Dihydroxynitrobenzaldehydes and Hydroxymethoxynitrobenzaldehydes: Synthesis and Biological Activity as Catechol-*O*-methyltransferase Inhibitors

Rosa A. Pérez, Eldiberto Fernández-Alvarez,* Ofelia Nieto,* and F. Javier Piedrafita

Instituto de Química Orgánica General (CSIC), Juan de la Cierva, 3, 28006 Madrid, Spain

Received March 11, 1992

A series of nitro derivatives of dihydroxy- and hydroxymethoxybenzaldehyde was synthesized and tested as potential inhibitors of partially purified pig liver catechol-*O*-methyltransferase (COMT). All the dihydroxynitrobenzaldehydes prepared were potent inhibitors of COMT, but only one hydroxymethoxynitrobenzaldehyde (3-hydroxy-4-methoxy-5-nitrobenzaldehyde) showed activity as a COMT inhibitor. Although previously reported data showed that the presence of electron-withdrawing substituents at position 5 seemed to be very important for activity as COMT inhibitor, our results suggest that the requirement necessary to enhance the activity of the dihydroxynitrobenzaldehyde derivatives toward COMT is the presence of the nitro group in a position ortho with respect to one hydroxyl group. The assayed compounds showed a reversible inhibition of COMT, which was mixed for all the dihydroxynitro derivatives but noncompetitive for 3-hydroxy-4-methoxy-5-nitrobenzaldehyde when pyrocatechol was the variable substrate and uncompetitive in all the inhibitors with respect to *S*-adenosyl-L-methionine.

1. Introduction

Catechol-*O*-methyltransferase (EC 2.1.1.6, COMT) is a magnesium-requiring enzyme that catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to a hyroxyl group of a catechol substrate.[1-3] The enzyme plays an important role in the extraneural inactivation of endogenous catecholamines and the detoxification of many xenobiotic catechols.[4,5] The alteration of normal levels of physiologically active catecholamines has been related with several clinical disorders, among them the Parkinson's disease. In the current therapy of Parkinson's disease,[6] the drug of choice is still L-dopa in combination with peripheral decarboxylase inhibitors (carbidopa and benserazide),[7,8] as well as MAO inhibitors (L-deprenyl)[9-11] and direct dopamine receptor agonists (bromocriptine, lisuride, pergolide).[12-14] However, when dopa decarboxylase is inhibited, the 3-O-methylation route becomes dominant and 3-*O*-methyldopa (3-OMD) is accumulated in the peripheral systems.[15] Several clinical observations have shown that high plasma levels of 3-OMD are associated with a poor response to L-dopa. Thus, the inhibition of COMT would have a beneficial effect in the treatment of Parkinson's disease, by improving the bioavailability of L-dopa and reducing the plasma 3-OMD levels.[16] Since the discovery of COMT, many compounds that inhibit the enzyme in vitro and in vivo have been described.[4,17-19] However, most of them were shown to be not very effective and in general highly toxic in vivo. Thus, there were no COMT inhibitors with acceptable pharmacological properties for clinical use.[4] Recently two research groups independently reported the development of potent and selective COMT inhibitors with a 5-nitrocatechol structure.[20,21] It is known that electron-accepting substituents, especially strong π-electron acceptors at positions 1 and

* Address correspondence to Dr. Ofelia Nieto or Dr. E. Fernández-Alvarez, Instituto de Química Orgánica General (CSIC), Juan de la Cierva, 3, 28006 Madrid, Spain. Phone: (34-1) 5622900. Fax: (34-1) 5644853.

(1) Axelrod, J.; Tomchick, R. Enzymatic O-methylation of epinephrine and other catechols. *J. Biol. Chem.* **1958**, *233*, 702-705.

(2) Molinoff, P. B.; Axelrod, J. Biochemistry of catecholamines. *Annu. Rev. Biochem.* **1971**, *40*, 465-500.

(3) Borchardt, R. T. N- and O-methylation [reactions with enzymes]. *Enzymatic Basis of Detoxication*; Jacoby, Williams, Eds.; Wiley: New York, 1980; Vol. 2 pp 43-63.

(4) Guldberg, H. C.; Marsden, C. A. Catechol-O-methyltransferase: Pharmacological aspects and physiological role. *Pharmacol. Rev.* **1975**, *27*, 135-206.

(5) Kopin, I. J. Catecholamine metabolism: Basic aspects and clinical significance. *Pharmacol Rev.* **1985**, *37*, 335-364.

(6) Da Prada, M.; Keller, H. H.; Pieri, L.; Kettler, R.; Haefely, W. E. The pharmacology of Parkinson's disease: Basic aspects and recent advances. *Experientia* **1984**, *40*, 1165-1172.

(7) Bartholini, G.; Pletscher, A. Decarboxylase inhibitors. *Pharmacol. Ther.* **1975**, *1*, 407-421.

(8) Papavasiliou, P. S.; Cotzias, G. C.; Duby, S. E. Levodopa in Parkinsonism: Potentiation of central effects with a peripheral inhibitor. *N. Engl. J. Med.* **1972**, *285*, 8-14.

(9) Birkmayer, W. Long-term treatment with L-deprenyl. *J. Neural Transm.* **1978**, *43*, 239-244.

