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INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE BY 1-VINYL DERIVATIVES OF NITROCATECHOLS AND NITROGUAIACOLS

KINETICS OF THE IRREVERSIBLE INHIBITION BY 3-(3-HYDROXY-4-METHOXY-5-NITRO BENZYLIDENE)-2,4-PENTANEDIONE

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Abstract—It is well known that activated alkene derivatives react with thiol groups according to a Michael's addition reaction. On the basis of the presence of at least one thiol group essential for the activity of catechol-*O*-methyltransferase (COMT), several 1-vinyl derivatives of nitrocatechol and nitroguaiacol were synthesized and tested as potential irreversible active site-directed inhibitors of COMT. All the synthesized products were potent inhibitors of partially purified pig liver COMT. However, the inhibition was reversible in most cases, with the exception of 3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentanedione (compound **2**) which inhibited COMT in an irreversible manner. When the inhibition of COMT was measured as a function of the length of time of pre-incubation with **2**, biphasic kinetics were observed, suggesting the modification of at least two thiol groups which are essential for COMT activity. The analysis of the two parts of the inhibition curve as a function of the inhibitor concentration showed that compound **2** modified the more reactive group in a non-specific manner, while it behaved as an active site-directed inhibitor on the second slow-reacting thiol group. Importantly, a saturating concentration of *S*-adenosyl-L-methionine (AdoMet) in the pre-incubation mixture gave pseudo-first order kinetics, suggesting total protection of one thiol group. Magnesium ions had no effect on the protection of COMT by AdoMet. In the presence of 3,5-dinitrocatechol (DNC) slight protection of COMT was observed; when the inactivation of both groups was analysed independently, protection of the specifically modified group was detected, while the reaction with the other group was faster in the presence of DNC. When both AdoMet and DNC were present, inactivation of COMT by **2** was not observed, suggesting that both reacting groups are located at or near the active site.

Catechol-*O*-methyltransferase (EC 2.1.1.6; COMT‡) plays an important role in the extraneuronal inactivation of catecholamine neurotransmitters and exogenous catechol compounds [1, 2]. The lack of COMT inhibitors for clinical use, mainly in the treatment of Parkinson's disease [3, 4], has generated considerable research interest in the study of COMT inactivation. In addition, the study of COMT inhibition should improve our knowledge of the structure and composition of the active site of the enzyme.

At present, Parkinson's disease is alleviated by combination therapy with levodopa (L-Dopa), an active dopamine precursor, together with a peripheral inhibitor of L-Dopa decarboxylase (as carbidopa or benserazide) [5, 6]. However, in this case the major plasma metabolite of levodopa is the O-methylation product, 3-*O*-methyldopa. The accumulation of this metabolite in plasma may be detrimental to patients because it competes with the active transport of L-Dopa through the intestinal mucosa and the blood–brain barrier [7–9]. Thus, selective inhibition of COMT would improve the bio-availability of L-Dopa and its transport into the brain. The administration of a peripheral COMT inhibitor in combination with L-Dopa and carbidopa (or benserazide) would notably reduce the effective dose of L-Dopa and the concentration of 3-*O*-methyldopa in plasma [10].

Since the existence of *O*-methyltransferase activity was first reported [11] several compounds have been described to inhibit COMT, but most of them were not very effective and showed high toxicity *in vivo* [1]. The inactivation of COMT by several sulfhydryl reagents suggested the presence of at least one thiol group essential for COMT activity [11]. Several active site-directed inhibitors that follow pseudo-first order kinetics have been described [12–17]. The inhibition of COMT by *N*-ethylmaleimide suggested the existence of at least two thiol groups that were modified with different rate constants [18]. On the basis of these studies, several maleimide, succinimide, maleamic acids and succinamic acid derivatives were synthesized and assayed as COMT inhibitors, and some of the maleimide derivatives were shown to act as irreversible active site-directed inhibitors of the more reactive thiol group [19, 20].

Recently, two research groups have reported independently the synthesis of potent and selective

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; COMT, catechol-*O*-methyltransferase; DMSO, dimethyl sulfoxide; DNC, 3,5-dinitrocatechol; DTT, dithiothreitol.

COMT inhibitors bearing a 1-substituted 5-nitrocatechol structure [21, 22]. Previously reported data indicated that the presence of electron withdrawing substituents at position 5 was very important for the activity of the dihydroxybenzaldehyde derivatives [22, 23]. However, we have observed that substitution at this position is not essential, instead substitution at an *ortho* position relative to one of the two hydroxyl groups increases the activity of the compound as a COMT inhibitor [24]. Moreover, the presence of a carbonyl group at position 1, directly conjugated with the aromatic ring or through a carbon–carbon double bond, increased the potency of the nitrocatechol derivatives as inhibitors of COMT isolated from rat brain [21].

Having these previous works in mind, our goal was to synthesize new, activated 1-vinyl derivatives of nitrocatechol and nitroguaiacol and to test their potential activity as irreversible active site-directed inhibitors of partially purified pig liver COMT. The activated vinyl group conjugated with the benzene ring would be able to react covalently with SH group(s) present at, or near, the active site of COMT, through a Michael-type addition reaction. The catechol or guaiacol structure was substituted at different positions by a nitro group, in order to increase the potency and selectivity of the compounds towards COMT.

