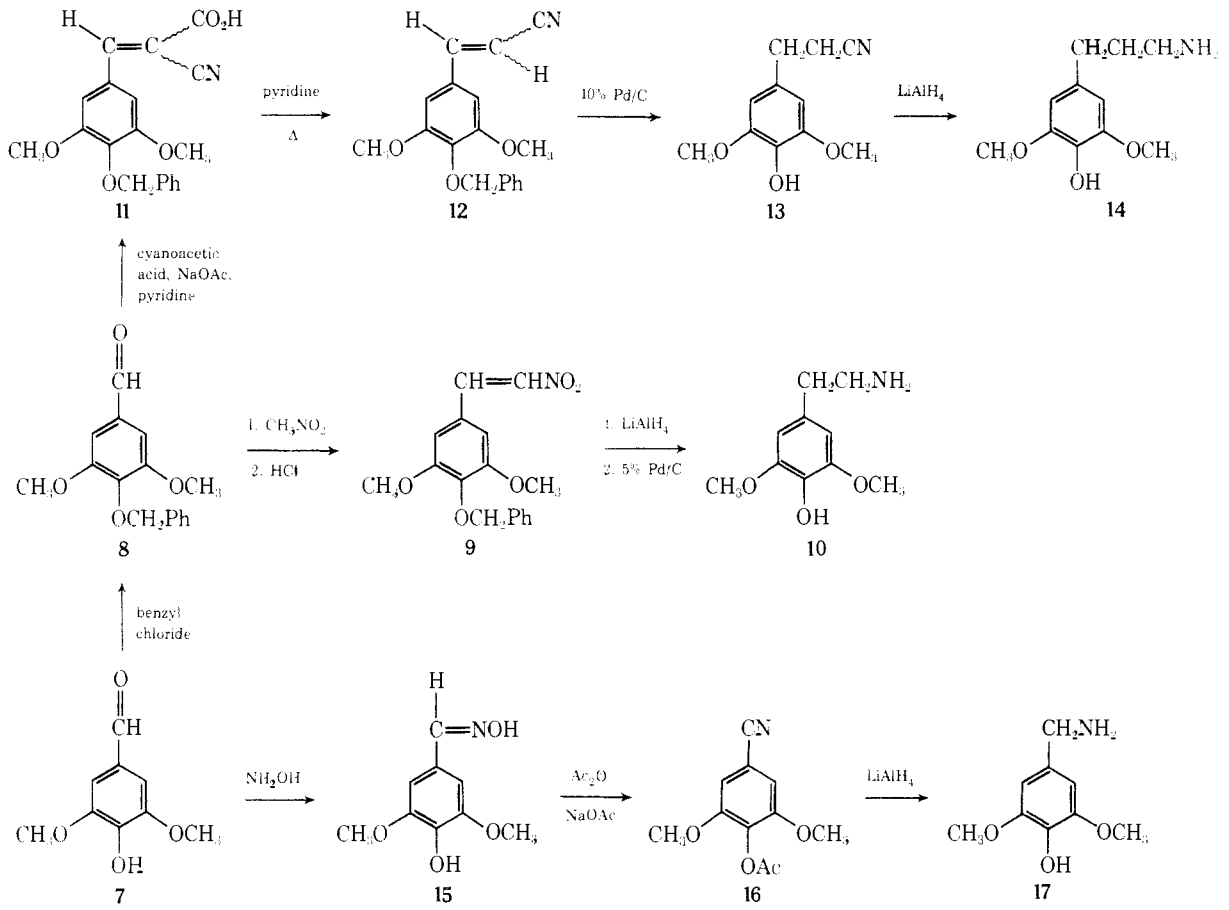
The nitrile 13 (0.8 g, 2.69 mmol) was dissolved in dry Et_2O–THF (50:1) and reduced using $LiAlH_4$ (0.5 g, 12.8 mmol) by a procedure similar to that used for the synthesis of 10. The amine 14 was converted to the hydrochloride salt and was precipitated from methanol by adding Et_2O to obtain 0.15 g (22.5%), mp 200.5–202°. *Anal.* ($C_{11}H_{18}ClNO_3$) C, H, N.

***N*-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylpropylamine (5).** 3,5-Dimethoxy-4-hydroxyphenylpropylamine (14, 0.089 g, 0.37 mmol) was treated with iodoacetic acid (0.064 g, 0.37 mmol) and DCC (0.07 g, 0.37 mmol) in acetonitrile using conditions similar to those described for the synthesis of 4. The product was purified by preparative thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 7:93) and crystallized ($CHCl_3$–hexane) to yield 0.045 g (32%) of 5, mp 98–99.5°. *Anal.* ($C_{13}H_{18}INO_4$) C, H, N.