(10) Lees, A. J.; Kohout, L. J.; Shaw, K. M.; Stern, G. M.; Elsworth, J. D.; Sandlerk, M.; Youdim, M. B. H. Deprenyl in Parkinson's disease. *Lancet* **1977**, *2*, 65-69.

(11) Rinne, U. K.; Siirtola, T.; Sonninen, V. L-Deprenyl treatment of on-off phenomena in Parkinson's disease. *J. Neural Transm.* **1978**, *43*, 253-262.

(12) Fahn, S.; Cote, L. J.; Snider, S. R.; Barrett, R. E.; Isgreen, W. P. Role of bromocriptine in the treatment of Parkinsonism. *Dopaminergic ergot derivatives and motor function*; Fluxe, K., Calne, D. B., Eds.; Wenner-Gren Center International Symposium, Pergamon Press: Oxford, 1979; Vol. 31, pp 303-312.

(13) Lieberman, A.; Goldstein, M.; Neophytides, A.; Kupersmith, M.; Leibowitz, M.; Zasorin, N.; Walker, R.; Kleinberg, D. Lisuride in Parkinson disease: Efficacy of lisuride compared to levodopa. *Neurology* **1981**, *31*, 961-965.

(14) Lieberman, A.; Goldstein, M.; Leibowith, M.; Neophytides, A.; Kupersmith, M.; Pact, V.; Kleinberg, D. Treatment of advanced Parkinson disease with pergolide. *Neurology* **1981**, *31*, 675-682.

(15) Nutt, J. G.; Fellman, J. H. Pharmacokinetics of levodopa. *Clin. Neuropharmacol.* **1984**, *7*, 35-49.

(16) Männistö, P. T.; Kaakkola, S. Rationale for selective COMT inhibitors as adjuncts in the drug treatment of Parkinson's disease. *Pharmacol. Toxicol.* **1990**, *66*, 317-323.

(17) Nikodejevic, B.; Senoh, S.; Daly, J. W.; Creveling, C. R. Catechol-O-methyltransferase. II. A new class of inhibitors of catechol-O-methyltransferase; 3,5-dihydroxy-4-methoxybenzoic acid and related compounds. *J. Pharm. Exp. Ther.* **1970**, *174*, 83-93.

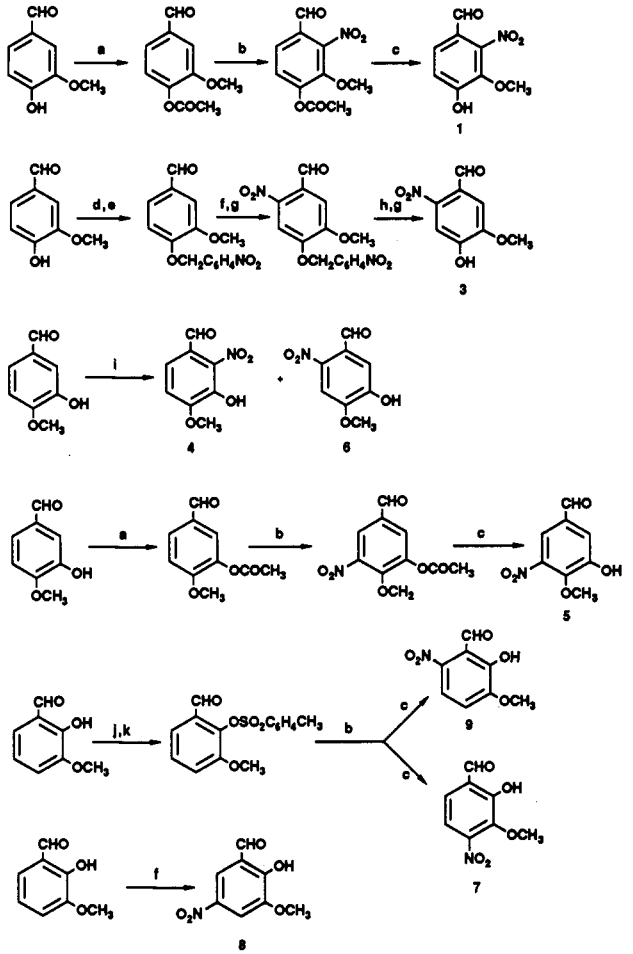
(18) Borchardt, R. T. Catechol-O-methyltransferase. 4. *In vitro* inhibition by 3-hydroxy-4-pyrones, 3-hydroxy-2-pyridones and 3-hydroxy-4-pyridones. *J. Med. Chem.* **1973**, *16*, 581-583.

(19) Borchardt, R. T.; Huber, J. A.; Houston, M. Catechol-O-methyltransferase. 10. 5-substituted 3-hydroxy-4-methoxybenzoic acids (isovanillin acids) and 5-substituted 3-hydroxy-4-methoxybenzaldehydes (isovanillins) as potential inhibitors. *J. Med. Chem.* **1982**, *25*, 258-263.

(20) Bäckström, R.; Honkanen, E.; Pippuri, A.; Kairisalo, P.; Pystynen, J.; Heinola, K.; Nissinen, E.; Linden, I. B.; Männistö, P. T.; Kaakkola, S.; Pohto, P. Synthesis of some novel potent and selective catechol-O-methyltransferase inhibitors. *J. Med. Chem.* **1989**, *32*, 841-846.