MATERIALS AND METHODS

Biological methods

COMT isolation. COMT was partially purified from pig liver according to Nikodejevic *et al.* [25]. All the steps were performed at 4°. The tissue was first homogenized in 2% KCl (w/v) and centrifuged at 12,000 *g* for 30 min. The pellet was discarded and the supernatant was adjusted to pH 5.0 with 1 M acetic acid and stirred for 15 min. The mixture was centrifuged as above and the new supernatant was carefully neutralized with 1 M NaOH, then fractionated with 30–50% of $(NH_4)_2SO_4$. The precipitated protein was sedimented at 17,000 *g* for 20 min, resuspended in 10 mM phosphate buffer (pH 7.0) and desalted through a Sephadex G25 column, previously equilibrated with the same buffer. The eluted protein was concentrated by ultrafiltration to about 50 mL and applied to a Sephadex G200 column (5 × 90 cm); the protein was then eluted and tested for COMT activity and UV absorbance at 280 nm. The fractions which showed COMT activity were pooled and stored at −30°. Protein concentration was determined using the Benedict reagent [26].

COMT assay. Enzyme activity was determined using the one-step radiochemical method described by Zürcher and Da Prada [27] slightly modified by us [20]. Briefly, in a model assay the reaction mixture (0.25 mL) contained: enzyme (1 U is defined as the amount of protein that catalyses the transformation of 1 nmol of substrate per min), 20 mM pyrocatechol (Fluka, Buchs, Switzerland), 0.9 mM [^{3}H]*S*-adenosyl-L-methionine (AdoMet) (1.6 Ci/mol) (Amersham, U.K., Boehringer Mannheim, Germany), 1.5 mM $MgCl_2$ and 2.5 mM dithiothreitol (DTT) in 125 mM phosphate buffer pH 7.6. Blanks were prepared without enzyme. The mixture was incubated at 37° for 10 min and 0.25 mL of 1 M ice-cold citric acid was added to stop the reaction. Then, 1.5 mL of hexane–toluene (4:1), containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyl oxazolyl)]benzene, was added and the mixture was vortexed vigorously for 30 sec. The radioactivity present in the organic phase was directly counted in a LKB Rackbetta scintillation counter.

The production of guaiacol was shown to be linear with protein concentration and with incubation time. In all cases, the amount of transformed substrate was not higher than 5% of the initial concentration, ensuring that initial velocities were measured. Suitable controls were always performed.

Analysis of the kinetic data. The kinetic data were first analysed graphically according to a Lineweaver–Burk representation. A linear relationship was obtained in all cases. The kinetic constants were then calculated on a personal computer using the Fortran IV program described by Cleland [28].

The analysis of the rate constants for the irreversible inactivation of COMT was performed according to the equation described by Ray and Koshland [29] and the graphical approximation used by Borchardt and Thakker [18]. Once the values of k_1, k_2 and F were estimated, a non-lineal regression computer program was used to calculate the real values of the rate constant of inactivation.

Chemical methods

TLC was performed on aluminium sheets precoated with silica gel (Merck, Darmstadt, Germany; Kieselgel 60, F254). Column chromatography separations were carried out on silica gel (Merck, Kieselgel 60, 230–240 mesh) under pressure. Melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Infrared spectra were obtained with a Perkin–Elmer 681 spectrometer and the frequencies are given in cm^{-1} units. ^{1}H-NMR spectra were recorded in a Varian XL-300 (300 MHz), and ^{13}C-NMR spectra were recorded on a Bruker AM-200 (50 MHz) or a Bruker WP-80 (20 MHz). δ values are in ppm relative to internal standard tetramethylsilane; coupling constants (J) are in Hz. The compounds were dried over P_2O_5 *in vacuo* and analysed on a Perkin–Elmer 240. The correct elemental analysis for compounds **4, 8** and **11** could not be obtained; however, their spectral data (I.R., ^{1}H-NMR and ^{13}C-NMR) were satisfactory. The syntheses of compounds **1, 6, 10** and **12** were as described elsewhere [24].

3-(3-*Hydroxy*-4-*methoxy*-5-*nitrobenzylidene*)-2,4-*pentanedione* (**2**). A mixture of 5-nitroisovanillin (0.5 g, 2.54 mmol) [30] and 2,4-pentanedione (0.26 mL, 2.54 mmol) in tetrahydrofuran (3 mL) was saturated with hydrogen chloride. After stirring overnight at 4°, the solvent was removed to dryness *in vacuo*. The crude product was chromatographed by flash chromatography on silica gel with ethyl acetate: hexane (2:1) to afford **2** (0.49 g, 69%). m.p. 167–169°. i.r.: 3500–3000, 1710, 1645, 1615, 1540. ^{1}H-NMR (DMSO-d_6): 2.29 (s, 3H), 2.43 (s, 3H), 2.92 (s, 3H) 7.21 (d, 1H, J = 2.0 Hz), 7.47 (d, 1H, J = 2.0 Hz), 7.62 (s, 1H). ^{13}C-NMR (20 MHz, CD_3COCD_3): 26.28, 62.08, 117.72, 121.31, 130.17,

137.61, 143.37, 144.74, 145.76, 152.74, 197.60. Anal. calcd for $C_{13}H_{13}O_6N$: C 55.91, H 4.65, N 5.01; found C 55.83, H 4.90, N 5.29.

