5-[4-(Benzyloxy)phenyl]-1,3,4-oxadiazol-2(3*H*)-one Derivatives and Related Analogues: New Reversible, Highly Potent, and Selective Monoamine Oxidase Type B Inhibitors

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Received December 30, 1992

Thirty-three new 5-[4-(benzyloxy)phenyl]-1,3,4-oxadiazol-2(3*H*)-one derivatives including related analogues, designed as inhibitors of monoamine oxidase type B (MAO B), were synthesized and investigated both in vitro and ex vivo for their abilities to inhibit selectively rat brain MAO B over MAO A. Three inhibitors were found to act as reversible, highly potent, and selective MAO B inhibitors, namely the nitrile derivative 5-[4-(benzyloxy)phenyl]-3-(2-cyanoethyl)-1,3,4-oxadiazol-2(3*H*)-one (**12a**) and two closely related homologues, the corresponding oxadiazolethione **13a** and the alcohol **14b**. Their IC_{50}(MAO B) values are in the low nanomolar range of 1.4–4.6 nM and their selectivities, estimated by the ratio of IC_{50} values (A/B), are from 3200 to >71 400. Compound **12a** exhibited the highest activity against MAO B. Its IC_{50} was evaluated to be 1.4 nM with a quasitotal selectivity (>71 400) toward this enzyme. In ex vivo studies, **12a** showed a reversible and short duration of action. MAO B was markedly inhibited with the oral dose of 1 mg/kg without any alteration of MAO A, and the inhibition almost did not exceed 24 h. Its ED_{50} (1 h after oral administration) was evaluated to be 0.56 mg (1.7 μmol)/kg. Remarkably, MAO A was not affected at doses as high as 1500 mg/kg, po. In addition, no apparent toxicity or behavioral anomaly was observed during the treatment even at the maximum administrated dose. SAR studies emphasize the existence of three binding sites to the enzyme with a special importance of the terminal phenyl. Analysis of the inhibition kinetics indicated that **12a** acts in a two-step mechanism as a competitive, slow, and tight-binding inhibitor of MAO B with a K_i value of 0.22 μM and an overall K_i* value at equilibrium of 0.7 nM.

Introduction

Monoamine oxidase (MAO; EC 1.4.3.4) is a FAD-containing enzyme[1] located in the outer membrane of mitochondria.[2] On the basis of specificity for substrates and sensitivity to inhibitors, two MAO forms, called MAO A and MAO B, have been distinguished.[3,4] The two forms catalyze the oxidative deamination of a wide variety of biogenic monoamines[5] as well as xenobiotics.[6]

Recently, it has been shown that the two isoenzymes are distinct with about 70% homology in their primary sequence[7] and are coded by two different genes located on the X chromosome.[8] However, the region of the active site has not yet been identified and it still remains in question whether or not the catalytic differences between the two enzymes are due to different active site sequences.

Both enzymes present a considerable pharmacological interest because of their key role in the metabolism of monoamine neurotransmitters like serotonin, catecholamines, and dopamine and their possible involvement in many neuropsychiatric disorders.[9] For example, motor disability in Parkinsonism is linked to a deficit of dopamine in striatum, resulting from degeneration of the dopamine nigrostriatal neurones which project to striatum.

According to a current concept which is gaining acceptance, MAO's of glial cells neighboring nerve terminals play an important role in the metabolism of certain amine neurotransmitters as well as other amines such as false transmitters or intruding monoamines.[10] In particular, the mixed substrate dopamine is thought to be substantially metabolized in the extraneural compartment, e.g. in these MAO B rich glial cells. In line with this hypothesis, recent studies revealed no trace of MAO B and just small amounts of MAO A in dopaminergic neurons of the substantia nigra.[11]

The origin of Parkinsonism is not yet known. It has been suggested that hydroxyl radicals •OH are implicated in the genesis of the disease.[12] Due to a deficit of glutathione in substantia nigra in Parkinsonian brains, accumulation of H_2O_2 from dopamine deamination with concommitant increase of Fe^{2+} would lead, via Fenton reaction, to an overproduction of •OH and nigral degeneration. Alternatively, proliferation of glial cells in replacement of dead neurons[13] may contribute to the breakdown of the neurotransmitter.

In modern Parkinson therapy, one major approach to overcome the lack of dopamine is substitution by L-dopa with adjunction of a MAO B inhibitor,[14] either alone or with an antioxidant.[15] From controlled clinical trials with L-deprenyl (1), a preferential MAO B inhibitor,[4] it has been confirmed that selective inhibitors of MAO B either alone[16] or as adjuncts to L-dopa[17] are beneficial in Parkinson management, especially in the early stages of the disease.

Currently, only a few examples of selective MAO B inhibitors are available.[10,18] Historically, first effective

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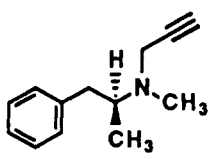
and selective inhibitors in vivo were mechanism-based inhibitors such as the propargylamines L-deprenyl (1) and



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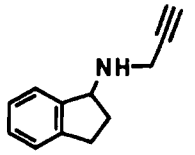

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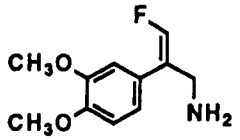

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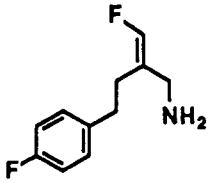

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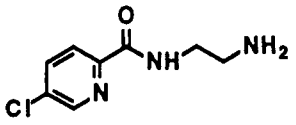
AGN 1135[19] (2), and the fluoroallylamine MDL 72,145[20] (3). As proved by the recently developed MDL 72,974 A[21] (4), net progress has been made in terms of activity and selectivity. However, the selectivity in vivo of this type of inhibitors is still to be improved, especially with regard to the novel reversible ones.[10,22]

During the last decade, more attention has been focused on the development of reversible inhibitors, believed to be advantageous in therapeutic uses.[23] The recently developed Ro 16-6327[21] (5) and its less potent analogue



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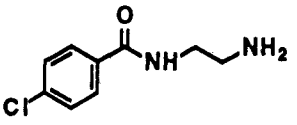

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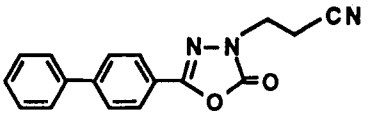
Ro 16-6491[24] (6) are the only two reversible MAO B inhibitors available. Interestingly, 5 has even proved to surpass the "suicide" inhibitors both in activity and selectivity.[10,22]

Another new promising class of reversible MAO B inhibitors are the 1,3,4-oxadiazol-2(3*H*)-one derivatives[25] with 7 as the representative compound. Our basic studies



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on these oxadiazolones and related analogues[26] highlighted, in particular, the crucial role of the semirigid terminal phenyl both in activity and selectivity of the inhibitors.