0022-2623/92/1835-4584$03.00/0

Scheme I[a]



[a] (a) KOH, Ac_2O; (b) fuming HNO_3; (c) Hydrolysis; (d) $BrCH_2C_6H_4pNO_2$, K_2CO_3; (e) H_2O; (f) HNO_3, AcOH; (g) H_2O, 0 °C; (h) H_2SO_4; (i) 1.2 equiv of fuming HNO_3, acetone; (j) NaOH, H_2O; (k) $CH_3C_8H_4SO_2Cl$.

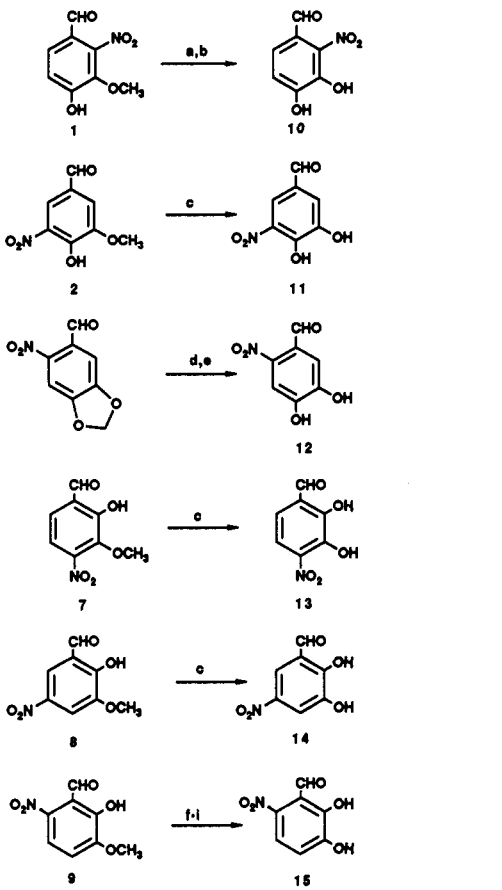
5, increase the activity as inhibitor and the selectivity toward COMT.[19,21]

Our purpose was to synthesize and evaluate the biological activity of several dihydroxy- and hydroxymethoxynitrobenzaldehyde derivatives and investigate how the position of the nitro and aldehyde groups, relative to both hydroxyl groups, influences the in vitro activity as COMT inhibitors. The results obtained show that all the nitrocatechol derivatives were active as COMT inhibitors, but the presence of the nitro group in a position ortho with respect to one of both hydroxyl groups increases the activity of these compounds toward COMT. In the case of the guaiacol analogues, only the 3-hydroxy-4-methoxy-5-nitrobenzaldehyde showed activity as COMT inhibitor (Schemes I and II).

2. Chemistry

The benzaldehydes studied as COMT inhibitors have been prepared as described in the literature or by standard procedures; 4-hydroxy-3-methoxy-5-nitrobenzaldehyde has been obtained from a commercial supplier. The starting material for the synthesis of all the compounds was the appropriate hydroxymethoxybenzaldehyde. The

Scheme II[a]



[a] (a) $AlCl_3$, pyr, CH_2Cl_2; (b) HCl, H_2O; (c) HBr, AcOH, 150 °C; (d) $AlCl_3$, $Cl_2(CH_2)_2$; (e) HBr; (f) CH_2Cl_2; (g) H_2O; (h) NaOH; (i) HCl.

hydroxymethoxynitrobenzaldehydes were synthesized by direct nitration of the proper benzaldehyde or by protection of the hydroxyl group, followed by nitration and cleavage of the protecting group, as detailed below. An additional process of demethylation gave the corresponding dihydroxy derivative.

2.1. Hydroxymethoxynitro Derivatives. 3-Hydroxy-4-methoxy-2-nitrobenzaldehyde[24] (4), 3-hydroxy-4-methoxy-6-nitrobenzaldehyde[24] (6), and 2-hydroxy-3-methoxy-5-nitrobenzaldehyde[26] (8) were obtained by direct nitration of the corresponding aldehyde.

By acetylation of the hydroxyl group of 4-hydroxy-3-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, and 2-hydroxy-3-methoxybenzaldehyde, the respective acetyl derivatives were obtained. Nitration of these and subsequent deacetylation gave 4-hydroxy-3-methoxy-2-nitrobenzaldehyde[22] (1), 3-hydroxy-4-methoxy-5-nitrobenzaldehyde[24] (5), and 2-hydroxy-3-methoxy-5-nitrobenzaldehyde[26] (7), respectively.

Protection of 4-hydroxy-3-methoxybenzaldehyde with 4-nitrobenzyl bromide, nitration, and removal of the protecting group gave the 6-nitro derivative[23] (3).

(21) Borgulya, J.; Bruderer, H.; Bernauer, K.; Zürcher, G.; Da Prada, M. Catechol-O-methyltransferase-inhibiting pyrocatechol derivatives: synthesis and structure–activity studies. *Helv. Chim. Acta* **1989**, *72*, 952–968.

(22) Julia, M.; Manoury, P.; Voillaume, C. *Bull. Soc. Chim. Fr.* **1965**, 1417–1423.

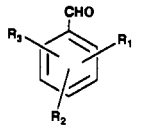
(23) Velayudhan Nair, P.; Robinson, R. The nitration of O-benzylvanillin. *J. Chem. Soc.* **1932**, 1236–1239.