[2-(3-*Hydroxy*-4-*methoxy*-5-*nitrophenyl*)*vinyl*]-*phenyl ketone* (**3**). The reaction of 5-nitroisovanillin and acetophenone was carried out in a similar manner as described for compound **2**, but with methanol as solvent. After stirring overnight at 4°, a precipitate was obtained, which was filtered and dried affording **3** (67%). m.p. 180–182°. i.r.: 3600–3120, 1660, 1600, 1540. ^{1}H-NMR (DMSO-d_6): 3.90 (s, 3H), 7.53 (d, 1H, J = 2.0 Hz), 7.57 (t, 2H, J = 7.4 Hz), 7.64 (d, 1H, J = 16 Hz), 7.67 (t, 1H, J = 7.4 Hz), 7.89 (d, 1H, J = 16 Hz), 7.94 (d, 1H, J = 2.0 Hz), 8.14 (dd, 2H, J = 1.3 Hz, J = 7.4 Hz). ^{13}C-NMR (20 MHz, DMSO-d_6): 61.29, 113.81, 120.92, 123.39, 128.49, 128.69, 130.75, 133.17, 137.33, 141.55, 145.20, 151.67, 188.89. Anal. calcd for $C_{16}H_{13}O_5N$: C 64.21, H 4.35, N 4.68; found: C 64.36, H 4.34, N 5.11.

(3-*Hydroxy*-4-*methoxy*-5-*nitrobenzylidene*)*nitro-acetic acid* (**4**). The condensation of 5-nitroisovanillin and ethyl nitroacetate was also carried out in methanol as reported for **3**. The mixture was stirred at 25° for 10 days. The reaction mixture then was diluted with water and extracted with ethyl acetate. The organic phase was dried (Na_2SO_4), filtered and the solvent was removed *in vacuo*. The residue was crystallized three times from methanol–water, to afford **4** (17%). m.p. 138–141°. i.r. 3650–3000, 1530, 1350, 1320. ^{1}H-NMR (DMSO-d_6): 3.87 (s, 3H), 7.43 (d, 1H, J = 1.8 Hz), 7.45 (d, 1H, J = 1.8 Hz), 8.09 (s, 1H), 10.63 (bs, 1H), 11.43 (bs, 1H). ^{13}C-NMR (50 MHz, CD_3COCD_3): 62.14, 114.17, 116.50, 130.56, 142.68, 147.38, 152.83.

2,5-*Bis*(3-*hydroxy*-4-*methoxy*-5-*nitrobenzylidene*)-*cyclopentanone* (**5**). The reaction of 5-nitrovanillin (1.01 mmol) and cyclopentanone (0.72 mmol) was performed as described for compound **3** to afford **5** (0.205 g, 46%) as a yellow solid. m.p. desc. 290°. i.r. 3650–3050, 1625, 1540, 1325. ^{1}H-NMR (DMSO-d_6): 2.49 (s, 4H), 3.91 (s, 6H), 7.33 (s, 2H), 7.47 (d, 2H, J = 2 Hz) 7.57 (d, 2H, J = 2 Hz). ^{13}C-NMR (50 MHz, DMSO-d_6): 25.68, 61.20, 116.38, 121.50, 130.49, 131.08, 138.50, 141.01, 144.93, 151.76, 194.79. Anal. calcd for $C_{21}H_{18}N_2O_9$: C 57.01, H 4.07, N 6.33; found C 56.86, H 4.14, N 6.41.

[2-(3,4-*Dihydroxy*-2-*nitrophenyl*)*vinyl*]*phenyl ketone* (**7**). The condensation reaction of 3,4-dihydroxy-2-nitrobenzaldehyde [31] and acetophenone was carried out as reported for **3**. The crude product was crystallized from methanol–water affording **7** (75%) as a red solid. m.p. 170–173°. i.r. 3480, 3320–2900, 1650, 1590, 1565, 1530. ^{1}H-NMR (DMSO-d_6): 7.01 (d, 1H, J = 8.5 Hz), 7.32 (d, 1H, J = 15.3 Hz), 7.56 (td, 2H, J = 7.3 Hz, J = 1.5 Hz), 7.62 (d, 1H, J = 8.5 Hz), 7.67 (tt, 1H, J = 7.3 Hz, J = 1.3 Hz), 7.81 (d, 1H, J = 15.3 Hz), 8.09 (dd, 2H, J = 7.3 Hz, J = 1.3 Hz). ^{13}C-NMR (50 MHz, CD_3COCD_3): 118.02, 119.99, 120.30, 124.29, 129.27, 129.54, 133.80, 137.50, 138.79, 139.45, 141.63, 149.44. Anal. calcd for $C_{15}H_{11}O_5N$: C 63.16, H 3.86, N 4.91; found C 63.11, H 3.81, N 4.90.

(3,4-*Dihydroxy*-2-*nitrobenzylidene*)*nitroacetic acid* (**8**). This compound was prepared from 3,4-dihydroxy-2-nitrobenzaldehyde and ethyl nitroacetate as in the case of compound **4**. After stirring the reaction mixture at 25° for 10 days, the crude obtained was washed with water and filtered, giving **8** (32%). m.p. desc. 190°. i.r.: 3490, 3475–3100, 1550, 1250. ^{1}H-NMR (DMSO-d_6): 6.93 (d, 1H, J = 8.5 Hz), 6.98 (d, 1H, J = 8.5 Hz), 7.87 (s, 1H), 10.60 (bs, 1H), 10.61 (bs, 1H), 11.33 (s, 1H). ^{13}C-NMR (50 MHz, CD_3COCD_3): 117.64, 117.88, 120.05, 139.59, 144.68, 148.36.