3,5-Dimethoxy-4-hydroxybenzaldoxime (15). To a solution of 3,5-dimethoxy-4-hydroxybenzaldehyde (7, 1.0 g, 5.46 mmol) in 75 ml of EtOH was added hydroxylamine hydrochloride (1.15 g, 16.6 mmol) and sodium acetate (1.15 g, 14.0 mmol), which were dissolved in 10 ml of water. The reaction mixture was refluxed for 3 hr after which time the solvent was removed and the residual oil was crystallized (EtOH–H_2O) to afford 0.95 g (89%) of the desired product, mp 128–129°. *Anal.* ($C_9H_{11}NO_4$) C, H, N.

3,5-Dimethoxy-4-acetoxyphenylnitrile (16). Oxime 15 (0.9 g, 4.57 mmol) was dissolved in 5 ml of Ac_2O to which was added 5 mg of sodium acetate. The reaction mixture was refluxed for 18 hr after which time the excess Ac_2O was removed under reduced

Scheme I



pressure and the solid residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was filtered to remove sodium acetate and the product was crystallized ($CHCl_3$–EtOAc–hexane) to afford 0.87 g (87%), mp 138–140°. *Anal.* ($C_{11}H_{11}NO_4$) C, H, N.

***N*-Iodoacetyl-3,5-dimethoxy-4-hydroxybenzylamine (6).** To a solution of $LiAlH_4$ (0.47 g, 12 mmol) in 40 ml of dry Et_2O was added, over a 90-min period, the nitrile 16 (0.75 g, 3.4 mmol) dissolved in 120 ml of dry Et_2O. The reaction mixture was refluxed for 2 hr after which time the excess $LiAlH_4$ was decomposed and the product was isolated as the hydrochloride salt as previously described for the preparation of **10**. The hydrochloride salt of 17 was then dissolved in H_2O and the aqueous solution extracted with $CHCl_3$ and Et_2O. The aqueous solution was neutralized with dilute NH_4OH and extracted with $CHCl_3$. The $CHCl_3$ solution was dried ($MgSO_4$) and filtered and the solvent removed to yield 0.075 g (12%) of an oil, which was the desired amine 17: nmr ($CDCl_3$) δ 6.60 (m, 2 H, aromatic), 3.90 (m. 11 H. $ArCH_2$–, $-OCH_3$, $-NH_2$, $-OH$); a drop of D_2O reduces integration of δ 3.90 peak to 8 protons; ir (KBr) 3500 and 3100 cm^{-1}. Compound 17 was sufficiently pure and was carried onto the next step.

3,5-Dimethoxy-4-hydroxybenzylamine (17, 0.07 g, 0.38 mmol) was treated with iodoacetic acid (0.064 g, 0.38 mmol) and DCC (0.078 g, 0.38 mmol) in acetonitrile under conditions similar to those described for the preparation of 4. The product was purified by preparative thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 7:93) and crystallized ($CHCl_3$–hexane) to yield 0.013 g (10%): mp 145–146.5°; nmr ($CDCl_3$) δ 6.55 (s, 2 H, aromatic), 6.3 (m, 2 H, -NHCO- and phenol), 4.4 (d, 2 H, $ArCH_2NH$–, J = 7 Hz), 3.9 (s, 6 H, $-OCH_3$), 3.75 (s, 2 H, $-COCH_2I$). *Anal.* ($C_{11}H_{14}INO_4$) C, H, N.

COMT Inactivation Experiments. A typical COMT inactivation experiment consisted of the following components (in μmol) added in this sequence: water, so that the final volume was 3.20 ml; magnesium chloride (4.80); phosphate buffer, pH 7.60 (400); inhibitor (variable); and 541 μg of 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 3,4-dihydroxybenzoate (DHB, 0.50 μmol) to a final volume of 0.25 ml. The assay mixtures were incubated for 5 min at 37° and the reaction was stopped by addition of 0.10 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 the appropriate DHB blank. The per cent activity remaining at any given time was calculated relative to zero-time activity. The pseudo-first-order kinetic constants of inactivation, K_{app}, were calculated from the slope of plots of log of per cent of activity remaining *vs.* time.[4,9]

Results and Discussion

Chemistry. The *N*-acyl-3,5-dimethoxy-4-hydroxyphenylalkylamines **1–6**, which were synthesized for this study, are listed in Chart I. These potential affinity labeling reagents and their synthetic intermediates were characterized by several methods, including their spectral and chromatographic properties and elemental analyses. The general approach used in the synthesis of the desired compounds involved the preparation of the phenylalkylamines **10**, **14**, and **17**, followed by acylation of the amines with the appropriate acids. In Scheme I is shown the pathways used for the synthesis of the parent amines.