(24) Pschorr, R.; Stöhrer, W. *Ber. Dtsch. Chem. Ges.* **1902**, *35*, 4393–4399.

(25) Dey, B. B.; Ammalu Kutti, V. The nitration and halogenation of 8-methoxy- and 8-hydroxy-coumarins. *Proc. Natl. Inst. Sci. India* **1940**, *VI*, No. 4, 641–669.

(26) Davies. *J. Chem. Soc.* Substitution in vicinal trisubstituted benzene derivatives. Part I. **1923**, *123*, 1583.

Table I. Structure and COMT Inhibitory Activity of the Compounds Synthesized



compd	R_1	R_2	R_3	ref	% yield	mp, °C	lit. mp, °C	formula	anal.	COMT inhibitory activity, IC_{50}, M
1	2-NO_2	3-OCH_3	4-OH	22	55	134–136	135	$C_8H_7O_5N$	C,H,N	
2	5-NO_2	3-OCH_3	4-OH	*a*			177–179	$C_8H_7O_5N$		
3	6-NO_2	3-OCH_3	4-OH	22, 23	37	208	209	$C_8H_7O_5N$	C,H,N	
4	2-NO_2	3-OH	4-OCH_3	24	27	143–145	148–149	$C_8H_7O_5N$	C,H,N	
5	5-NO_2	3-OH	4-OCH_3	24	30	110–113	113	$C_8H_7O_5N$	C,H,N	3.4×10^{-5}
6	6-NO_2	3-OH	4-OCH_3	24	51	185	189	$C_8H_7O_5N$	C,H,N	
7	4-NO_2	2-OH	3-OCH_3	25[b]	26	89–92	92–93	$C_8H_7O_5N$	C,H,N	
8	5-NO_2	2-OH	3-OCH_3	26	63	137–138	140	$C_8H_7O_5N$	C,H,N	
9	6-NO_2	2-OH	3-OCH_3	22[b]	37	101–103	104	$C_8H_7O_5N$	C,H,N	
10	2-NO_2	3-OH	4-OH	27	41[c]	172–175	175–178	$C_7H_5O_5N$	C,H,N	1.5×10^{-6}
11	5-NO_2	3-OH	4-OH	28[b]	61	145–146	145–148	$C_7H_5O_5N$	C,H,N	2.0×10^{-6}
12	6-NO_2	3-OH	4-OH	28	88	196–198	203–204	$C_7H_5O_5N$	C,H,N	8.5×10^{-6}
13	4-NO_2	2-OH	3-OH	*b*	17[c]	128–130		$C_7H_5O_5N$	C,H,N	1.5×10^{-6}
14	5-NO_2	2-OH	3-OH	29	47[c]	230–233	230–233	$C_7H_5O_5N$	C,H,N	2.1×10^{-6}
15	6-NO_2	2-OH	3-OH	*b*	8[c]	120–124		$C_7H_5O_5N$	C,H,N	1.0×10^{-5}

[a] Commercial, recrystallized. [b] Experimental Section. [c] Yield from the corresponding hydroxymethoxybenzaldehyde.

2-Hydroxy-3-methoxy-4-nitro- and 2-hydroxy-3-methoxy-6-nitrobenzaldehydes[25,22] (7 and 9, respectively) were prepared from 2-hydroxy-3-methoxybenzaldehyde, by protection with *p*-toluenesulfonyl chloride, nitration, and cleavage of the tosyl group. All these reactions are shown in Scheme I.

2.2. Dihydroxynitro Derivatives. These compounds have been prepared from the appropriate hydroxymethoxynitrobenzaldehyde precursor, by cleavage of the ether group using known methods,[30] except for 3,4-dihydroxy-6-nitrobenzaldehyde[28] (2), which was obtained by deprotection of the methylenedioxy group of 6-nitropiperonal (Scheme II).

3. Biological Results

The in vitro COMT inhibitory activities of the synthesized compounds were determined with a partially purified enzyme isolated from pig liver, with pyrocatechol as the methyl-acceptor substrate. In a first scanning, the inhibition percentage was measured as a function of the inhibitor concentration, without and with preincubation, for 15 min at 37 °C, of a mixture of enzyme and inhibitor. Table I shows the structure of the compounds and the IC_{50} values for the in vitro COMT inhibition by the active compounds. All the hydroxymethoxynitrobenzaldehyde derivatives, with the exception of 5, showed no inhibition of COMT at a concentration of 5×10^{-5} M and were rejected for additional studies. The results obtained with the active derivatives showed that the inhibition of COMT by all the compounds was not time-dependent, suggesting a reversible process; this assumption was confirmed by dialysis of a preincubated mixture of enzyme and inhibitor (not shown).