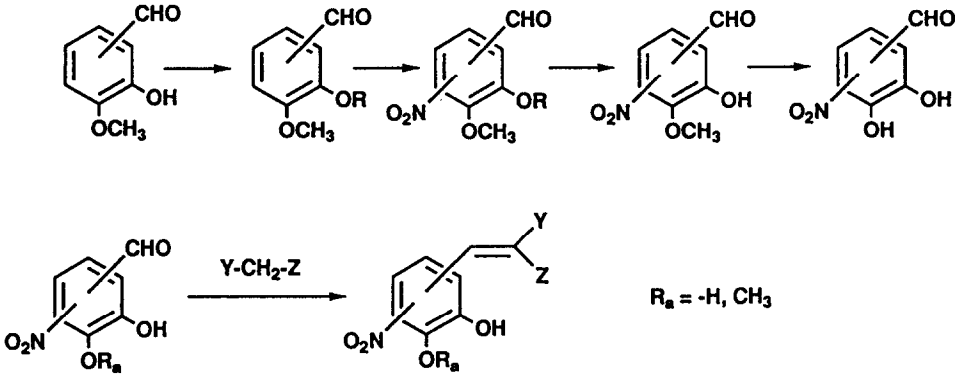
2,5-*Bis*(3,4-*dihydroxy*-2-*nitrobenzylidene*)*cyclo-pentanone* (**9**). The reaction was carried out using 3,4-dihydroxy-2-nitrobenzaldehyde as described for compound **5**. The reaction was finished after stirring at 4° for 30 min. This process gave a precipitate which was filtered and dried, affording **9** (75%) as an orange solid. m.p. desc. 250°. i.r. 3700–3000, 1650–1560, 1545. ^{1}H-NMR (DMSO-d_6): 3.00 (s, 4H), 6.95 (s, 2H), 7.05 (d, 2H, J = 8.7 Hz), 7.19 (d, 2H, J = 8.7 Hz). ^{13}C-NMR (50 MHz, CD_3COCD_3): 26.97, 117.44, 120.48, 121.97, 125.75, 139.38, 140.00, 142.19, 148.53, 194.65. Anal. calcd for $C_{19}H_{14}O_9N_2$: C 55.07, H 3.38, N 6.76; found C 55.33, H 3.75, N 6.54.

(2,3-*Dihydroxy*-4-*nitrobenzylidene*)*nitroacetic acid* (**11**). The reaction of 2,3-dihydroxy-4-nitrobenzaldehyde [15] and ethyl nitroacetate was carried out as reported for **4**, except that the mixture was stirred at room temperature for 7 days. The crude product was crystallized from water to give **11** (78%). m.p. 185–187°. i.r.: 3600–3100, 1535, 1170. ^{1}H-NMR (CD_3COCD_3): 7.16 (d, 1H, J = 9 Hz), 7.61 (d, 1H, J = 9 Hz), 8.46 (s, 1H). ^{13}C-NMR (50 MHz, CD_3COCD_3): 115.47, 119.68, 123.92, 135.56, 145.05, 148.07, 149.72.

(3,4-*Dihydroxy*-5-*nitrobenzylidene*)-2,4-*pentanedione* (**13**). This compound was obtained as reported previously [8] from 3,4-dihydroxy-5-nitrobenzaldehyde and 2,4-pentanedione. m.p. 175–178° (reported m.p. 175–178°).

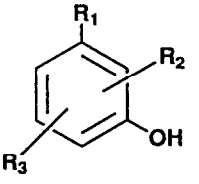
RESULTS AND DISCUSSION

A series of 1-vinyl derivatives of catechol and guaiacol, substituted with a nitro group at the *ortho* position with respect to one of the hydroxyl groups (catechol derivatives) or the methoxy group (guaiacol analogues), were synthesized by condensation of the adequate nitrobenzaldehydes with an activated methyl or methylene group under acidic conditions [21]. The starting nitrobenzaldehydes were obtained by protection of the hydroxyl group of the appropriate hydroxymethoxybenzaldehyde, followed by nitration and cleavage of the protective group [24]; the dihydroxynitrobenzaldehyde derivatives were obtained by cleavage of the ether group using published methods [24]. The vinyl derivatives of nitrocatechol or nitroguaiacol were obtained by condensation of the corresponding nitro-benzaldehydes with a compound carrying an active methyl or methylene group. Scheme 1 shows the general synthetic process of these products. The compounds thus obtained have an activated vinyl group at position 1 conjugated with the aromatic ring, which can be expected to react with thiol groups.



Scheme 1.