In a new approach, we investigated the influence of a conformationally nonrestrained terminal phenyl. Various derivatives of the lead compound in which the CH_2O group was introduced as a conformationally flexible linker to the terminal phenyl (Figure 1) were synthesized and evaluated as MAO B inhibitors.

In this paper, the SAR are discussed. Three new potent and selective MAO B inhibitors both in vitro and ex vivo are described and their mechanism of action is investigated. Of relevance is probably the finding of a reversible and highly potent inhibitor of MAO B with a quasiabsolute selectivity and without apparent toxic effects. A two-step mechanism of inhibition is depicted. The kinetic

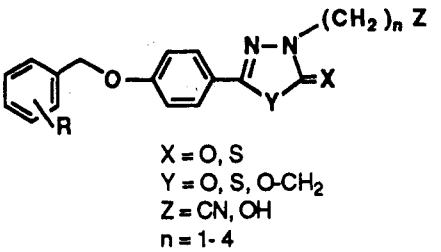


Figure 1.

parameters of this inhibitor as well as quantitation of the MAO B in mitochondria are reported.

Chemistry

The synthesis of 1,3,4-oxadiazol-2(3*H*)-one derivatives and analogues was performed as illustrated in Schemes I and II. Ester **8** was converted into esters **9a–h** by treatment with sodium hydride in dry dimethylformamide followed by reaction with appropriate benzyl chloride derivatives. Treatment of **9a–h** with hydrazine hydrate afforded hydrazides **10a–h**. A Michael reaction between hydrazides **10a–h** and acrylonitrile gave 1,2-disubstituted hydrazines **11a–h**. Byproducts formed in traces from a double Michael addition were not isolated. Hydrazines **11a–h** were converted into oxadiazolones **12a–h** and thiadiazolones **13a–h** by treatment with phosgene or thiophosgene, respectively.[27]

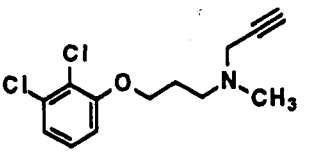
Cyclization of **10a** with phosgene gave the oxadiazolone **14a** which upon treatment with NaOH followed by reaction with appropriate chloride derivatives provided oxadiazolones **14b–g**. Oxadiazinones **15a–h** were synthesized by successive treatment of **11a–h** with chloroacetyl chloride and sodium carbonate.[28] Oxadiazinethione **16** was synthesized from reaction of phosphorus pentasulfide with oxadiazinone **15a** in pyridine.

The thioanalogue **18** of **11a** was obtained by nucleophilic attack of (2-cyanoethyl)hydrazine on [[[4-(benzyloxy)-phenyl](thiocarbonyl)]thio]acetic acid (**17**), which was prepared according to the classical method of Kjaer[29] (Scheme II). Treatment of **18** with phosgene or thiophosgene gave thiadiazolone **19** or thiadiazolethione **20**.

The physicochemical characteristics of the new compounds are given in Tables I and II. Satisfactory NMR and IR spectra as well as analytical data were obtained for all compounds.

Results and Discussion

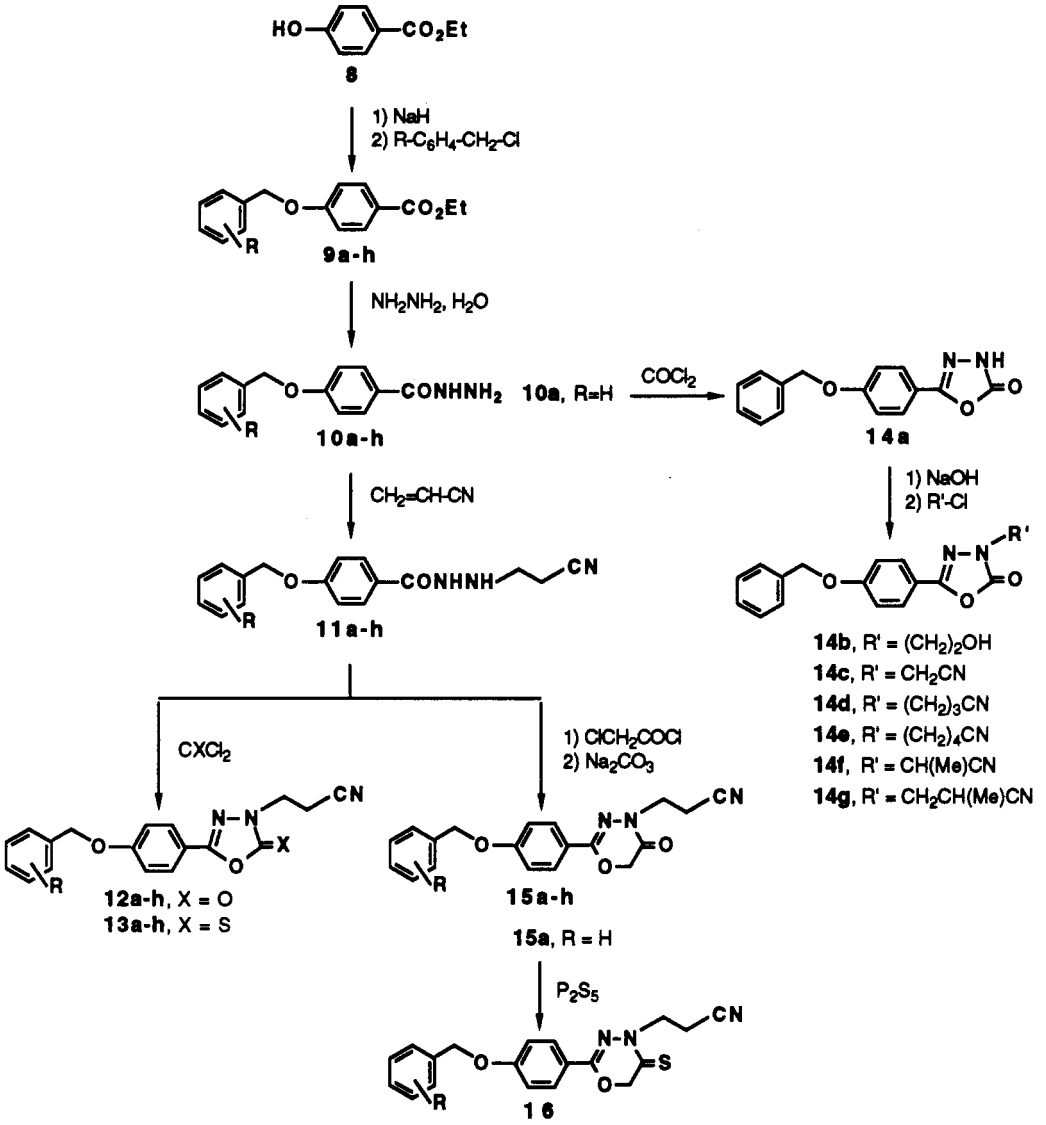
In Vitro Evaluation. Thirty-three oxadiazolone derivatives and related analogues were tested in vitro for their inhibitory effects on rat brain MAO types A and B (Table II). Clorgyline (**21**) and L-deprenyl (1) were used



21

as inhibitor references for the A and B forms, respectively. Most of the inhibitors acted preferentially against MAO B with IC_{50} values in the range of 0.69 μM-2.2 nM. Their selectivity, as estimated by the ratio of IC_{50}(MAO A) to IC_{50}(MAO B), is ranked from 4 to >45 450.

