Notes

Catechol O-Methyltransferase. 11. Inactivation by 5-Hydroxy-3-mercapto-4-methoxybenzoic Acid

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5-Hydroxy-3-mercapto-4-methoxybenzoic acid was synthesized as a potential affinity-labeling reagent for catechol O-methyltransferase (COMT, EC 2.1.1.6). This compound was shown to produce noncompetitive inhibition of COMT when assayed in the presence or absence of the reducing agent, dithiothreitol (DTT). If COMT was assayed in the absence of DTT, the compound was shown to be a more potent inhibitor (K_{is} = 59.9 ± 15.9 μM; K_{ii} = 30.2 ± 5.8 μM) than if the assays were conducted in the presence of the reducing agent (K_{is} = 1140 ± 233 μM; K_{ii} = 743 ± 141 μM). The potent inhibitory effects produced in the absence of DTT could be partially reversed by the addition of DTT to the incubation mixture or by dialysis of the modified enzyme against DTT-containing buffer. These data suggest that in the absence of DTT, 5-hydroxy-3-mercapto-4-methoxybenzoic acid serves as an affinity-labeling reagent for COMT by reaction of the 3-mercapto group of the ligand with an active-site sulfhydryl group. This ligand–protein disulfide bond can be reduced with DTT with subsequent reversal of the inhibitory effects.

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). This enzyme 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]

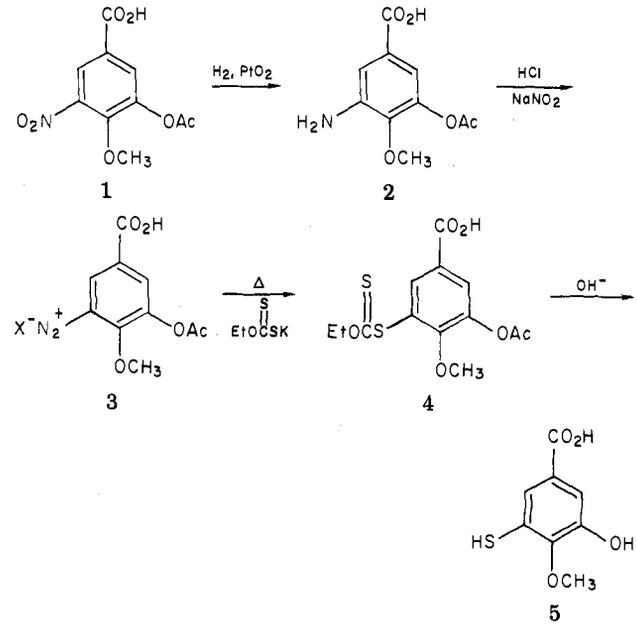
In 1972, Lutz et al.[2] reported the COMT inhibitory effects of 3-mercaptotyramine, a sulfhydryl-containing analogue of dopamine. 3-Mercaptotyramine was found to inhibit COMT by a time-dependent process. However, the inhibition could be reversed or even prevented by inclusion of dithiothreitol (DTT), a disulfide reducing agent. Thus, Lutz et al.[2] proposed that the sulfhydryl group of the inhibitor was reacting with an enzyme active-site sulfhydryl group to form a disulfide bond, accounting for the compound's ability to produce "pseudoirreversible" inactivation of the enzyme.

During the course of our studies of 5-substituted isovanillic acids and isovanillins as potential inhibitors of COMT,[3] we prepared 5-hydroxy-3-mercapto-4-methoxybenzoic acid (**5**). The potential COMT inhibitory activity of **5** was of particular interest because of its structural similarity to 3-mercaptotyramine, the affinity-labeling reagent previously identified by Lutz et al.[2] In the present paper we describe the synthesis of **5** and provide evidence to suggest that **5** is an affinity-labeling reagent for COMT.