Table 1. Inhibition of COMT by several nitrocatechol and nitroguaiacol 1-substituted derivatives



Compound	R_1	R_2	R_3	IC_{50} (M)
1*	—CHO	4-OCH_3	5-NO_2	3.4×10^{-5}
2	—CH = $C(COCH_3)_2$	4-OCH_3	5-NO_2	5.5×10^{-5}
3	—CH = $CHCOC_6H_5$	4-OCH_3	5-NO_2	1.5×10^{-5}
4	—CH = $C(NO_2)COOH$	4-OCH_3	5-NO_2	1.9×10^{-5}
5	—CH = CCH_2CH_2C(=CO) = CH— —C_6H_2-3-OH-4-OCH_3-5-NO_2	4-OCH_3	5-NO_2	8.0×10^{-7}
6*	—CHO	4-OH	2-NO_2	1.5×10^{-6}
7	—CH = $CHCOC_6H_5$	4-OH	2-NO_2	3.2×10^{-7}
8	—CH = $C(NO_2)COOH$	4-OH	2-NO_2	1.1×10^{-6}
9	—CH = CCH_2CH_2C(=CO) = CH— —C_6H_2-2,3-$(OH)_2$-4-NO_2	4-OH	2-NO_2	2.3×10^{-7}
10*	—CHO	2-OH	4-NO_2	1.5×10^{-6}
11	—CH = $C(NO_2)COOH$	2-OH	4-NO_2	1.1×10^{-6}
12*	—CHO	4-OH	5-NO_2	2.0×10^{-6}
13	—CH = $C(COCH_3)_2$	4-OH	5-NO_2	8.0×10^{-7}

* Ref. 24.

The IC_{50} values were calculated from a plot of the percentage of inhibition as a function of inhibitor concentration. Results are mean values of two experiments.

These products were evaluated as potential inhibitors of partially purified COMT from pig liver; all of them behaved as COMT inhibitors. Table 1 shows the IC_{50} values obtained for the inhibition of COMT by these compounds, without pre-incubation of the enzyme with the inhibitor, under the standard conditions for the COMT assay, except that DTT was absent. The IC_{50} values obtained with the 1-vinyl nitrocatechol and nitroguaiacol derivatives were similar or lower than those obtained for the correspondent aldehyde precursors [24], with the exception of 3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentanedione (compound **2**); this result is in agreement with that reported in the literature for a similar substitution in 5-nitrocatechols, which generally increases the activity of the compounds as COMT inhibitors [21]. Remarkably, the condensation of the corresponding nitrobenzaldehyde with cyclopentanone, resulting in the bis-adducts **5** and **9**, led approximately to a 100-fold decrease in the IC_{50} for these compounds towards COMT. However, the condensation of the aldehyde group with ethylnitroacetate (**4**, **8** and **11**) did not produce a lower IC_{50} value. Comparing these results with

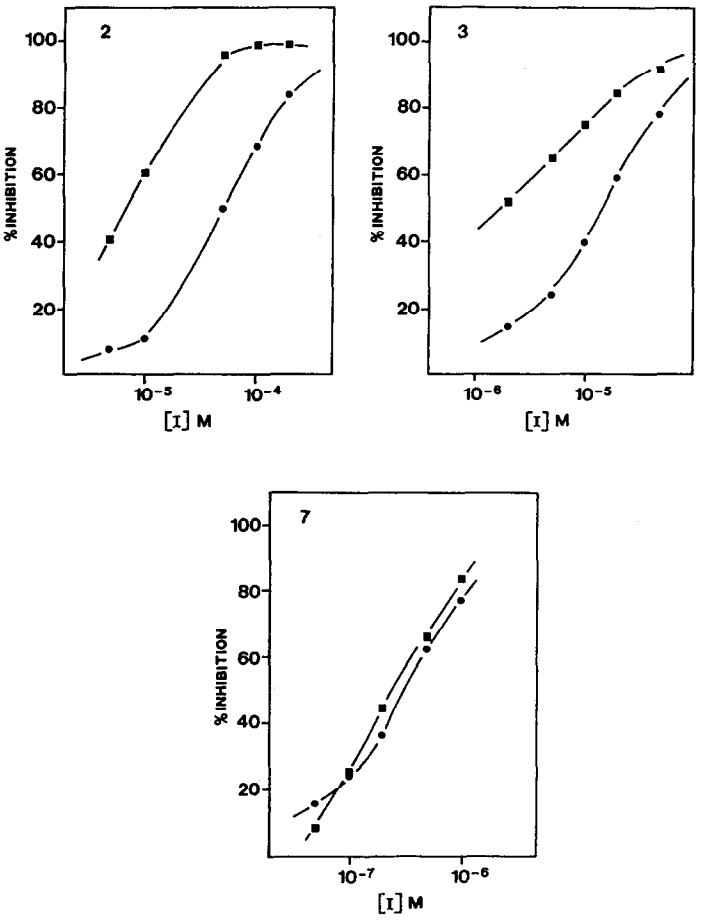


Fig. 1. Inhibition of COMT as a function of inhibitor concentration for compounds **2**, **3** and **7**. The inhibition of COMT was determined at different concentrations of inhibitor without pre-incubation (●) or with pre-incubation for 1 hr at 37° of a 62-fold concentrated mixture of enzyme and inhibitor (■). Plotted data are the mean values of two determinations.

those reported [21] for other 5-nitro derivatives with related structures, we have observed higher IC_{50} values; the same phenomena was reported with several dihydroxybenzaldehyde derivatives [24]. This may be due to a lower affinity of these compounds for the pig liver enzyme, used in this work, as compared with enzymes from other sources. In fact, the enzyme from pig liver shows a significantly lower affinity for catechol as the substrate ($K_m = 2 \times 10^{-3}$ M) [20] than the enzyme isolated from rat liver ($K_m = 4 \times 10^{-4}$ M) [27] or human placenta ($K_m = 4 \times 10^{-5}$ M) [32].