Scheme I



The oxadiazolone **12a** and its analogues **13a** and **14b** rise as the most efficient MAO B inhibitors in these series. Like the mechanism-based L-deprenyl, they act in a time-dependent manner as evidenced by the IC_{50} time dependency (Table III). An apparent equilibrium was attained within 1 h for **12a** and **13a**, and within 20 min for **14b**. At 1 h of preincubation, the IC_{50} values and selectivities were 1.4 nM and >71 400 for **12a**, 3.7 nM and 3200 for **13a**, and 4.7 nM and 30 400 for **14b**.

Ex Vivo Evaluation. Drugs of interest were explored ex vivo at 5 mg/kg on rat brain MAO (Table IV). As in vitro, compounds **12a**, **13a**, and **14b** were found to be selective and the most orally active in vivo, producing (1 h after administration) 87, 84, and 83% MAO B inhibition, respectively. Moreover, the inhibitory effects, which lasted at least 4 h, had almost disappeared 24 h later, indicating a short-lasting action and thus a rather reversible mechanism of inhibition. It is noteworthy that classical irreversible inhibitors of MAO B, like L-deprenyl (1), whose action depends on the enzyme turnover, lasted up to 8 days.[30]

At the lower dose of 1 mg/kg, **12a** remained fully active with a significant peak inhibition of 83% (Table IV). Its ED_{50}(MAO B), 1 h after oral administration, was evaluated to be 0.56 mg (1.7 μmol)/kg (data not shown). Also its remarkable selectivity toward MAO B was well-confirmed since MAO A was not affected with increasing doses of 1, 5, 100, and 1500 mg/kg (Table IV).

At the higher doses of 100 and 1500 mg/kg, about 70% of MAO B inhibition persisted 24 h after administration of the drug (Table IV). However, washing mitochondria of the inhibited homogenates resulted in full recovery of the enzyme activity (Table IV), confirming a reversible process of inhibition. A retention of the lipophilic inhibitor, for example in fatty tissues, is probably responsible for the prolonged effects in the case of high doses.

Perhaps the most striking observation was the absence of any apparent toxic or behavioral effects, even upon administration of **12a** at very high doses (a single dose of 1500 mg/kg, po, or a daily dose of 100 mg/kg, po, for nine days).

Structure–Activity Relationships. In most cases, the adjunction of the flexible CH_2O bridge to the terminal phenyl dramatically increased the activity and selectivity of the inhibitors, with respect to the parent analogues.[25,26] This is particularly true for **12a** and **14b**, which emerge as being typical B inhibitors with a quasiabsolute selectivity.

Introducing either electrodonating or -withdrawing groups on the phenyl terminal led in both cases to a

Scheme II

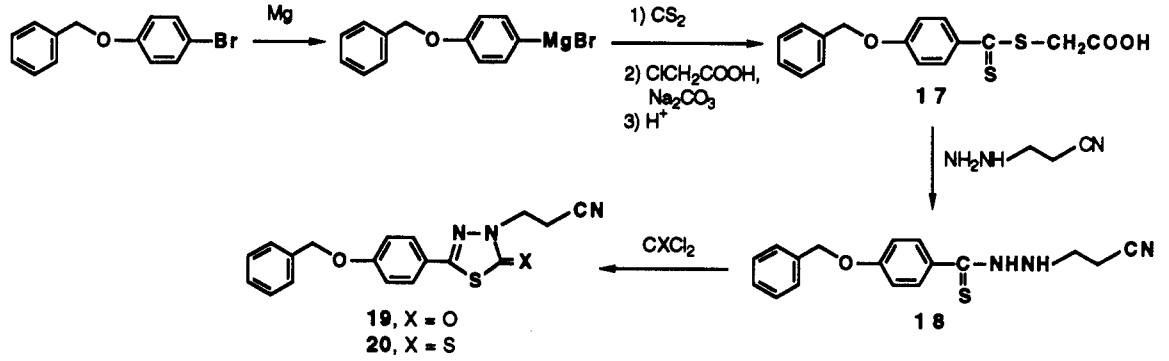
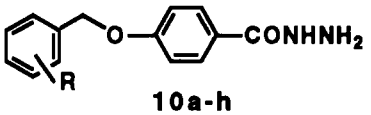
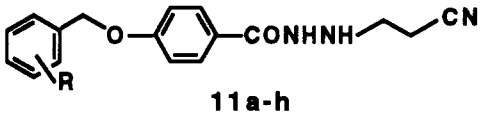


Table I. Physicochemical Data of Substituted [4-(Benzyloxy)benzoyl]hydrazines



10a-h



11a-h

no.	R	mp, °C	rec solv[a]	% yield[b]	formula[c]
10a	H	140	A	74	$C_{14}H_{14}N_2O_2$
10b	3-MeO	130	A	80	$C_{15}H_{16}N_2O_3$
10c	4-MeO	152	A	86	$C_{15}H_{16}N_2O_3$
10d	3-Me	127	A	78	$C_{15}H_{16}N_2O_2$
10e	4-Me	150	A	95	$C_{15}H_{16}N_2O_2$
10f	3-Cl	128	A	96	$C_{14}H_{13}ClN_2O_2$
10g	4-Cl	164	A	85	$C_{14}H_{13}ClN_2O_2$
10h	4-NO_2	201	B	60	$C_{14}H_{13}N_3O_4$
11a	H	139.5	A	68	$C_{17}H_{17}N_3O_2$
11b	3-MeO	96	A	57	$C_{18}H_{19}N_3O_3$
11c	4-MeO	168	A	77	$C_{18}H_{19}N_3O_3$
11d	3-Me	131	C	74	$C_{18}H_{19}N_3O_2$
11e	4-Me	157	A	79	$C_{18}H_{19}N_3O_2$
11f	3-Cl	120	A	50	$C_{17}H_{16}ClN_3O_2$
11g	4-Cl	162	A	95	$C_{17}H_{16}ClN_3O_2$
11h	4-NO_2	128	A	70	$C_{17}H_{16}N_4O_4$

[a] Recrystallization solvent: A = ethanol, B = butanol, C = methanol. [b] Yields were not optimized. [c] All compounds had C, H, N elemental analyses within ±0.4% of theoretical values.

significant decrease in the inhibitory effects. Maximal efficiency in vitro and ex vivo was obtained with inhibitors bearing an unhindered terminal phenyl (**12a** vs **12b–h**, **13a** vs **13b–h** and **15a** vs **15b–h**). This clearly confirms the prominent role of the terminal phenyl in the binding to the enzyme.