The inhibition of COMT activity by compounds 11 and 14 was previously described in the literature[20,21] and we use them to refer to the activity of our new COMT inhibitors. Comparing the data from Table I with the published results, we could observe a great difference in the IC_{50} values for 11 and 14: 2×10^{-6} M in contrast to 2×10^{-8} and 8×10^{-8} M for the compound 11[20,21] and 2×10^{-6} M in contrast to 1.6×10^{-7} M for 14.[21] This variation in the IC_{50} values could be because of the different source of enzyme used in our study, pig liver instead of rat liver. We have determined a K_m for the catechol substrate of $2.2 \times 10^{-3} \pm 0.2$ M,[31] a value 5 times higher than that observed with rat liver COMT,[32] the enzyme used in similar inhibition studies,[20,21] and 50-fold that obtained with the human placental enzyme.[33] Table II shows the kinetic constants for AdoMet and several catechol substrates for COMT from different sources, according to different authors. Moreover, the nature of the catechol substrate may influence the IC_{50} value; compare the IC_{50} determined for compound 11 with that of 3,4-dihydroxybenzoic acid as substrate for COMT (2×10^{-8} M)[20] and pyrocatechol (8×10^{-8} M).[21]

Table II. Kinetic Constant Values for AdoMet and the Catechol Substrate Obtained in Different Studies

K_m, M				
AdoMet	catechol	catechol substrate	COMT	ref
10^{-4}	2×10^{-3}	catechol	pig liver	31
4×10^{-4}	4×10^{-4}	catechol	rat liver	32
5×10^{-5}	4×10^{-5}	catechol	human placenta	33
3×10^{-6}	4×10^{-4}	adrenaline	rat liver	34
10^{-4}	6×10^{-4}	adrenaline	rat liver	35
3×10^{-5}	9×10^{-5}	3,4-dihydroxybenzoic acid	rat liver	36
2×10^{-5}	7×10^{-5}	3,4-dihydroxybenzoic acid	rat brain	37
9×10^{-6}	3×10^{-4}	dopamine	human brain	38
3×10^{-6}	3×10^{-6}	dopamine	human brain[a]	39

[a] Membrane-bound COMT.

The inhibition pattern was then examined for the active nitrobenzaldehyde derivatives, as a function of the py-

(27) Lee, F. G. H.; Dickson, D. E.; Suzuki, J.; Zirnis, A.; Manian, A. A. Synthesis of 5,7- and 6,7-disubstituted typtamines and analogs (1). *J. Heterocycl. Chem.* **1973**, *10*, 649–654.
(28) Hayduck, F. *Ber. Dtsch. Chem. Ges.* **1903**, *36*, 2930–2935.
(29) Kemp, D. S.; Wang, S. W.; Mollan, R. C.; Hsia, S. L.; Confalone, P. N. Peptide synthesis with benzisoxazolium salts I. Properties of substituted 2-ethyl-benzisoxazolium salts. *Tetrahedron* **1974**, *30*, 3677–3688.
(30) Bhatt, M. V.; Kulkarni, S. U. Cleavage of ethers. *Synthesis* **1983**, 249–282.
(31) Piedrafita, F. J.; Fernández-Alvarez, E.; Nieto, O.; Tipton, K. F. Kinetic and inhibition studies on catechol-O-methyltransferase. Affinity labeling by N-(3,4-dihydroxyphenyl)maleimide. *Biochem. J.* **1992**, *286*, part 3, 951–958.
(32) Zürcher, G.; Da Prada, M. Rapid and sensitive single-step radiochemical assay for catechol-O-methyltransferase. *J. Neurochem.* **1982**, *38*, 191–195.
(33) Nic a'Bháird, N.; Tipton, K. F. Behavior and properties of catechol-O-methyltransferase from human placenta. *J. Neural. Transm.* (Suppl.) **1990**, *32*, 359–368.

Table III. Reversible Inhibition of COMT by Nitrobenzaldehydes Derivatives

inhibitor	AdoMet[c]	catechol[c]	inhibn[a]	inhibn constant ± SE,[b] M
	variable	saturating	UC	$K_I = 1.35 \times 10^{-5} \pm 0.07$
5	saturating	variable	NC	$K_I = 1.72 \times 10^{-5} \pm 0.23$
	variable	saturating	UC	$K_{IS} = 6.78 \times 10^{-7} \pm 0.29$
10	saturating	variable	M	$K_{IS} = 4.97 \times 10^{-7} \pm 1.78$
				$K_{II} = 1.68 \times 10^{-7} \pm 0.27$
	variable	saturating	UC	$K_{IS} = 6.61 \times 10^{-7} \pm 0.22$
11	saturating	variable	M	$K_{IS} = 8.16 \times 10^{-7} \pm 0.20$
				$K_{II} = 1.38 \times 10^{-7} \pm 0.96$
	variable	saturating	UC	$K_{II} = 3.93 \times 10^{-6} \pm 0.14$
12	saturating	variable	M	$K_{IS} = 2.69 \times 10^{-6} \pm 0.36$
				$K_{II} = 9.21 \times 10^{-7} \pm 0.70$
	variable	saturating	UC	$K_{IS} = 5.38 \times 10^{-7} \pm 0.21$
13	saturating	variable	M	$K_{IS} = 4.78 \times 10^{-7} \pm 0.84$
				$K_{II} = 1.45 \times 10^{-7} \pm 0.12$
	variable	saturating	UC	$K_{IS} = 7.77 \times 10^{-6} \pm 0.54$
14	saturating	variable	M	$K_{IS} = 1.20 \times 10^{-6} \pm 0.17$
				$K_{II} = 2.31 \times 10^{-7} \pm 0.13$
	variable	saturating	UC	$K_{IS} = 1.79 \times 10^{-6} \pm 0.13$
15	saturating	variable	M	$K_{IS} = 4.77 \times 10^{-6} \pm 1.36$
				$K_{II} = 9.09 \times 10^{-7} \pm 1.07$