The synthesis of 3,5-dimethoxy-4-hydroxyphenylethylamine (**10**) was modeled after a general procedure for the preparation of phenylethylamines[7,8] and involved initially the reaction of benzaldehyde **8** with nitromethane to yield the β-nitrostyrene **9**. Reduction of **9** with $LiAlH_4$ and removal of the benzyl ether protecting group by catalytic hydrogenation afforded the desired amine **10**, which was isolated as the hydrochloride salt.

The synthesis of 3,5-dimethoxy-4-hydroxyphenylpropylamine (**14**) was achieved by the initial reaction of benzaldehyde **8** with cyanoacetic acid to yield a mixture of the *cis*- and *trans*-β-carboxycinnamonitrile **11**, which was de-

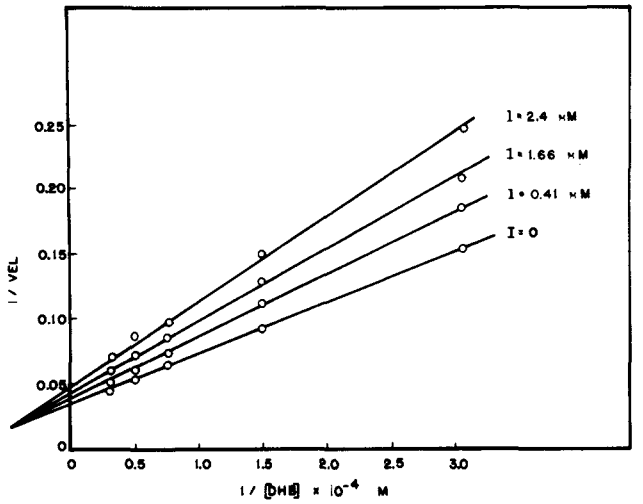
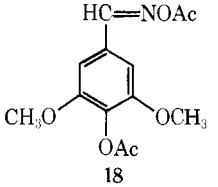


Figure 1. Inhibition of COMT by *N*-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (1). Reciprocal plots with 3,4-dihydroxybenzoate as the variable substrate. Assay conditions outlined in the Experimental Section except SAM concentration, 1.0 m*M*. Vel = nmol of product/mg of protein/min.

carboxylated by heating in pyridine to the cinnamonitrile **12**. The nmr spectrum of **12** indicated a mixture of approximately 70% of the trans isomer and 30% of the cis isomer. Catalytic hydrogenation (10% palladium on carbon) of cinnamonitrile **12** resulted in reduction of the double bond and removal of the benzyl ether protecting group and afforded a good yield of 3,5-dimethoxy-4-hydroxyphenylethylnitrile (**13**). Reduction of the nitrile **13** with $LiAlH_4$ yielded the desired phenylpropylamine **14**, which was isolated as the hydrochloride salt.

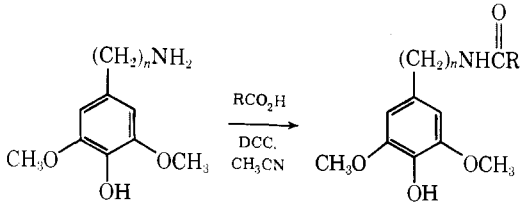
3,5-Dimethoxy-4-hydroxybenzylamine (**17**) was prepared by reduction of the corresponding nitrile **16** using $LiAlH_4$. The nitrile **16** was prepared by heating the oxime **15** in acetic anhydride containing a trace amount of sodium acetate. The general procedure[10] for conversion of an oxime to a nitrile involves heating the appropriate oxime in acetic anhydride alone. However, when the oxime **15** was heated in only acetic anhydride, a mixture of the nitrile **16** and the *O*-acetyloxime **18** was obtained. Apparently the *O*-acetyloxime **18** is an intermediate in the formation of the desired nitrile, since addition of a trace amount of sodium acetate to the reaction mixture resulted in complete conversion to the nitrile **16**.



18

The phenylalkylamines **10**, **14**, and **17** were converted to the appropriate *N*-acylphenylalkylamines **1–6** by the general reaction shown in Scheme II. This reaction involved