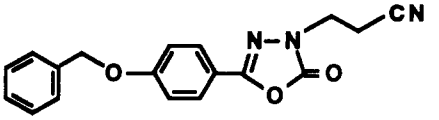
As compared to the six-membered heterocycles, the contribution of five-membered heterocycles is substantially higher (**12a** vs **15a** and **13a** vs **16**). On the other hand, the activity seems to follow the electronegativity of the heteroring group YC=X or C=X: SC=S (**20**) < SC=O (**19**) < OC=S (**13a**) < OC=O (**12a**), and C=S (**16**) < C=O (**15a**). The highest activities were observed with the oxadiazolone ring.

In parallel to the specific substrates benzylamine and β-phenylethylamine, the inhibitors elicit potent and selective interactions with MAO B only when the side chain has one or two unsubstituted carbons (**14c** and **12a** vs **14d** and **14e** and vs **14f** and **14g**). The importance of a nonmodified ethano link in the side chain has been also reported for other reversible inhibitors like the (2-aminoethyl)benzamide derivatives **5** and **6**.[31] Therefore, it becomes clear that the inhibitors behave as substrate analogues. In particular, the CN group (at least for **12a**, see below) should compete with the NH_2 group of the substrate for binding to an essential site of the enzyme, probably via H bonds with the SH group of the essential cysteine.[32]

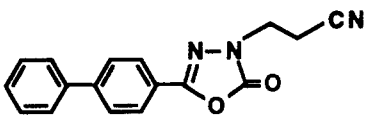
Altogether, these observations correlate with our previous results[25] and highlight in particular the existence of three anchoring sites on the enzyme: a hydrophobic pocket for the interactions with the terminal phenyl, a nucleophilic site for interactions with the heteroring, probably with the electron-deficient N-4 atom adjacent to the electronegative group YC=X, and finally an essential group for the interactions with the CN group.

Mechanism of Action of 12a. Full recovery of MAO B activity occurred after washing of the mitochondria partially inhibited by **12a**, **13a**, or **14b**, indicating a reversible mechanism of the inhibition as predictable from the ex vivo results. In contrast, not any gain of activity was obtained with the irreversible inhibitor L-deprenyl (**1**) (Table V).

Compound **12a** was chosen to investigate the type of



12a



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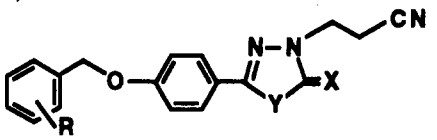
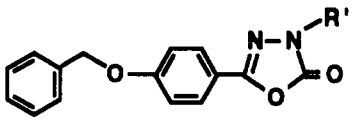
inhibition. Firstly, the very close structural homology with

Table II. Physicochemical Data and MAO Inhibitory Properties in Vitro for Oxadiazolones **12a–h** and **14b–g**, Their Sulfur Analogues **13a–h**, **19**, and **20**, Oxadiazinones **15a–h**, and Oxadiazinethione **16**



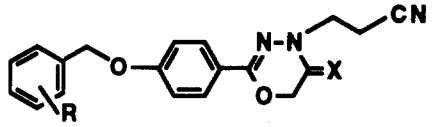
12a-h
13a-h
19, 20

no.	X	Y	R	mp, °C	rec solv[a]	% yield[b]	formula[c]	IC_{50},[d] μM MAOA	IC_{50},[d] μM MAOB	B selectivity[e]
12a	O	O	H	152	A	78	$C_{18}H_{15}N_3O_3$	10%[f]	0.0022	>45450
12b	O	O	3-MeO	134	A	57	$C_{19}H_{17}N_3O_4$	19	0.029	722
12c	O	O	4-MeO	137	A	43	$C_{19}H_{17}N_3O_4$	16	0.027	522
12d	O	O	3-Me	148	A	89	$C_{19}H_{17}N_3O_3$	20	0.008	2500
12e	O	O	4-Me	154	A	89	$C_{19}H_{17}N_3O_3$	25	0.015	1666
12f	O	O	3-Cl	150	A	84	$C_{18}H_{14}ClN_3O_3$	7.4	0.012	617
12g	O	O	4-Cl	169	A	66	$C_{18}H_{14}ClN_3O_3$	3.3	0.0075	440
12h	O	O	4-NO_2	196	A	40	$C_{18}H_{14}N_4O_5$	11	5.5	200
13a	S	O	H	117	A	89	$C_{18}H_{15}N_3O_2S$	12	0.0044	2727
13b	S	O	3-MeO	135	B	57	$C_{19}H_{17}N_3O_3S$	8.1	0.1	81
13c	S	O	4-MeO	139	B	34	$C_{19}H_{17}N_3O_3S$	4.8	0.91	5
13d	S	O	3-Me	95	A	85	$C_{19}H_{17}N_3O_2S$	14	0.066	212
13e	S	O	4-Me	143	B	64	$C_{19}H_{17}N_3O_2S$	10	0.4	25
13f	S	O	3-Cl	134	A	81	$C_{18}H_{14}ClN_3O_2S$	11	0.16	69
13g	S	O	4-Cl	152	B	63	$C_{18}H_{14}ClN_3O_2S$	2.7	0.69	4
13h	S	O	4-NO_2	180	B	46	$C_{18}H_{14}N_4O_4S$	2.6	4.3	0.06
19	O	S	H	143	D	62	$C_{18}H_{15}N_3O_2S$	45	0.008	5625
20	S	S	H	138	E/F	51	$C_{18}H_{15}N_3OS_2$	160	0.016	10000



14a-g

no.	R′	mp, °C	rec solv[a]	% yield[b]	formula[c]	IC_{50},[d] μM MAOA	IC_{50},[d] μM MAOB	B selectivity[e]
14a	H	117	D	76	$C_{15}H_{12}N_2O_3$			
14b	$(CH_2)_2OH$	156	A	67	$C_{17}H_{16}N_2O_4$	140	0.0037	37800
14c	CH_2CN	206	A	90	$C_{17}H_{13}N_3O_3$	NI[g]	0.06	>1666
14d	$(CH_2)_3CN$	172	C	42	$C_{19}H_{17}N_3O_3$	NI[g]	0.41	>244
14e	$(CH_2)_4CN$	129	A	34	$C_{20}H_{19}N_3O_3$	NI[g]	0.49	>204
14f	CH(Me)CN	210	A	62	$C_{18}H_{15}N_3O_3$	4%[f]	48	2
14g	$CH_2CH(Me)CN$	156.5	A	40	$C_{19}H_{17}N_3O_3$	13%[f]	2.4	>42