[a] UC, uncompetitive inhibition; NC, noncompetitive inhibition; M, mixed. [b] SE refers to the standard error of the calculated values. [c] The K_m values for catechol and AdoMet determined with the enzyme used in this study were $2.2 \times 10^{-3} \pm 0.2$ M and $1.0 \times 10^{-4} \pm 0.1$ M, respectively.[31]

rocatechol and AdoMet concentrations. As Table III shows, all the compounds exhibited uncompetitive inhibition patterns when measured as a function of AdoMet concentration. The guaiacol analogue, compound 5, showed a noncompetitive inhibition of COMT in regard to catechol. Surprisingly, the catechol analogues showed a mixed inhibition pattern with respect to the pyrocatechol substrate. The presence of one or two nitro groups in the catechol ring does not alter the competitive inhibition pattern of COMT (data not shown). Thus, our results with the dihydroxynitrobenzaldehydes might be due to the presence of an aldehyde group at position 1 of the aromatic ring, which can alter the mode of interaction of the inhibitor with the active site of COMT.

Our results on the kinetic behavior of 3-hydroxy-4-methoxy-5-nitrobenzaldehyde (5) are in disagreement with those of Borchardt et al.,[19] which showed a competitive inhibition of partially purified rat liver COMT with respect to 3,4-dihydroxybenzoic acid as substrate. Under our conditions, compound 5 showed a noncompetitive inhibition of pig liver COMT with pyrocatechol as substrate (Figure 1A). This result is in agreement with that obtained with other analogues of the product guaiacol,[17,19,31] which showed a noncompetitive or mixed interaction mode with COMT. As in the other cases, compound 5 showed an uncompetitive inhibition of the enzyme in regard to AdoMet (Figure 1B).

The kinetic data obtained for these nitrobenzaldehydes showed that all the dihydroxynitrobenzaldehydes synthesized were potent inhibitors of COMT and their activity was dependent on the position of the nitro group. The substitution of the position 5 by a nitro group is not critical for a maximal activity, and we have shown that the compounds with the nitro group in a position ortho relative to one hydroxyl group (10, 11, 13) were more active as COMT inhibitors than other derivatives tested.

4. Experimental Section

4.1. Biological Methods. 4.1.1. COMT Isolation. COMT was obtained from pig liver according to the methods of Nikodejevic et al.[17] The whole process was performed at 0–4 °C.

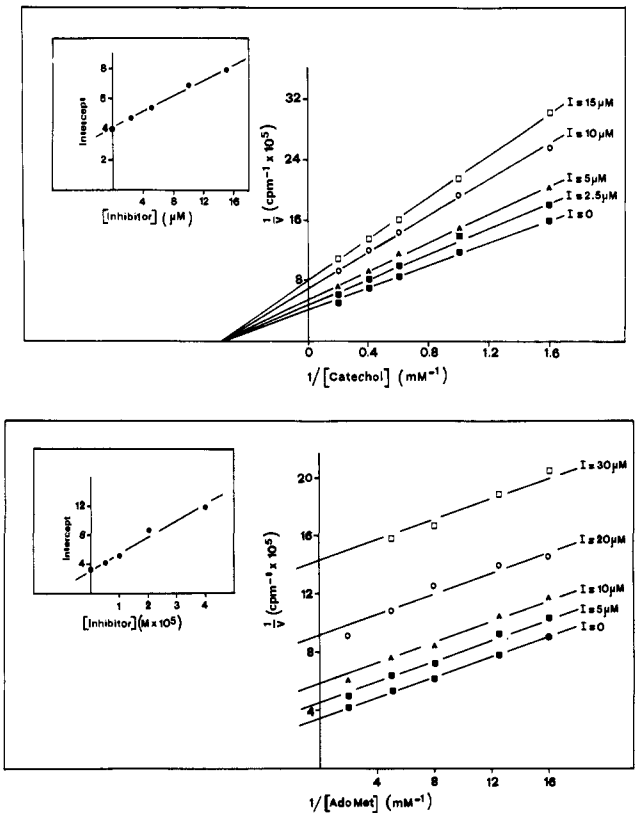


Figure 1. Inhibition of COMT by 3-hydroxy-4-methoxy-5-nitrobenzaldehyde (5). (A, top) Reciprocal plot of initial velocity versus the catechol concentration at saturation of AdoMet and different concentrations of inhibitor. Assay conditions are outlined under Experimental Section. (B, bottom) Double-reciprocal plot with AdoMet as the variable substrate and saturation by catechol.

The tissue was homogenized in 2% KCl (w/v) and centrifuged at 12000*g* for 30 min. After discarding the pellet, the supernatant was adjusted to pH 5.0 with 1 M acetic acid and stirred for 15 min before centrifugation as above. The new supernatant was neutralized with 1 M NaOH and fractionated with 30–50% of $(NH_4)_2SO_4$. The protein precipitated at 50% ammonium sulfate was sedimented at 17000*g* for 20 min, resuspended in 10 mM phosphate buffer, pH 7.0, and desalted through a Sephadex G25 column equilibrated with the same buffer. Finally, the eluate was concentrated to about 50 mL and applied to a Sephadex G200 column (5 × 90 cm); the protein was eluted with 10 mM phosphate buffer, pH 7.0, and tested for COMT activity and UV absorbance at 280 nm. This COMT preparation was stable for several months, if frozen at −30 °C. The protein concentration was measured using the procedure of Goa.[40]