Results

Synthesis. The synthesis (Scheme I) of 5-hydroxy-3-mercapto-4-methoxybenzoic acid (**5**) was initiated from the 3-nitro analogue of isovanillic acid (**1**), which was readily available.[4] The reduction of the nitro compound **1** to the corresponding amine **2** was accomplished using catalytic hydrogenation conditions. Compound **2** was diazotized using sodium nitrite with a small excess of acid to minimize polymerization of the diazonium intermediate **3**, yet not acidic enough to decompose potassium xanthate in the conversion of **3** to **4**. The xanthate **4** was hydrolyzed to the desired mercapto compound **5** using mild alkaline conditions. Sublimation of **5** gave a product free of the disulfide dimer as determined by mass spectral analysis. So as to minimize contamination by the disulfide dimer, further purification of **5** was not undertaken.

Scheme I. Synthetic Pathway



Biology. Experiments with **5** and purified COMT showed that the compound was 20 times more potent as an inhibitor of the enzyme when evaluated in the absence of the reducing agent dithiothreitol (DTT). If the inhibitory effects of **5** were determined in the presence of DTT (4 mM), the observed inhibition constants were K_{is} = 1140 ± 233 μM and K_{ii} = 743 ± 141 μM. In the absence of DTT, the observed inhibition constants were K_{is} = 59.9 ± 15.9 μM and K_{ii} = 30.2 ± 5.8 μM (Figure 1).

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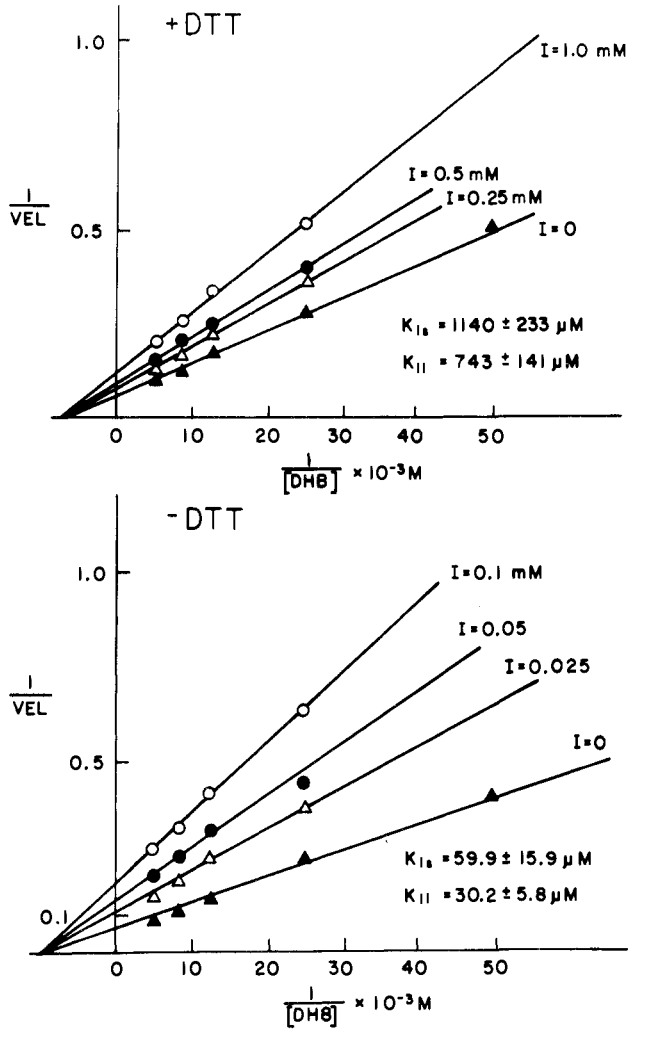

Figure 1. Inhibition of COMT by 5-hydroxy-3-mercapto-4-methoxybenzoic acid (5). Reciprocal plots with 3,4-dihydroxybenzoic acid (DHB) as the variable substrate. Assay conditions are outlined under Experimental Section, except for DHB concentration, 20–200 μM, and SAM concentration, 20 μM. If DTT was included, the final concentration was 4.0 mM.