15a-h, 16

no.	X	R	mp, °C	rec solv[a]	% yield[b]	formula[c]	IC_{50},[d] μM MAOA	IC_{50},[d] μM MAOB	B selectivity[e]
15a	O	H	140.5	A	64	$C_{19}H_{17}N_3O_3$	160	0.018	8889
15b	O	3-MeO	136	B	48	$C_{20}H_{19}N_3O_4$	32	0.068	470
15c	O	4-MeO	158	B	45	$C_{20}H_{19}N_3O_4$	45	0.088	511
15d	O	3-Me	129	B	86	$C_{20}H_{19}N_3O_3$	40	0.031	1290
15e	O	4-Me	144	B	31	$C_{20}H_{19}N_3O_3$	250	0.32	78
15f	O	3-Cl	127	B	94	$C_{19}H_{16}ClN_3O_3$	13	0.013	1000
15g	O	4-Cl	146	B	54	$C_{19}H_{16}ClN_3O_3$	3.4	0.1	34
15h	O	4-NO_2	151	A	45	$C_{19}H_{16}N_4O_5$	2	0.3	6
16	S	H	136.5	C	76	$C_{19}H_{17}N_3O_2S$	25	0.14	179
					MAOI's references				
L-deprenyl (1)							0.8	0.008	100
clorgyline (21)							0.0012	1.9	0.006

[a] Recrystallization solvent: A = ethanol, B = methanol, C = 1-propanol, D = 1-butanol, E = ethyl acetate, F = petroleum ether (bp 40–60 °C). [b,c] See corresponding footnotes in Table I. [d] IC_{50} value refers to the assay concentration of drug which produced 50% inhibition of enzyme activity. The values were obtained graphically from –log concentration/MAO inhibition plots based on 9–12 concentrations. Data represent means of three separate experiments carried out in duplicates. Standard errors, not shown, were 10–25%. [e] Selectivity for the B form was estimated by the ratio of IC_{50}(MAO A) to IC_{50}(MAO B). [f] Percent inhibition at 100 μM drug concentration. [g] No inhibition at 100 μM drug concentration.

the parent analogue **7**, which behaves in a competitive manner,[25] strongly suggests that **12a** would act in the same fashion.

Indeed, **12a** only differs from the parent analogue by the CH_2O moiety in which the active oxygen atom could give, at most, weak bonds (like H bonds) with the enzyme, and consequently would not change the nature of the inhibition.

Secondly, considering (a) the low concentrations at which **12a** acts at equilibrium (CI_{50} = 1.4 nM, Table III),

Table III. Time Dependence of IC_{50}(MAO)[a] for **12a**, **13a**, **14b**, and L-Deprenyl (1)

no.	IC_{50}, μM MAOB 0 min	20 min	30 min	60[d]	MAO A[b] 60 min	B selectivity[c] 60 min
12a	0.025	0.0027	0.0022	0.0014	>100	>71400
13a	0.047	0.0066	0.0047	0.0037	12	3200
14b	0.033	0.0046	0.0044	0.0046	140	30400
L-deprenyl (1)	0.160	0.0099	0.0094	0.007	0.7	100

[a] IC_{50} values at indicated preincubation periods were determined as described in footnote *d* of Table I. Variablility in determinations is 5–15%. [b] For inhibition of MAO A by **12a**, **13a**, and **14b**, values were the same with or without the preincubation. [c] See footnote *e* in Table II. [d] IC_{50}(MAO B) values for **12a**, **13a**, **14b**, and L-deprenyl (1) were the same at preincubation times of 60 and 120 min.

Table IV. Ex Vivo Time Course of Rat Brain MAO Inhibition by Oxadiazolones and Related Analogues[a]

no.	dose, mg/kg	% MAO B inhibition[b,c] 0.5 h	1 h	4 h	24 h	48 h
12a	1	34 ± 6**	86 ± 1***	75 ± 3***	18 ± 13 NS	
	5	69 ± 4***	87 ± 3***	83 ± 2***	18 ± 7	
	100[d]		90 ± 1***		73 ± 3***	
					3 ± 5[e]	
	1500		91 ± 3***		74 ± 6***	
					2 ± 3[e]	
12b	5	12 ± 5	19 ± 6**	6 ± 4	−3 ± 5	−7 ± 5
12d	5	6 ± 6	16 ± 6	15 ± 17	−3 ± 4	−3 ± 4
12e	5	9 ± 5	22 ± 4***	14 ± 4*	−3 ± 5	1 ± 4
12f	5	1 ± 5	36 ± 7**	29 ± 11*	−7 ± 5	4 ± 7
12g	5	30 ± 6***	54 ± 2***	47 ± 6***	−6 ± 4	−6 ± 4
13a	5	68 ± 13	84 ± 2	74 ± 3	21 ± 7	8 ± 9
19	5	44 ± 4	52 ● 4	45 ± 2	15 ± 4	9 ± 8
20	5	27 ± 7	18 ± 5	8 ± 10	8 ± 7	10 ± 6
14b	5	65 ± 7	82 ± 6	70 ± 4	8 ± 7	
15a	5	21 ± 6	31 ± 6	22 ± 5	23 ± 7	16 ± 6
16	5	12 ± 17	3 ± 8	5 ± 9	−4 ± 10	9 ± 15

[a] Compounds were given by oral gavage. Unless indicated otherwise, a single dose was administered. After treatment and at indicated times, groups of four animals were sacrificed and brain MAO activity was assayed as described in the Experimental Section. [b] The results are expressed as percent inhibition vs the control. Values represent means ± SEM of determinations in four homogenates. In all drug tests, any inhibition of MAO A was detected. [c] Statistical significance is indicated by asterisks. For **12a**: at 1 mg/kg, ***$p < 0.001$, **$p < 0.01$ (Bonferroni's test), NS, not significant (Wilcoxon's test); at 5, 100, and 1500 mg/kg, ***$p < 0.001$ (Student's test). For the rest of the molecules: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (Bonferroni's test). [d] Given once per day for 9 days. [e] Results of the reversibility test for the 24 h-inhibited homogenates (after the last dose), proceeding as described in the Experimental Section.

very close to the enzyme concentration (1.1 nM, see below), (b) its reversible (Table V) and time-dependent action (Figure 2A), and (c) the change of the Lineweaver–Burk plot from competitive (without preincubation with the enzyme, Figure 3A) to "pseudo" noncompetitive patterns with upward parabolic secondary replot (upon preincubation with the enzyme, Figure 3B), the inhibitor seems to fulfill the model of competitive and tight-binding inhibitors.[33,34] The tight-binding behavior is strongly supported by the Hendersen plot,[35] giving parallel lines (Figure 4).

On the other hand, as shown in Figure 2A, time dependency of the inhibition found at concentrations of **12a** (0.2–2 μM) much higher than that of the enzyme (8.8 nM) clearly indicates a slow-binding behavior.