4.1.2. COMT Assay. The activity of COMT was monitored using the one-step radiochemical method of Zürcher and Da Prada[32] modified by us.[31] In a final volume of 0.25 mL, the

(34) Flohe, L.; Schwabe, K. P. Kinetics of purified catechol-O-methyltransferase. *Biochim. Biophys. Acta* **1970**, *220*, 469–476.
(35) Corward, J. F.; Slisz, E. P.; Wu, F. Y.-H. Kinetic studies on catechol-O-methyl transferase. Product inhibition and the nature of the catechol binding site. *Biochemistry* **1973**, *12*, 2291–2297.
(36) Borchardt, R. T. Catechol-O-methyltransferase. 1. Kinetics of tropolone inhibition. *J. Med. Chem.* **1973**, *16*, 377–382.
(37) Tunnicliff, G.; Ngo, T. T. Kinetics of rat brain soluble catechol-O-methyltransferase and its inhibition by substrate analogs. *Int. J. Biochem.* **1983**, *15*, 733–738.
(38) Jeffery, D.; Roth, J. A. Purification and kinetic mechanism of human brain soluble catechol-O-methyltransferase. *J. Neurochem.* **1985**, *44*, 881–885.
(39) Rivett, A. J.; Roth, J. A. Kinetic studies of the O-methylation of dopamine by human brain membrane-bound catechol-O-methyltransferase. *Biochemistry* **1982**, *21*, 1740–1742.
(40) Goa, J. A microburet method for protein determination. Determination of total protein in cerebrospinal fluid. *Scand. J. Clin. Lab. Invest.* **1953**, *5*, 218–222.

reaction mixture contained, unless otherwise stated, enzyme (1 unit, defined as the amount of enzyme which catalyzes the transformation of 1 nmol of substrate/min at saturation for both substrates and magnesium ions), 20 mM pyrocatechol (Fluka), 0.9 mM [^{3}H]AdoMet 15 Ci/mmol (Amersham, Boehringer Mannheim), 1.5 mM $MgCl_2$, 2.5 mM DTT, and 125 mM phosphate buffer, pH 7.6. The reaction mixture was incubated at 37 °C for 10 min and stopped by the addition of 0.25 mL of 1 M ice-cold citric acid. Blanks were prepared without enzyme. A 1.5-mL portion of hexane–toluene (4:1), containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene, was then added to each mixture, which was vigorously vortexed for 30 s. The radioactivity present in the organic phase was directly counted in a LKB Rackbetta scintillation counter.

It was verified that in all the experiments reported in this paper the percent conversion of the substrates to products was always less than 5%, and generally less than 4%, of the substrate used. Moreover, the formation of product was shown to be linear with the protein (enzyme) concentration and the time of incubation above described, ensuring that initial velocities were measured and the Michaelis–Menten assumptions were satisfied. The pyrocatechol, AdoMet, and Mg^{2+} concentrations given above represent saturating conditions.

4.1.3. Analysis of the Kinetic Data. All kinetic data were first analyzed graphically, by plotting the reciprocal velocities against reciprocal of the substrate concentrations. In all cases, a linear relationship was obtained. The calculation of the kinetic constants was performed on a personal computer using the Fortran IV program described by Cleland.[41]

4.2. Chemical Methods. Column chromatography was performed with silica gel 60 (230–240 mesh, Merck). Melting points are uncorrected and were determined with a Kofler thermopan. IR spectra were recorded with a Perkin-Elmer 681 spectrometer and are reported in cm^{-1}. ^{1}H-NMR spectra were measured with Varian EM-390 (90 MHz) and Varian XL-300 (300 MHz) instruments; δ values are in ppm relative to the internal standard TMS; coupling constants (J) are in Hz. MS were determined with a Perkin-Elmer 240 spectrometer. Reagents and products were dried over P_2O_5 in vacuo. 4-Hydroxy-3-methoxy-5-nitrobenzaldehyde **2** was obtained from Aldrich Chemical Co.

4.2.1. 2-Hydroxy-3-methoxy-4-nitro- and 2-Hydroxy-3-methoxy-6-nitrobenzaldehydes (7 and 9). 3-Methoxy-2-[(*p*-Tolylsulfonyl)oxy]benzaldehyde (16). To a stirred solution of 2-hydroxy-3-methoxybenzaldehyde (3 g, 19.7 mmol) in water (10 mL) and sodium hydroxide (1 g) was added dropwise *p*-toluenesulfonyl chloride (4.5 g) in dichloromethane (6 mL). The mixture was stirred at room temperature for 24 h. After separation of the phases, the organic layer was washed with 6 N hydrochloric acid (2 × 5 mL), a saturated solution of sodium hydrogen carbonate (1 × 5 mL), and brine (1 × 5 mL). The combined aqueous layer was extracted with dichloromethane (5 × 10 mL), and the combined organic solutions were dried and evaporated. The residue was purified by flash chromatography on silica gel with ethyl acetate–hexane (4:1) to afford the corresponding tosyl derivative (5.31 g, 88%). Mp: 93–95 °C. IR: 1700, 1200, 1380, 1180. ^{1}H NMR (90 MHz, $CDCl_3$): 2.4 (s, 3 H), 3.6 (s, 3 H), 7.0–7.9 (m, 7 H), 10.1 (s, 1 H).