When COMT was incubated with compound **5** (500 μM) in the presence of DTT, partial inhibition (39%) of enzymatic activity was observed at time zero with no further inactivation of the enzyme after preincubation for 30 min at 37 °C (Figure 2). In the absence of DTT, compound **5** produced 91% inhibition at time zero, and the degree of inhibition increased to 99% with preincubation for 30 min at 37 °C. If DTT was added to this latter sample (with or without further incubation), a substantial amount of the inhibition could be reversed, with the COMT activity approaching that seen in the original DTT-containing sample.

The ability of DTT to reverse the COMT inhibitory effects of compound **5** was further demonstrated by the dialysis experiments outlined in Table I. If COMT is preincubated with compound **5** in the presence of DTT, partial inhibition is observed (experiment 1, Table I). Dialysis of this preincubated sample in the presence of DTT results in nearly complete recovery of enzyme activity (experiment 2, Table I). In contrast, preincubation of COMT and compound **5** in the absence of DTT (experiment 3, Table I) causes nearly complete inactivation of the enzyme. This inhibition can be reversed by dialysis in the presence of DTT (experiment 4, Table I). If the enzyme inactivated by preincubation with compound **5** is dialyzed in the absence of DTT (experiment 5, Table I), enzyme activity cannot be recovered.

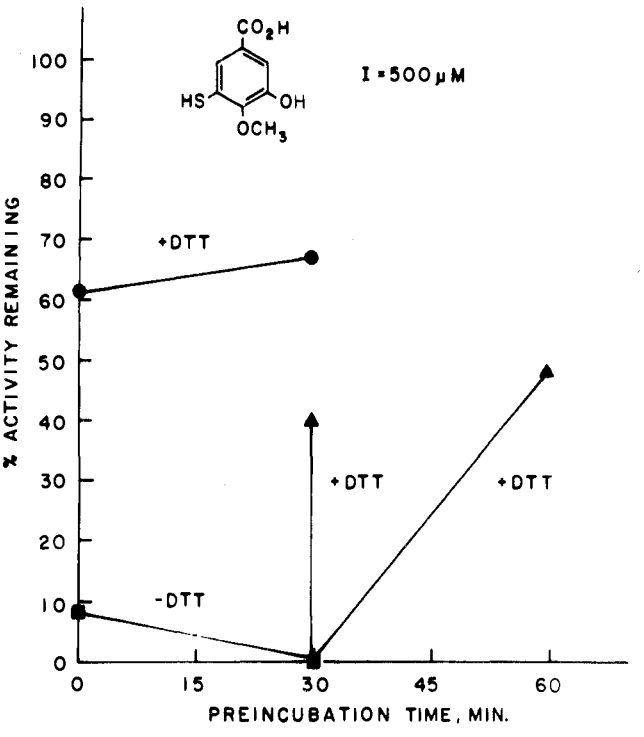

Figure 2. Effect of dithiothreitol (DTT) on the COMT inhibitory effects of 5-hydroxy-3-mercapto-4-methoxybenzoic acid (5). Incubation mixtures consisting of compound 5 (500 μM) were incubated (37 °C) with an aliquot of COMT in the presence (4 mM) or absence of DTT. Aliquots were removed at zero time and 30 min, and residual COMT activity was determined as described under Experimental Section. To samples not originally containing DTT which had been incubated for 30 min was added DTT to a final concentration of 4 mM. One aliquot of this sample was immediately assayed for COMT activity and another sample was incubated at 37 °C for an additional 30 min, and then residual COMT activity was determined.