On the basis of these results, a two-step interaction between **12a** and MAO B can be assumed according to eq 1,[34] where E and I represent MAO B and **12a**. According to this mechanism, E and I combine rapidly to form a reversible complex EI which isomerizes slowly to a tighter complex EI*.

Table V. Reversibility Test for **12a**, **13a**, **14b**, and L-Deprenyl (1)[a]

	% MAO B inhibition before the test	after the test
control (nmol/min per mg of protein)	0.901 ± 0.016	0.200 ± 0.003
12a (6.25 nM)	35 ± 1	102 ● 2
13a (12.5 nM)	40 ± 1.5	101 ± 4
14b (12.5 nM)	39 ± 3	105 ± 3
L-deprenyl (1) (12.5 nM)	21 ● 1	21 ● 0.5

[a] The results are means of SE of three separate experiments. For details, consult the Experimental Section.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \quad (1)$$

rapid slow

Evaluation of the Kinetic Parameters for 12a. The relation between the dissociation constant K_i, the overall equilibrium constant K_i^* and the other rate constants may be described by the following equations:

$$K_i = \frac{k_2}{k_1} \quad (2)$$

$$K_i^* = \frac{k_2}{k_1}\frac{k_4}{k_3 + k_4} = K_i\frac{k_4}{k_3 + k_4} \quad (3)$$

$$\frac{k_3}{k_4} = \frac{K_i}{K_i^*} - 1 \quad (4)$$

For a competitive tight-binding inhibitor that reacts slowly with the enzyme, the rate of inhibition under equilibrium conditions is independent of the substrate concentration and follows eq 5,[33] adapted from the

$$\frac{[I]_o}{1 - \frac{v_i}{v_o}} = K_i^*\frac{v_o}{v_i} + [E]_o \quad (5)$$

Henderson equation[35] where $[I]_o$ and $[E]_o$ are the initial inhibitor and enzyme concentrations, respectively; v_o and v_i are the velocities determined in the absence and presence of the inhibitor.

According to this equation, a plot of $[I]_o/(1 - v_i/v_o)$ against v_o/v_i at different $[E]_o$ will give parallel lines of slope K_i^* cutting the y axis at $[E]_o$. As can be seen in Figure 4, this seems to be true for the inhibition of MAO B by **12a**. Special care was taken to determine v_o since the first term of the equation considerably amplifies errors on v_o. Determinations were carried out at two relatively low protein concentrations in the assays (0.11 and 0.22 mg of protein/mL). From this analysis, the K_i^* value was estimated to be 0.66 ± 0.01 nM. On the other hand, the concentration of MAO B amounted to 4.55 ± 0.05 pmol/mg of protein, which is in agreement with the reported value of 4.2 pmol/mg of protein found with L-deprenyl (1) titration.[36] Thus, assuming a concentration of MAO B of 4.55 pmol/mg of protein in mitochondrial membranes, [MAO B] at preincubation would be 1.1 nM in the standard assay.

At IC_{50}, eq 5 turns into a simple linear equation (eq 6),[33]

$$IC_{50} = 0.5[E]_o + K_i^* \quad (6)$$

which represents an alternative to estimate K_i^*. According

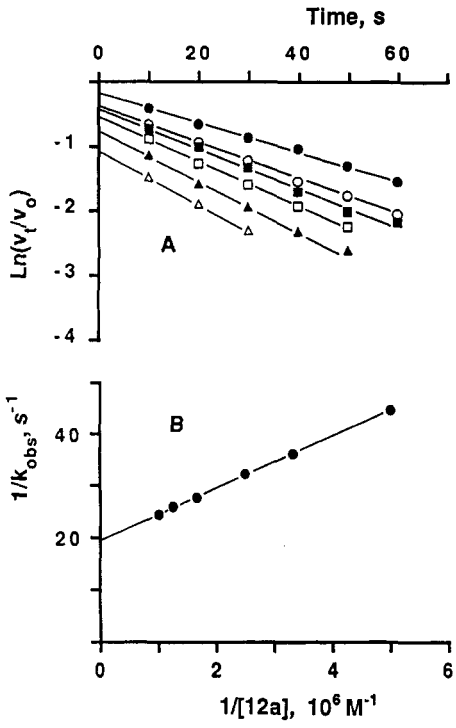


Figure 2. Time-dependent inhibition of MAO B by **12a**. Mitochondrial fractions were preincubated at 37 °C with various concentrations of **12a**. At indicated times, 160-μL aliquots were removed and assayed for MAO B activity by addition of 40 μL of PEA (final concentration of 50 μM, about 10-fold the K_m). The reaction time was shortened to 1 min. The protein concentration at preincubation was set to 1.94 mg/mL (8.8 nM MAO B). The results represent means of determinations in three homogenates. (A): Pseudo-first-order plot. Preincubation concentrations of **12a** were 0.2 (●), 0.3 (○), 0.4 (■), 0.6 (□), 0.8 (▲), and 1 μM (△). (B): Double-reciprocal plot of the rate constants obtained in A with varying concentrations of **12a**. $k_3 = 0.05\ s^{-1}$, $K_s = 0.26\ \mu M$.