We decided to synthesize the 3-(3,4-dihydroxy-5-nitro-benzilidene)-2,4-pentanedione **13** (nitecapone), previously reported [8], and to assay it as inhibitor of COMT from pig liver under the conditions for compounds in Table 1. Compound **13** showed the same IC_{50} value as compound **5** and similar values as **7** and **9** (Table 1). This value was 800 nM, i.e. 40-fold higher than the value reported when rat brain COMT was used with 3,4-dihydroxybenzoic acid as acceptor substrate (IC_{50} = 18 nM) [21].

Our goal was to obtain new irreversible and selective inhibitors of COMT. To test if the inhibition of COMT was time dependent, the compounds were pre-incubated with the enzyme for 15 min at 37° and the remaining activity was determined. Surprisingly, the results showed no time dependence for most of the compounds (with the exception of **2** and **3**), suggesting a reversible inhibition of COMT. To confirm the reversibility of the process, a dilution assay was then carried out. According to these results, all compounds inhibited COMT reversibly, with the exception of **2** and **3** which behaved as apparent irreversible inhibitors. Figure 1 shows the results with some of these compounds (**2**, **3** and **7**).

To confirm that **2** and **3** inhibition of COMT was irreversible, the activity of a pre-incubated mixture of enzyme and inhibitor was measured before and after filtration through a Sephadex G25 column. While the inhibition afforded by **2** persisted after the gel filtration, we observed a partial recovery of the activity of COMT that had been pre-incubated with **3** (data not shown). These results suggest that **2** behaves as an irreversible inhibitor, while **3** seems

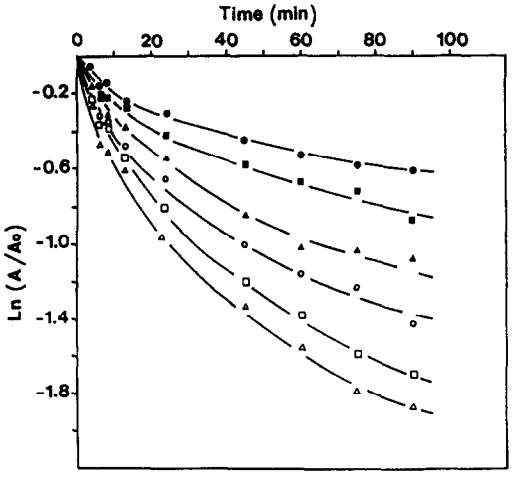


Fig. 2. Inactivation of COMT by **2** as a function of incubation time. COMT was incubated at 37° in 150 mM phosphate buffer pH 7.6 with different concentrations of **2**. Aliquots of 15 μL (containing 1 U of enzyme) were removed at determined time intervals and assayed for the remaining activity of COMT. The inhibitor concentrations were: (●) 0.07 mM; (■) 0.10 mM; (▲) 0.15 mM; (○) 0.20 mM; (□) 0.25 mM; (△) 0.30 mM. Plotted data are the mean values of four determinations.

to act as a reversible slow-dissociating inhibitor. Our interest was then centered on the inhibition of COMT by **2**.

Kinetics of the irreversible inhibition of COMT by **2**

In order to study the mechanism of the irreversible inactivation of COMT by **2**, the enzyme was pre-incubated with the inhibitor in the absence of DTT, and the remaining activity was determined after different lengths of time of pre-incubation after a 25-fold dilution. Figure 2 shows the inhibition of COMT as a function of the pre-incubation time in the presence of several concentrations of **2**. When the logarithm of the net percentage of remaining activity was plotted versus the pre-incubation time, biphasic kinetics were observed. According to Ray and Koshland [29], these kinetics suggest the reaction of **2** with two or more essential thiol groups [18–20]. However, it is also possible that the inhibitor modifies essential residues of different forms of COMT which could exist in pig liver, as has been shown to be the case in rat liver [33]. These kinetics obey the equation:

$$\ln A/A_0 = (1\text{-}F)e^{-(k_1+k_2)t} + Fe^{-k_2t}$$

where A is the remaining activity at time t and A_0 is the activity with no pre-incubation; k_1 and k_2 are the first-order apparent rate constants for modification of groups 1 and 2, respectively, and F is the fraction of active enzyme when the more reactive group (1) is completely modified.

The values of k_1 and k_2 were first estimated according to the graphical method of Borchardt and Thakker [18] and then calculated using a non-linear regression computer program. When the values of k_1 were plotted against the inhibitor concentration, a straight line with a positive intercept on the x-axis was obtained (Fig. 3A). This result suggested that the most reactive group was modified following a non-specific, random mechanism:

$$\text{COMT} + \text{I} \xrightarrow{k_a} \text{COMT*–I}$$

where k_a is the first-order rate constant of inactivation of group 1. Under our experimental conditions, the apparent first-order rate constant will be given by $k_1 = k_a[\text{I}]$, and k_a could be calculated from the slope of a plot of k_1 against inhibitor concentration (Table 2). The positive intercept on the x-axis may be due to the reaction of **2** with other thiol groups present on COMT (which are not essential for COMT activity) or with other contaminant proteins in the enzymatic preparation that are able to react with **2**. However, when the k_2 values were plotted against the concentration of inhibitor, saturation kinetics were observed (Fig. 3B), suggesting that **2** modified the slow-reacting group according to a specific mechanism, through the formation of an intermediate non-covalent complex:

$$\text{COMT} + \text{I} \overset{K_I}{\rightleftharpoons} \text{COMT–I} \xrightarrow{k_b} \text{COMT*–I}.$$