Table I. Effect of Dialysis on the Inhibitory Properties of 5-Hydroxy-3-mercapto-4-methoxybenzoic Acid

expt no.	DTT addition[a]	act. determination[b]	% act. remaining
1	+	before dialysis	70
2	+	after dialysis (+DTT)	87
3	–	before dialysis	7
4	–	after dialysis (–DTT)	1
5	–	after dialysis (+DTT)	76

[a] Preincubation mixtures contained 0.2 M phosphate buffer, pH 7.6, ± DTT (8.0 mM) and 5-hydroxy-3-mercapto-4-methoxybenzoic acid (1 mM). Samples were incubated at 37 °C for 30 min prior to analysis for COMT activity or dialysis. [b] Dialysis was carried out using 0.2 M phosphate buffer, pH 7.6, containing DTT (8.0 mM).

Discussion

Based on the results described above, it would appear that 5-hydroxy-3-mercapto-4-methoxybenzoic acid (**5**) inhibits COMT through a mechanism similar to that reported earlier for 3-mercaptotyramine by Lutz et al.[2] COMT appears to have a weak affinity for compound **5** when assayed in the presence of DTT. This inhibition probably results through a mechanism involving binding of the inhibitor to the substrate binding site in a reversible fashion. In the absence of a reducing agent it would appear that compound **5** binds to COMT, followed by formation of a disulfide bond with an active-site sulfhydryl residue. This ligand–protein disulfide bond can be reduced, by dialysis in buffer containing DTT, with subsequent reversal of the inhibitory effects of compound **5**. The mechanism

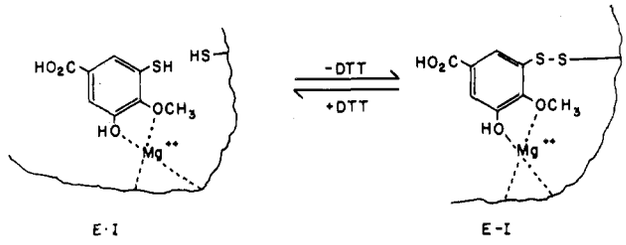

Figure 3. Proposed mechanism for the inactivation of COMT by 5-hydroxy-3-mercapto-4-methoxybenzoic acid.

shown in Figure 3 is modified after that proposed by Lutz et al.,[2] to explain the inactivation of COMT by 3-mercaptotyramine. Borchardt and Thakker[5] had previously provided evidence for two sulfhydryl groups at the active site of COMT. These groups could be selectively modified by functional-group reagents or affinity-labeling reagents.[6] In the case of compound **5** and COMT, the ligand–protein disulfide bond probably forms because of the close juxtaposition of the sulfhydryl groups of the inhibitor and protein during the initial reversible binding process. It would appear that an active-site sulfhydryl group is involved because of the fact that the inhibitory effects of compound **5** can be partially prevented by inclusion of the catechol substrate (data not shown). Therefore, 5-hydroxy-3-mercapto-4-methoxybenzoic acid (**5**) can be added to the list of affinity-labeling reagents that modify a sulfhydryl group at the active site of COMT.[6]

Experimental Section

Materials. *S*-Adenosyl-L-(*methyl*-^{14}C)methionine (SAM-$^{14}CH_3$, New England Nuclear Corp., 50–60 mCi/mmol) was diluted to a concentration of 8.8 μCi/mL and stored at −20 °F. SAM chloride (Sigma Chemical Co.) was stored as a 10 mM aqueous stock solution at the same temperature. 3,4-Dihydroxybenzoic acid was obtained from Aldrich Chemical Co., and dithiothreitol (DTT) was obtained from Sigma Chemical Co.

COMT Purification and Assay. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) using a modification[7,8] of the procedure described by Nikodejevic et al.[9] The enzyme preparation used in the kinetic experiments was purified through the calcium phosphate step, resulting in a 48-fold increase in specific activity as compared to the crude supernatant. This enzyme preparation had a specific activity of 49.6 nmol of product (mg of protein)$^{-1}$ min^{-1} using 3,4-dihydroxybenzoic acid as the substrate. The enzyme activity was determined using SAM-$^{14}CH_3$ (55 Ci/mol) and 3,4-dihydroxybenzoic acid as substrates according to a previously described radiochemical assay.[7] Upon prolonged storage, some loss of enzyme activity was detected. Therefore, prior to use, COMT was routinely reactivated by preincubation for 40 min at 37 °C in phosphate buffer (pH 7.6) containing 4 mM DTT.