Scheme II



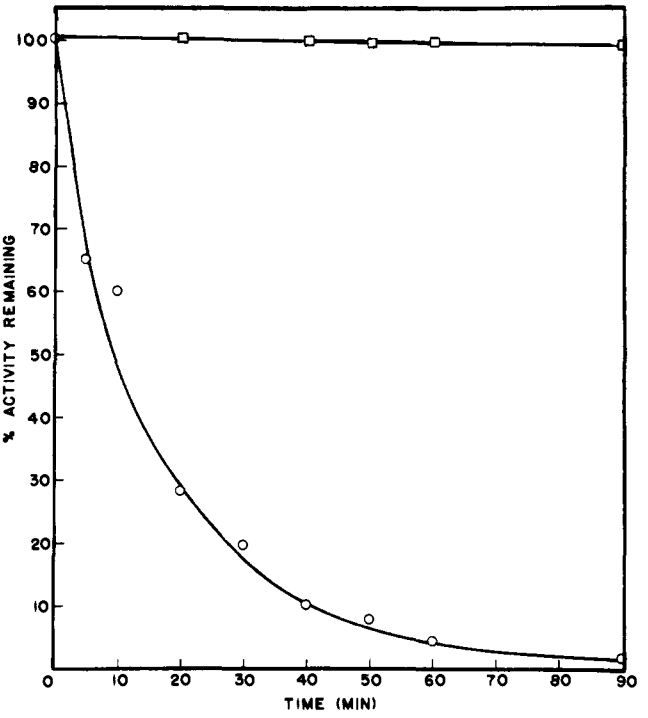


Figure 2. Effect of *N*-acyl-3,5-dimethoxy-4-hydroxyphenylethylamines 1 and 4 on COMT activity. Purified COMT was preincubated with inhibitors and the activity of the enzyme was monitored as a function of time as described in the Experimental Section. Inhibitor concentration, 0.415 m*M*. O—O, *N*-iodoacetyl derivative 4; □—□, *N*-acetyl derivative 1.

the *N*,*N*′-dicyclohexylcarbodiimide catalyzed condensation of the appropriate phenylalkylamines and carboxylic acids. In all cases the major products were the desired *N*-acylphenylalkylamines **1–6** and not the corresponding *O*-acyl derivatives.

COMT Inactivation Studies. In an effort to establish whether these *N*-acyl-3,5-dimethoxy-4-hydroxyphenylalkylamines **1–6** have an inherent affinity for the active site of COMT and initially form a reversible complex with the enzyme, we have determined the kinetic patterns for inhibition of COMT by *N*-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1**). This analog does not have a chemically reactive moiety; therefore, its interaction with the enzyme should be of a reversible nature. As shown in Figure 1, when 3,4-dihydroxybenzoate was the variable substrate and compound **1** the inhibitor, a linear noncompetitive pattern of inhibition of COMT was observed. From these data a K_{il} = 3.58 ± 0.69 m*M* and a K_{is} = 8.22 ± 4.19 m*M* were calculated. When *S*-adenosylmethionine was the variable substrate, an uncompetitive pattern of inhibition was observed. These kinetic patterns are similar to those observed previously for various other reversible dead-end type inhibitors of COMT.[5]

In Figure 2 is shown the effects on COMT activity when the enzyme was preincubated with the N-acetyl derivative **1** or the *N*-iodoacetyl derivative **4**. From these data it is apparent that the *N*-acetyl derivative **1** does not cause inactivation of COMT as would be expected; however, the *N*-iodoacetyl derivative produces a dramatic decrease in enzyme activity as a function of time. This inactivation of COMT by the *N*-iodoacetyl derivative **4** is completely irreversible, since enzyme activity cannot be recovered after dialysis or gel filtration on Sephadex G-25.

In an effort to determine the reactivity of the amino acid group being modified in this reaction, the time courses for inactivation of this enzyme by various *N*-acylphenylethylamines (**1–4**) were determined and the results are