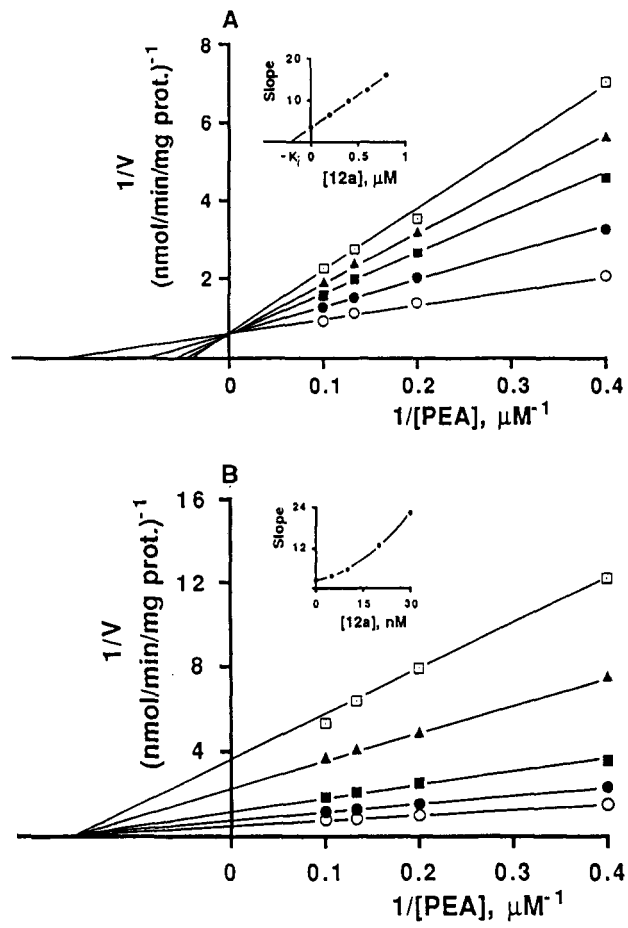


Figure 3. Lineweaver–Burk plot of the inhibition of MAO by **12a**. Mitochondrial fractions were set to an assay concentration of 1.55 mg protein/mL. MAO B activity was measured with 2.5, 5, 7.5, and 10 μM PEA over a reaction period of 1 min. (A): Samples were assayed without preincubation in the absence (○) or presence of 0.2 (●), 0.4 (■), 0.6 (▲), and 0.8 (⊡) μM **12a**. Final DMSO concentration was 2%. (B): Samples were assayed with preincubation of the enzyme for 10 min at 37 °C in the absence (○) or presence of 6.25 (●), 12.5 (■), 25 (▲), and 37.5 (⊡) nM **12a**. All values represent means of duplicate determinations in three homogenates. Secondary plots of the slopes against inhibitor concentration are shown as figure insets. From Figure 3A, K_i = 0.22 μM.

to this equation, when IC_{50} values are determined under equilibrium conditions at different $[E]_o$, a plot of IC_{50} against $[E]_o$ will give a straight line which cuts the IC_{50} axis at K_i^* and the $[E]_o$ axis at $-2K_i^*$. As shown in Figure 5B, a linear relationship between IC_{50} and protein concentration was obtained over most of the range of $[E]_o$. Graphical analysis gave a K_i^* value of 0.74 nM which compares favorably with the value of 0.66 nM obtained from the Henderson plot discussed above. Therefore, taking into account both procedures, a valid estimate of K_i^* would be 0.7 ± 0.04 nM.

However, the concentration of MAO B, as calculated from the slope × 2, amounted to 9.4 pmol/mg of protein, a value which is 2-fold higher than that given by the Henderson plot. Such a discrepancy may be connected to the procedure which accounts not only for specific but also for nonspecific binding. An increase in nonspecific binding with inhibitor concentration would lead to an overestimation of IC_{50}. If the partition ratio of the inhibitor between specific and nonspecific sites at IC_{50}'s does not vary much, then overestimates should be accentuated with protein concentration. From eq 6 and Figure 5B the net result would be an increase of the slope, thus of MAO B concentration, without much affecting the y intercept (K_i^*), which seems to be in line with the obtained data.

If the rate constant k_4 is much smaller than the other three rates (see eq 1) and if the free inhibitor concentration is not significantly depleted ($[I]_o \gg [E]_o$), then the improved steady-state assumption[37] can be applied to yield the rate equation (eq 8):

$$[E]_t = \frac{[E]_o}{1 + \frac{[I]_o}{K_s}} e^{-k_{obs}t} \qquad (8)$$

where

$$k_{obs} = \frac{k_3}{1 + \frac{K_s}{[I]_o}} \text{ and } K_s = \frac{k_2 + k_3}{k_1}$$

The rate of inhibition of the enzyme by **12a** may then be determined by measuring the amount of enzyme activity remaining after preincubation with **12a** for various times and at various inhibitor concentrations. When enzyme activity is proportional to free enzyme concentration, a plot of $\ln(v_t/v_0)$ versus preincubation time should yield straight lines with $-k_{obs}$ as slopes and with y intercepts varying with inhibitor concentration according to eq 9, where v_0 and v_t are the enzyme activities at 0 and t time, and k_{obs} is the pseudo-first-order rate constant of inhibition.

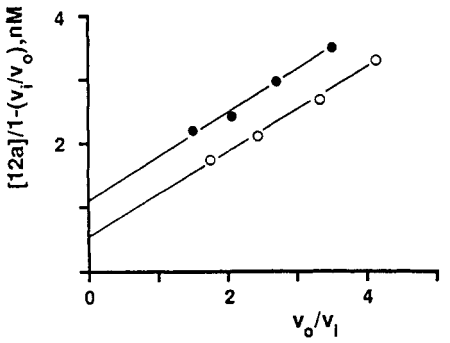


Figure 4. Hendersen plot for the inhibition of MAO B by **12a**. MAO B activity was assayed with 5 μM PEA following preincubation of the homogenate for 60 min at 37 °C in the absence or presence of 0.75, 1.25, 1.9, and 2.5 nM **12a**. Mitochondrial fractions were set to a preincubation concentration of 0.12 (●) or 0.24 (○) mg of protein/mL. The data represent means of duplicate determinations in three mitochondrial homogenates. K_i* = 0.66 nM, [MAO B] = 4.55 pmol/mg of protein.

$$\ln\frac{v_t}{v_0} = -k_{obs}t + \frac{1}{1+\frac{[I]_o}{K_s}} \quad (9)$$

The proportionality of enzyme activity with enzyme concentration will hold only if the EI complex is catalytically inactive. In this case, k_{obs} will vary hyperbolically with $[I]_o$, and a plot of $1/k_{obs}$ versus $1/[\mathbf{12a}]$ according to eq 10 should yield a straight line with an intercept on the

$$\frac{1}{k_{obs}} = \frac{K_s}{k_3[I]_o} + \frac{1}{k_3} \quad (10)$$

ordinate at $1/k_3$, the ratio of slope to intercept yielding K_s.

As shown in Figure 2, data are consistent with these predictions and validate the concept of a rapid equilibration followed by a slow interaction of the enzyme with the inhibitor, with K_s = 0.26 μM and k_3 = 0.05 s^{-1}. The half-life for association at a saturating inhibitor concentration, $t_{1/2(ass)}$, as given by (ln 2)/k_3 would be 0.23 min.

The rapid interaction of **12a** with MAO B was investigated by measuring inhibition when **12a** and the substrate were added simultaneously to the enzyme (without preincubation). The assay time was shortened to 1 min to minimize the interference of the slow second phase of inhibition. From Figure 3A, the first phase is purely competitive with K_i = 0.23 μM. The close concordance between the values of the thermodynamic constant K_i and the kinetic constant K_s validates the assumption that the complex EI is catalytically incompetent as well as $k_2 \gg k_3$.

From relationship 4, the rate constant k_4 may be estimated to be 1.6 × 10^{-4} s^{-1}. Therefore, in the absence of free **12a**, the half-life for dissociation of EI,* $t_{1/2(diss)}$, would be (ln 2)/k_4 = 72 min.

Conclusion

This study is the first evidence of nitrile and alcohol derivatives as effective MAO B inhibitors ex vivo. According to their activity and selectivity, nitriles **12a** and **13a**, and alcohol **14b** can be placed among the most powerful and selective inhibitors of MAO B reported to date. Of particular interest is the cyanide **12a** characterized as a reversible, apparently nontoxic, potent, and fully selective MAO B inhibitor with a competitive slow and tight-binding behavior (K_i* = 0.7 nM). For information, **12a** is 5 times more active and by far more selective than the classical L-deprenyl (**1**) (Table III).