Preparation of 5-Hydroxy-3-mercapto-4-methoxybenzoic Acid (5). A mixure of 3-acetoxy-4-methoxy-5-nitrobenzoic acid (**1**;[4] 510 mg, 2 mmol) and PtO_2 (100 mg) in absolute EtOH (100 mL) was hydrogenated (40 psi) for 1.5 h in a Parr shaker. The reaction mixture was then filtered, and the product was isolated as the HCl salt: mp 168 °C; NMR of the free base of **2** (acetone-d_6) δ 2.3 (s, 3, OAc), 3.7 (s, 3, OMe), 5.75 (NH_2, exchanges with D_2O), 6.9 and 7.4 (2 d, 1 each, Ar H). The material was sufficiently pure to be used in subsequent synthetic conversions.

The HCl salt of **2** (410 mg, 1.47 mmol) was dissolved in a mixture of 1–2 g of ice and 0.15 mL (1.8 mequiv) of concentrated HCl. When dissolution was complete, 135 mg (1.95 mmol) of $NaNO_2$ in 1.4 mL of cold water was added slowly so that the temperature remained below 10 °C. Addition of the $NaNO_2$ was stopped when an aliquot of the reaction mixture turned starch–iodine paper blue. Meanwhile, a separate solution containing 270 mg (1.7 mmol) of potassium ethyl xanthate in 0.70 mL of H_2O and 210 mg of sodium carbonate (2.0 mmol) in 1.4 mL of H_2O was prepared under N_2 and heated to 70 °C. The diazonium solution was maintained in the cold for 15 min and then filtered through glass wool into the hot, well-stirred xanthate solution. Stirring at 75 °C was continued for 1 h, after which 310 mg (7.8 mmol) of NaOH in 1.4 mL of H_2O was added. The mixture was refluxed for 3 h and then cooled in ice and acidified. Extraction with Et_2O (3 times) and evaporation of the Et_2O extract gave 70 mg (mp 170 °C) of crude product. Further extraction of the aqueous phase with EtOAc (2 times) gave 100 mg of additional product. Sublimation (130–150 °C, 0.01 mm) of a 70-mg sample of the crude material afforded 42 mg of a light yellow solid, mp 172–177 °C. The product exhibited a single spot (R_f 0.2) on thin-layer chromatography (silica gel; solvent: 95% CH_3Cl saturated with formic acid, 5% EtOH). When the thin-layer plates were sprayed with Ellman's reagent,[10] a characteristic yellow color developed: NMR (acetone-d_6) δ 7.5 and 7.35 (2 d, J = 3 Hz, 1 each, Ar H), 9.0–6.0 (br, 2, exchanges with D_2O, SH, OH), 3.85 (s, 3, OCH_3); IR (KBr) 3400, 2580, 1700, 1580 cm^{-1}; mass spectrum, m/e 200 (M^+). MS peak matching: calcd, 200.01427; found, 200.01415.

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.

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Antiestrogenic Properties of Substituted Benz[*a*]anthracene-3,9-diols

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The antiestrogenic potency of benz[*a*]anthracene-3,9-diol, as well as its 7- and 12-methyl derivatives, was evaluated by measuring the inhibition in the onset of estrus brought about by this compound in ovariectomized rats treated with 17β-estradiol. At a dose of 0.5 mg the 7,12-dimethyl derivative caused a decrease in the percentage of rats in estrus from 78 to 44%. This decrease is identical with that caused by 0.05 mg of nafoxidine.

There is much evidence to support the concept that estrogen is of primary importance in the development and growth of rat mammary cancer and that at least a part of these biochemical events occurs directly in the target or-

0022-2623/82/1825-0323$01.25/0