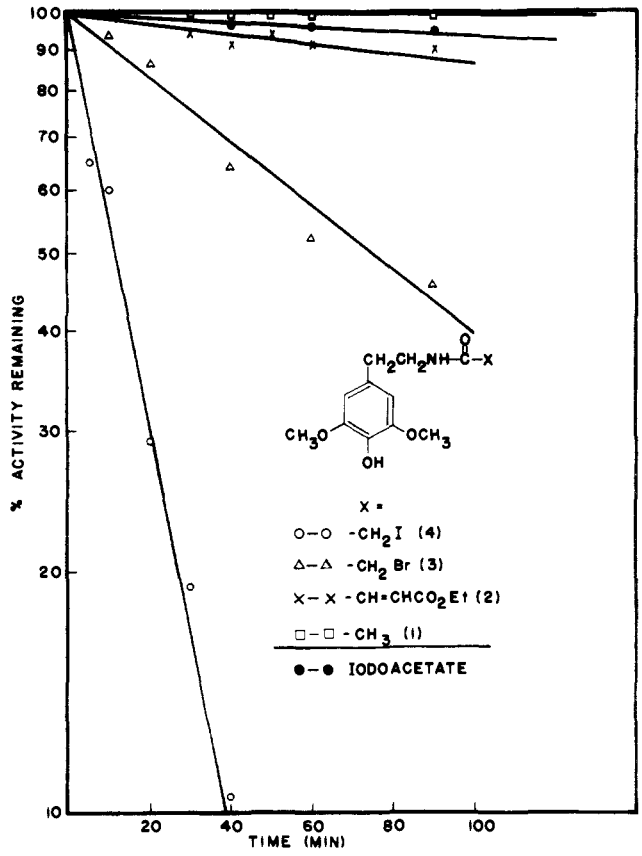


Figure 3. Effect of *N*-acyl-3,5-dimethoxy-4-hydroxyphenylethylamines 1–4 and iodoacetate on COMT activity. Enzyme activity remaining after the appropriate preincubation time was determined as outlined in the Experimental Section. Inhibitor concentration in all cases was 0.415 m*M*.

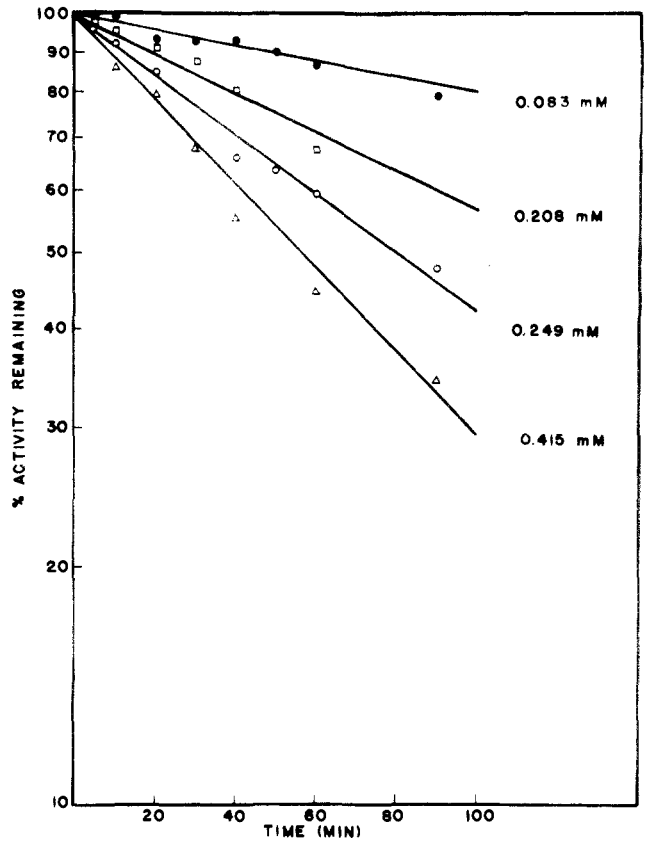


Figure 4. Effect of *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (3) concentration on the rate of inactivation of COMT. Inhibitor concentrations noted were those present in the preincubation mixture (see Experimental Section). The pseudo-first-order rate constants of inactivation, K_{app}, were calculated from the slopes for each concentration of inhibitor.

shown in Figure 3. Similar to our earlier observation with the *N*-iodoacetyl derivative 4,[4] a linear relationship between log of the per cent activity remaining *vs.* preincubation time was observed when COMT was preincubated with the *N*-bromoacetyl derivative 3. This linearity is characteristic of a first-order process with respect to the active enzyme remaining, which can be defined by an apparent first-order rate constant, K_{app}. Since iodide is a better leaving group than bromide in a nucleophilic reaction, it was not surprising to observe that the *N*-iodoacetyl derivative 4 inactivated COMT more rapidly than the *N*-bromoacetyl derivative 3. The *N*-acetyl derivative 1, the *N*-fumaryl derivative 2, and iodoacetic acid produced little or no inactivation of this enzyme. The substantial increase in reactivity of *N*-iodoacetyl derivative 4 over iodoacetic acid is characteristic of an active-site-directed alkylating reagent.

By studying the rate of enzyme inactivation as a function of inhibitor concentration, we have shown previously that the *N*-iodoacetyl derivative 4 inactivated COMT by a unimolecular reaction within a dissociable complex rather than by a nonspecific bimolecular reaction.[4] The model for this type of inactivation is shown in eq 1 and 2, where

$$E + I \rightleftharpoons E{\cdot}I \xrightarrow{k_2} E\text{–}I \qquad (1)$$

$$1/K_{app} = K_I/k_2[I] + 1/k_2 \qquad (2)$$

E·I is the reversible complex, E–I the inactive enzyme, k_2 the first-order rate constant, and K_I the steady-state constant of inactivation (K_I = [E][I]/[E·I]). Evidence to support this type of inactivation pathway (eq 1) can be obtained if a linear relationship is observed between the reciprocal of the pseudo-first-order rate constants ($1/K_{app}$) *vs.* the reciprocal of the inhibitor concentrations (1/[I]). If this reciprocal plot is linear the kinetic constants (k_2 and K_I) can be calculated using eq 2. (See ref 9 for the derivation of this equation.)