In this case, K_I is the steady-state constant for the dissociation of the reversible complex COMT–I and k_b is the first-order rate constant of the irreversible step. The apparent first-order rate constant (k_2) is related to the inhibitor concentration by the relationship: $k_2 = k_b[\text{I}]/(K_I + [\text{I}])$ [34]. This result is surprising in the sense that the group reacting more slowly is the one specifically modified. Contrary to this, our previous work [20] with a series of *N*-(4-hydroxy-3-methoxyphenylalkyl) maleimides showed that the more reactive group was or was not specifically modified, depending on the distance between and relative orientation of the aromatic ring and the reactive moiety of the inhibitor. However, the slow-reacting group was in all cases non-specifically modified [20]. Moreover, the reactivity of the maleimide ring with the essential thiol groups of COMT, measured in terms of the rate constant of modification, was much higher than the reactivity observed for **2**. This could be due to the different intrinsical properties of both SH-reacting double bonds. The differences in the reactivity of the two sulfhydryl groups with the above-mentioned maleimides and compound **2** seems difficult to explain. However, these compounds may be considered theoretically as analogues of the products of the enzymatic reaction with 3-O-methylation in the case of maleimide and 4-O-methylation in the case of compound **2**, bound to the enzyme with different spatial orientation of the reactive double bond.

Reaction of **2** *with DTT and reversible inhibition of COMT by the adduct DTT–***2**

The high reactivity of compound **2** toward thiol groups is shown by the fact that **2** reacts instantaneously and quantitatively with DTT, as observed by TLC, forming a stable adduct DTT–**2**.

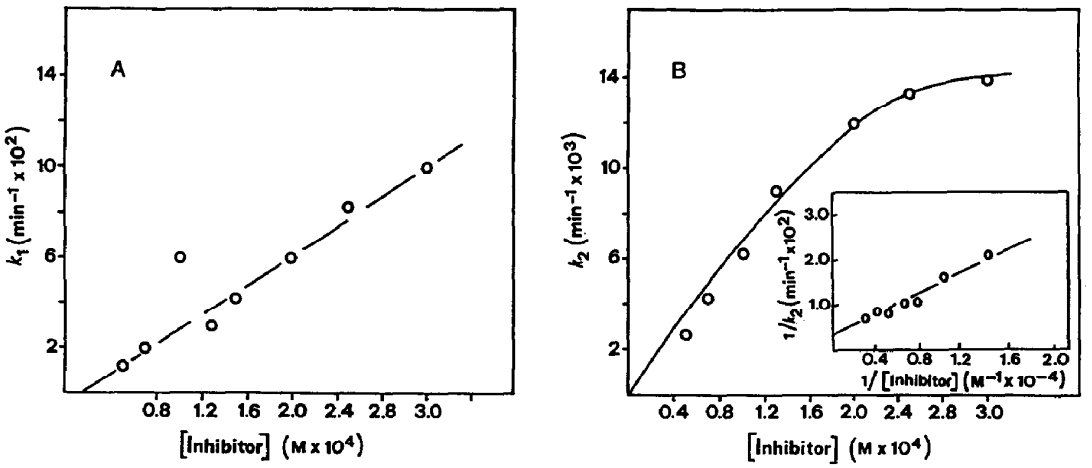


Fig. 3. Analysis of the first order rate constants for the modification of residues 1 and 2 as a function of the concentration of compound **2**. (A) Plot of k_{app} observed for group 1 (k_1) vs **2** concentration; k_a was calculated from the slope according to the equation $k_1 = k_a[I]$. (B) Plot of k_{app} for group 2 (k_2) vs **2** concentration; inset shows Lineweaver–Burk plot, and the values of k_b and K_I could be calculated from the ordinate axis intercept and the slope values, respectively.

Table 2. Irreversible inhibition of COMT by compound **2**

Group modified	Inhibition mechanism	Inhibition constant ± SE
1	Non-specific	$k_a = 5.57 \pm 0.34\ M^{-1} \times sec^{-1}$ $K_I = 4.73 \times 10^{-4} \pm 0.67\ M$
2	Specific	$k_b = 5.78 \times 10^{-4} \pm 0.58\ sec^{-1}$ $k_b/K_I = 1.22\ M^{-1} \times sec^{-1}$

Rate constants for the modification of both reactive groups of COMT by **2** were calculated from the data shown in fig. 2.

k_b/K_I gives an estimate of the global rate for the reversible and irreversible steps in the specific mechanism, for comparison with the rate constant observed for the non-specific process.

Data are the means of four experiments.