2-Hydroxy-3-methoxy-4-nitro- and 2-Hydroxy-3-methoxy-6-nitrobenzaldehydes (7 and 9). Tosyl derivative **16** (5.2 g, 17.0 mmol) was rapidly added to fuming nitric acid (52 mL, d = 1.5 g cm^{-1}) which was stirred at 0 °C. After 30 min the mixture was poured into ice–water. The precipitate was collected by filtration, washed with water, and dried, affording a mixture of nitro derivates. Column chromatography of this crude mixture, using ethyl acetate–hexane (1:1) as eluent, gave 3.26 g of 6-nitro tosyl derivative as a pale yellow solid and 2.70 g of a mixture of the 4-nitro derivative and products of its nitration on the tosyl group, as a light yellow oil.

The solution of the 6-nitro derivate (7.8 mmol) obtained in the previous process, in methanol (8 mL), was refluxed for 30 min. This process gave a red precipitate which was filtered and the solution was acidified with concentrated hydrochloric acid. The solid was collected by filtration, washed with water, and dried, affording 2-hydroxy-3-methoxy-6-nitrobenzaldehyde (**9**, 1.4 g, 91%). Mp: 101–103 °C. IR: 3640–3200, 1655, 1515, 1330. ^{1}H NMR (90 MHz, $CDCl_3$): 4.0 (s, 3 H), 7.0 (d, 1 H, J = 9 Hz), 7.7 (d, 1 H, J = 9 Hz), 10.5 (s, 1 H).

The same treatment of the 4-nitro derivatives mixture did not give a precipitate. The solution was diluted with water and acidified with hydrochloric acid. When the mixture was cooled, a yellow precipitate could be obtained, which was filtered and dried, giving 2-hydroxy-3-methoxy-4-nitrobenzaldehyde (**7**, 1.0 g). Mp: 89–92 °C. IR: 3640–3000, 1670, 1530, 1300, 1275. ^{1}H NMR (90 MHz, DMSO-d_6): 3.9 (s, 3 H), 7.5 (d, 1 H, J = 9 Hz), 7.6 (d, 1 H, J = 9 Hz), 10.3 (s, 1 H).

4.2.2. 3,4-Dihydroxy-5-nitrobenzaldehyde (11). The mixture formed by 4-hydroxy-3-methoxy-5-nitrobenzaldehyde (**2**, 2 g, 10 mmol), glacial acetic acid (52 mL), and hydrobromic acid (45%, 61 mL) was stirred at 150 °C for 19 h. After cooling to room temperature, it was diluted with water, partially neutralized with sodium hydrogen carbonate, and extracted with ethyl acetate (4 × 60 mL). The organic phase was dried and evaporated. The residue was crystallized from toluene, giving 3,4-dihydroxy-5-nitrobenzaldehyde (**11**, 1.1 g, 61%). Mp: 145–146 °C. IR: 3640–2990, 1690, 1625, 1550, 1350. ^{1}H NMR (90 MHz, DMSO-d_6): 7.5 (d, 1 H, J = 1.5 Hz), 8.0 (d, 1 H, J = 1.5 Hz), 9.8 (s, 1 H).

4.2.3. 2,3-Dihydroxy-4-nitrobenzaldehyde (13). This compound was obtained in a manner similar to that of **11** with 2-hydroxy-3-methoxy-4-nitrobenzaldehyde **7** as starting material. The residue was crystallized from water, affording **13** (65%) as a red solid. Mp: 128–130 °C. IR: 3500–3000, 1675, 1525, 1230. ^{1}H NMR (90 MHz, DMSO-d_6): 7.3 (d, 1 H, J = 9 Hz), 7.5 (d, 1 H, J = 9 Hz), 10.3 (s, 1 H).

4.2.4. 2,3-Dihydroxy-6-nitrobenzaldehyde (15). To a stirred solution of 2-hydroxy-3-methoxy-6-nitrobenzaldehyde (**9**, 100 mg, 0.5 mmol) in dry dichloromethane (2 mL), at −78 °C under an argon atmosphere, was added boron tribromide (2 mL, 2 mmol, 1 M in dichloromethane). The reaction mixture was allowed to warm to room temperature and checked for completeness of reaction by TLC on silica gel (ethyl acetate–hexane 2:1, R_f = 0.3). After being stirred for 65 h, the mixture was diluted with water and extracted with ether (2 × 10 mL). The organic phase was extracted with 1 N sodium hydroxide and the aqueous residue was acidified with hydrochloric acid and extracted with ether. The combined organic layer was dried and evaporated. Recrystallization of the residue from water gave **15** (20 mg, 22%) as a brown solid. Mp: 120–124 °C. IR: 3700–3000, 1650, 1530, 1340. ^{1}H NMR (300 MHz, DMSO-d_6): 7.03 (d, 1 H, J = 8.8 Hz), 7.54 (d, 1 H, J = 8.8 Hz), 10.26 (s, 1 H).

Acknowledgment. R. A. Pérez is a predoctoral fellowship of the Spanish Ministerio de Educación y Ciencia. This work was supported by the Comisión Asesora de Investigación Científica y Técnica (proyecto no. Far 88-0194/1) and the Consejería de Educación de la Comunidad de Madrid (proyecto no. C 126/91).

(41) Cleland, W. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **1979**, *63*, 103–138.