In order to compare more accurately the reactivities of the *N*-iodoacetyl derivative 4 and *N*-bromoacetyl derivative 3, the first-order rate constants at saturation (k_2) were determined. The variation of the rate of COMT inactivation as a function of the concentration of the *N*-bromoacetyl derivative 3 is shown in Figure 4 (pH 7.60 and 37°). As predicted, the inhibition is pseudo-first order in enzyme concentration in all cases. A plot of the reciprocal of the pseudo-first-order rate constants ($1/K_{app}$) *vs.* the reciprocal of the concentration of derivative 3 (1/[I]) produced a linear relationship as shown in Figure 5. Using eq 2,[9] a steady-state constant of inactivation, K_I = 0.334 ± 0.10 m*M*, and a first-order rate constant at saturation, k_2 = 0.020 ± 0.003 min^{-1}, were calculated for derivative 3. Also shown in Figure 5 is a similar reciprocal plot for the *N*-iodoacetyl derivative 4, from which a first-order rate constant, k_2 = 0.141 ± 0.075 min^{-1}, was calculated. The first-order rate constants (k_2) observed for these *N*-haloacetyl derivatives support the greater reactivity of the *N*-iodoacetyl derivative 4, as would be predicted in an alkylation type mechanism. Furthermore, the linearity observed in the reciprocal plots for both of these derivatives provides evidence for the formation of dissociable enzyme-inhibitor complexes prior to the alkylation step.

In an attempt to determine if the amino acid residue being modified by these affinity labeling reagents was part

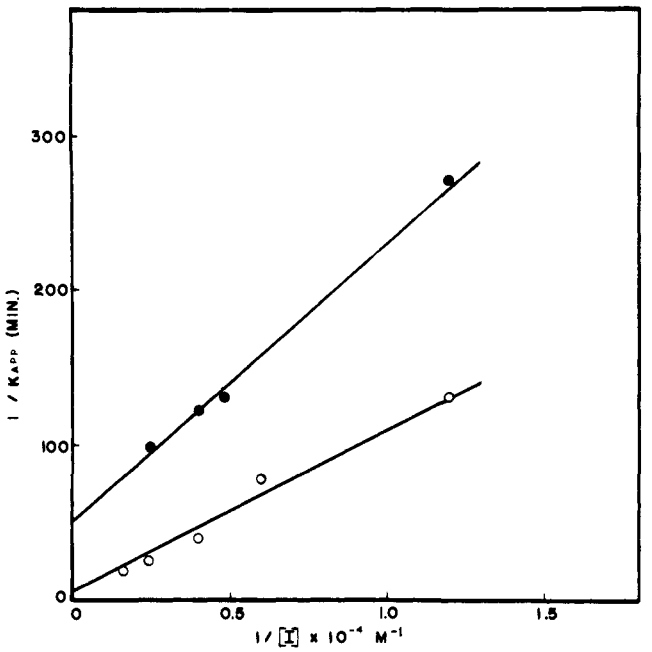


Figure 5. Double reciprocal plots of the pseudo-first-order rate constants of inactivation, K_{app}, *vs.* inhibitor concentrations: ●—●, *N*-bromoacetyl derivative 3; O—O, *N*-iodoacetyl derivative 4. Kinetic constants, k_2 and K_I, were calculated from the *Y* intecept and the slope, respectively, using the least-squares method.[4,9]

Table I. Kinetic Constants for the Inactivation of COMT by *N*-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylalkylamines 4–6

	Kinetic constants[a]	
Compd	$k_2 \pm$ SEM, min^{-1}	$K_I \pm$ SEM, m*M*
4	0.141 ± 0.075	2.54 ± 0.95
5	0.167 ± 0.075	2.05 ± 0.87
6	0.112 ± 0.052	1.65 ± 0.77

[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 2.[4,9]

of the site which binds the aromatic portion of these inhibitors or whether it was quite removed from the site and was not crucial in binding or transmethylation, several *N*-iodoacetylphenylalkylamines of varying side-chain length (4, 5, and 6) were prepared and evaluated for their ability to inactivate COMT. For each of these derivatives (4, 5, and 6) pseudo-first-order kinetics were observed for the time courses of inactivation and linear replots of the reciprocal of the pseudo-first order rate constants ($1/K_{app}$) *vs.* the reciprocal of the inhibitor concentrations ($1/[I]$) were obtained. 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 I. Since no statistically significant differences were observed in either the k_2's or K_I's for the *N*-iodoacetylphenylalkylamines 4, 5, and 6, it is our conclusion that the amino acid moiety being modified must be close to the site which binds the aromatic portion of these inhibitors. If the amino acid residue was far removed from this binding site one would expect the reactivity toward COMT to