The inhibition of COMT by **2** was studied in the presence of a great excess of DTT in the enzymatic assay. As expected, the adduct DTT-**2** inhibits COMT following a reversible process. A mixed pattern was observed when pyrocatechol was the variable substrate ($K_i = 5.32 \times 10^{-5} \pm 0.92$ M; $K_{is} = 2.27 \times 10^{-6} \pm 0.21$ M) and an uncompetitive pattern when AdoMet was the variable substrate ($K_{is} = 1.79 \times 10^{-5} \pm 0.09$ M). With the parent aldehyde **1** the inhibition was non-competitive with pyrocatechol as the variable substrate ($K_I = 1.71 \times 10^{-5} \pm 0.23$ M) and uncompetitive ($K_I = 1.35 \times 10^{-6} \pm 0.07$ M) with AdoMet as the variable substrate [24]. This experiment shows first that the presence of DTT in the enzymatic assay protects the enzyme from irreversible inhibition by **2** and second that compound **2** needs its reactivity towards thiol groups for inhibiting the enzyme irreversibly.

Substrate protection studies

Substrate protection studies were carried out in order to elucidate further the nature of the interaction between **2** and the active site of COMT. When the inactivation rate of COMT by **2** was determined in the presence of saturating AdoMet, first order kinetics were observed (Fig. 4), with a slope ($k_{app} = 0.78 \times 10^{-3}\ min^{-1}$) lower than that obtained for the modification of thiol groups 1 or 2 in the absence of this substrate ($k_1 = 1.1 \times 10^{-1}\ min^{-1}$, $k_2 = 5.8 \times 10^{-3}\ min^{-1}$). This result suggests that **2**, in the presence of AdoMet, modifies just one thiol group of COMT, presumably group 1, with a rate constant lower than that obtained in the absence of the methyl donor.

In contrast to what was observed with the inactivation of COMT by some maleimide derivatives [19, 20], Mg^{2+} ions had no significant effect on the protection afforded by AdoMet (Table 3). The total protection of one thiol group (presumably group 2) by the substrate against modification by **2** suggested that this group is located at, or near to, the binding site of AdoMet. However, in the presence of 3,5-

Table 3. Substrate protection of COMT from inactivation by compound **2**

AdoMet (mM)	$MgCl_2$ (mM)	Residual activity (%) ± SE*
—	—	14.70 ± 1.14
—	0.2	11.79 ± 2.33
0.01	—	21.51 ± 0.40
0.20	—	40.90 ± 1.48
0.01	0.2	23.98 ± 2.16
0.02	0.2	34.24 ± 4.01

COMT was pre-incubated at 37° for 2 hr with 0.135 mM **2** in the presence of several concentrations of AdoMet and/or $MgCl_2$ before determination of the remaining activity.

* Residual activity was calculated with respect to the activity without pre-incubation.

The mean values of four independent experiments are shown.

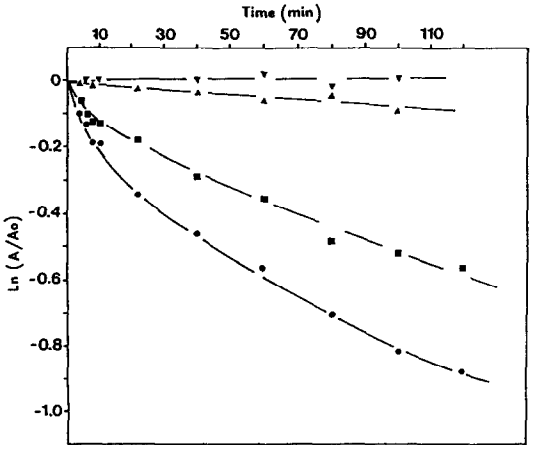


Fig. 4. Inactivation of COMT by compound **2** in the presence of AdoMet, DNC or both. COMT was pre-incubated as in Fig. 2, with 0.135 mM **2** alone (●) or in the presence of: 1.5 μM DNC (■); 2.5 mM AdoMet (▲) or 1.5 μM DNC plus 2 mM AdoMet (▼). Plotted data are the means of two determinations.

dinitrocatechol (DNC), a competitive and reversible inhibitor of COMT with respect to catechol as the variable substrate ($K_i = 30.8$ nM), some protection could be detected, although biphasic kinetics were still observed. The independent analysis of k_1 and k_2 showed a slight increase in k_1 (0.15 min^{-1}, in contrast to 0.1 min^{-1} in the absence of DNC), while the specifically slow-reacting thiol group was partially protected against modification by **2** ($k_2 = 3.9 \times 10^{-3}$ min^{-1}, compared with 5.8×10^{-3} min^{-1} with no DNC present). Again, the presence of Mg^{2+} ions had no effect on the result obtained (data not shown).

Remarkably, when the enzyme was incubated with **2** in the presence of both AdoMet and DNC (Fig. 4), either in the presence or in the absence of Mg^{2+}, inactivation of COMT was not observed; this suggested that not only the specifically slow-reacting thiol group but also the unspecifically modified one is located at, or near to, the active site of COMT. Thus, although compound **2** seems to "open" the active site making group 1 more easily accessible to the inhibitor while slightly protecting group 2, the presence of both AdoMet and DNC completely blocks the access of compound **2** to the active site of COMT. Similar results were obtained with *N*-(4-hydroxy-3-methoxyphenylalkyl)maleimides [20], suggesting that the same thiol groups were modified in both cases. A possible model of the active site of COMT would show group 2 located near the AdoMet binding site and group 1 located near the catechol or guaiacol binding sites. According to this, **2** could react specifically with group 2 after the formation of a reversible complex through the guaiacol site. However, once the reversible complex is formed, the double bond of **2** can not react with group 1, this reaction being only possible by a non-specific, random mechanism, with no formation of the reversible complex.

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