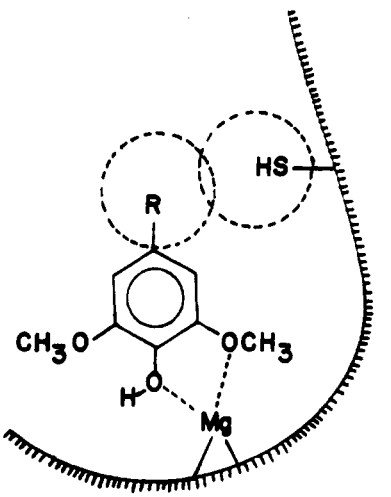


Figure 6. Proposed enzymatic binding of *N*-acyl-3,5-dimethoxy-4-hydroxyphenylalkylamines. Dotted circles are to denote proximity of the alkyl side chain of the inhibitor to the nucleophilic amino acid residue being modified.

increase with chain length, *i.e.*, be in the order of 5 > 4 > 6. However, if the amino acid moiety being modified was close to the site binding the aromatic ring, the side chain of these reagents could easily fold back to react with this nucleophilic group. In the latter case little or no difference in reactivity for 4, 5, and 6 would be expected, as was observed.

Conclusion

In the present study we have attempted to elucidate the mechanism and the specificity of COMT inactivation by *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (4). From these and earlier studies[4] we have shown that various *N*-haloacetyl-3,5-dimethoxy-4-hydroxyphenylalkylamines exhibit the general characteristics of affinity labeling reagents. 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. This conclusion was reached from a study of the kinetics of inactivation of COMT and from the observed reversible binding to COMT of the *N*-acetyl derivative 1 and of 3,5-dimethoxy-4-hydroxyphenylethylamine.[1] The inactivation of COMT by these affinity labeling reagents appears to result from alkylation of a crucial amino acid residue at or near the active site of this enzyme. The observation that the *N*-iodoacetyl derivative 4 inactivates COMT at a faster rate than the *N*-bromoacetyl derivative 3 would support an alkylation mechanism, since this would be the order of reactivity expected in a nucleophilic displacement reaction. Similar inactivations of COMT by nonspecific alkylating reagents have been observed previously.[11,12] Creveling, *et al.*,[12] have provided evidence for the presence of two nucleophilic residues at the active site of this enzyme. Because of the reactivity of these nucleophilic groups to *N*-ethylmaleimide, they are thought to be cysteine residues. Recent data from our laboratory[13] on the inactivation of COMT by 6-hydroxydopamine and its oxidation products further support the presence of important nucleophilic residues at the active site of this enzyme. An alkylation mechanism is also proposed to explain this inactivation process.

The location of the amino acid moiety which is being modified by these reagents is thought to be close to or part of the catechol binding site as is depicted in Figure 6. This conclusion was arrived at by the observation that changes in the number of methylene carbons separating

the aromatic ring and the iodoacetamide moiety in these reagents did not affect the first-order rate constants for inactivation of COMT. Also, the simple observation that modification of this amino acid moiety results in loss of enzymatic activity would imply a crucial role for this functional group in catalysis or binding. Our earlier observation[4] that inclusion of catechol substrate in the preincubation mixture protects the enzyme from inactivation by the *N*-iodoacetyl derivative 4 provides further evidence that an active site amino acid is being modified and that these compounds are specific active-site-directed alkylating reagents. Using the information obtained in this study, we are now carrying out incorporation studies and attempting to isolate and characterize the modified amino acid residues.

Acknowledgment. The authors gratefully acknowledge support of this project by a research grant from the National Institutes of Neurological Diseases and Stroke (NS-10918) and a Grant-in-Aid from the American Heart Association. Partial support was also provided by a University of Kansas General Research Grant. The excellent technical assistance of Mrs. Bi-Shia Wu is also gratefully acknowledged.

References

(1) B. Nikodejevic, S. Senoh, J. W. Daly, and C. R. Creveling, *J. Pharmacol. Exp. Ther.*, 174, 83 (1970).
(2) E. Shaw in "The Enzymes," Vol. 1, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1970, p 91.
(3) S. J. Singer, *Advan. Protein Chem.*, 22, 1 (1967).
(4) R. T. Borchardt and D. Thakker, *Biochem. Biophys. Res. Commun.*, 54, 1233 (1973).
(5) R. T. Borchardt, *J. Med. Chem.*, 16, 377, 382, 387, 581 (1973).
(6) C. F. H. Allen and G. W. Leubner in "Organic Syntheses," Collect. Vol. IV, N. Rabjohn, Ed., Wiley, New York, N.Y., 1963, p 866.
(7) F. A. Ramirez and A. Burger, *J. Amer. Chem. Soc.*, 72, 2781 (1950).
(8) F. Benington, R. D. Morin, and L. C. Clarke, *J. Amer. Chem. Soc.*, 76, 5555 (1954).
(9) P. H. Petra, *Biochemistry*, 10, 3163 (1971).
(10) J. S. Buck and W. S. Ide in "Organic Syntheses," Collect. Vol. II, A. H. Blatt, Ed., Wiley, New York, N.Y., 1943, p 622.
(11) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, 233, 702 (1958).
(12) N. D. Norris, F. McNeal, and C. R. Creveling, Abstracts of the 8th Middle Atlantic Regional Meeting of the American Chemical Society, Washington, D.C., Jan 1973, p 60.
(13) R. T. Borchardt, *Mol. Pharmacol.*, submitted for publication.

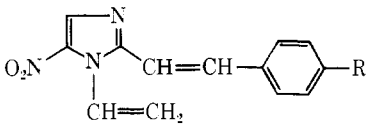
Antiparasitic Nitroimidazoles. 8. Derivatives of 2-(4-Formylstyryl)-5-nitro-1-vinylimidazole

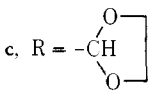
William J. Ross* and William B. Jamieson

Lilly Research Centre Limited, Erl Wood Manor, Windlesham, Surrey, England. *Received May 29, 1974*

A series of 33 thioacetals and hydrazones of 2-(4-formylstyryl)-5-nitro-1-vinylimidazole was prepared and examined for antitrypanosomal properties. The thioacetals were inactive as antitrypanosomal agents but three hydrazones derived from *N*-aminoguanidine, pyridylacetohydrazide chloride (Girard reagent P), and dimethylaminoacetohydrazide (Girard reagent D) displayed good activity against *Trypanosoma rhodesiense*.

In part 3[1] we described the antitrypanosomal properties of 2-(4-carboxystyryl)-5-nitro-1-vinylimidazole (**1a**) and a number of related compounds including the aldehyde **1b**.



1a, R = COOH
b, R = CHO
c, R = 

The latter compound was equiactive with **1a** against *Trypanosoma rhodesiense* infections in mice when dosed ip but was considerably less active when dosed orally.[1] However, it was shown[2] that **1b** is rapidly metabolized in the mouse to **1a** and excreted as its glucuronide.[3] We considered that if **1b** could be suitably derivatized we might accomplish two things: (a) increase the intrinsic activity of the compounds against trypanosomes; (b) prevent rapid metabolism and excretion of the compound.

Chemistry. It was already known[1] that **1c** had reasonable activity against a number of *Trypanosoma* species so we prepared by standard methods (see Experimental Section) a number of thioacetals (**2–7**) derived from **1b** which we considered would be metabolically and chemically more stable than **1c**. The hydrazones **8–19** were prepared by analogy with the many hydrazones derived from 5-nitrofurfuraldehyde and related compounds.[4,5] In particular, compounds **10**, **11**, and **12** were prepared as analogs of nitrofurazone, nitrofurantoin, and guanofuracin, respectively. Nitrofurazone has been shown to be effective against *Trypanosoma gambiense* infections in guinea pigs[6] and to inhibit a *Trypanosoma cruzi* infection in mice.[7] Compounds **13–16** can be considered as analogs of nifurtimox,[8] a promising compound for the treatment of acute and chronic Chagas disease (Table I).

The quaternary acylhydrazones **20** and **24** were prepared in an attempt to impart some water solubility to the compounds and also as analogs of 5-nitro-2-furfuraldehydetrimethylammonium acetylhydrazone chloride which has been shown to inhibit inections of *T. cruzi* in mice.[7]

Biological Results. All the compounds were tested against infections of *T. rhodesiense* in mice using the procedures described by Hawking.[9] If the compounds showed activity in this test they were tested against *T. cruzi*, *T. gambiense*, and *Trypanosoma congolense* by a similar procedure. Only compounds active against one or more of the above organisms are listed in Table II. None of the thioacetals **2–7** showed activity comparable to **1c** and this series was abandoned. The simple hydrazones **8** and **9** were both inactive as was **10**, the analog of nitrofurazone. The nitrofurantoin analog **11** exhibited marginal activity against *T. rhodesiense*, *T. gambiense*, and *T. congolense* but was inactive against *T. cruzi*. The guanofuracin ana-