From these results it can be concluded that **12a** may represent a valuable candidate for therapeutic investigations against Parkinsonism and other age-mediated neurodegenerative disorders.[9,38] Also, this tight binder, theoretically not altered by MAO, may be a powerful tomographic tool for diagnosis of these diseases.

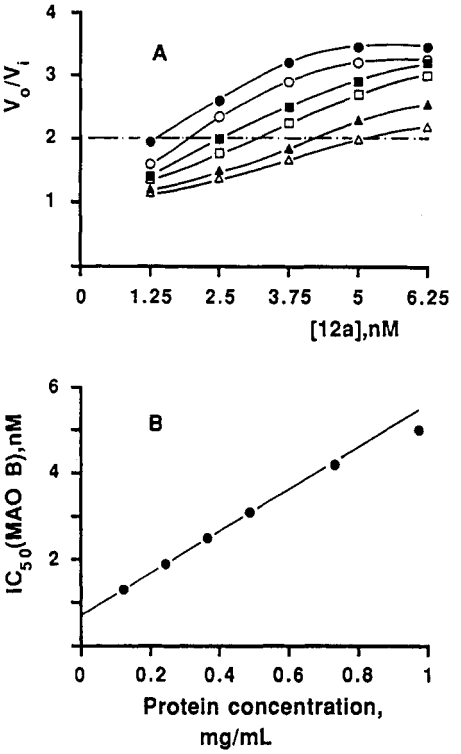


Figure 5. Enzyme concentration dependence of IC_{50}(MAO B) for **12a**. Mitochondrial fractions were preincubated at 37 °C for 60 min with various concentrations of **12a** and at different protein concentrations: 0.12 (●), 0.24 (○), 0.37 (■), 0.49 (□), 0.73 (▲), and 0.98 (△) mg of protein/mL. MAO B activity was assayed with 50 μM PEA (assay concentration). (A): Plot of v_o/v_i versus **[12a]**. Values represent averages of determinations in two mitochondrial fractions. (B): Plot of IC_{50}(MAO B) obtained in A with varying protein concentration. The IC_{50} values were calculated from individual curves in A. K_i* = 0.74 nM, [MAO B] = 9.4 pmol/mg of protein.

Experimental Section

Chemistry. Anhydrous DMF and THF were prepared by standard methods. All other solvents and reagents were reagent grade and used without purification, except for acrylonitrile, which was distilled before use. Melting points were determined in open capillary tubes on a Büchi 510 apparatus and are uncorrected. Column chromatography separation was carried out on Macherey-Nagel silica gel 60 (0.05–0.20 mm). Infrared spectra (KBr) were recorded on a Perkin-Elmer 1310 spectrophotometer. ^{1}H NMR were recorded at 90 MHz (Perkin-Elmer R32) with tetramethylsilane as the internal standard. Elemental analysis was performed at the Paris 6 structural chemistry department. Analytical data were within 0.4% of the calculated values.

Substituted Ethyl 4-(Benzyloxy)benzoates 9a–h. These known compounds were prepared following a procedure slightly different from that described in the literature.[39] To a stirred and cooled suspension of sodium hydride (2.4 g, 100 mmol) in dry DMF (20 mL) was added dropwise a solution of ethyl 4-hydroxybenzoate (**8**) (16.6 g, 100 mmol) in dry DMF (40 mL). The stirred mixture was allowed to stand for 30 min at room temperature. Then a solution of a suitable benzyl chloride

derivative (100 mmol) in dry DMF (10 mL) was added dropwise. The reaction mixture was stirred for 5 h. The solvent was evaporated and the crude ester was washed with 100 mL of ice/water and then filtered, dried, and recrystallized from EtOH.

[4-(Benzyloxy)benzoyl]hydrazine (10a). A mixture of **9a** (25.6 g, 100 mmol) and hydrazine hydrate (7.5 g, 150 mmol) in 1-propanol (150 mL) was stirred to reflux for 48 h. After cooling, compound **10a** crystallized. It was filtered, dried and recrystallized from EtOH to give 17.9 g (74%): mp 140 °C; IR (KBr) 3290, 3200, 1600 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 4.4 (s, 2 H), 5.15 (s, 2 H), 7.05 and 7.8 (2 d, 4 H), 7.4 (br s, 5 H), 9.6 (s, 1 H). Anal. ($C_{14}H_{14}N_2O_2$) C, H, N.

Compounds **10b–h**, shown in Table I, were synthesized by following the same procedure.

1-[4-(Benzyloxy)benzoyl]-2-(2-cyanoethyl)hydrazine (11a). A mixture of **10a** (4.84 g, 20 mmol) and acrylonitrile (1.32 g, 25 mmol) in EtOH (50 mL) was refluxed for 48 h. The solvent was removed in vacuo. The resulting crop was chromatographied twice, eluting with AcOEt/petroleum ether (9:1). The solvent was evaporated and the obtained solid was recrystallized from EtOH to give pure **11a** (4 g, 68%): mp 139.5 °C; IR (KBr) 3260, 3210, 2240, 1630 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 2.6 (t, 3 H), 3.05 (q, 2 H), 5.15 (s, 2 H), 5.4 (q, 1 H), 7.1 and 7.85 (2 d, 4 H), 7.4 (br s, 5 H), 9.9 (d, 1 H). Anal. ($C_{17}H_{17}N_3O_2$) C, H, N.

Compounds **11b–h**, shown in Table I, were synthesized by following the same procedure.

5-[4-(Benzyloxy)phenyl]-1,3,4-oxadiazol-2(3*H*)-one (12a). Compound **11a** (2.95 g, 10 mmol) was added to a freshly prepared solution of phosgene in toluene (10%, w/v, 30 mL) and the mixture was stirred for 3 h at room temperature. Evaporation to dryness under a hood gave a solid which was recrystallized from EtOH to give **12a** (2.5 g, 78%): mp 152 °C; IR (KBr) 2250, 1765, 1610 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3 (t, 3 H), 4.05 (t, 2 H), 5.2 (s, 2 H), 7.2 and 7.75 (2 d, 4 H), 7.45 (br s, 5 H). Anal. ($C_{18}H_{15}N_3O_3$) C, H, N.

Compounds **12b–h**, shown in Table II, were synthesized by following the same procedure.

5-[4-(Benzyloxy)phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (13a). To a solution of **11a** (0.59 g, 2 mmol) in dry chloroform (50 mL) was added freshly distilled thiophosgene (0.23 g, 2 mmol). The stirred mixture was heated under reflux for 3 h. The solvent was removed in vacuo and the resulting residue was chromatographied with CH_2Cl_2 as eluent then recrystallized from EtOH to give **13a** (0.6 g, 89%): mp 117 °C; IR (KBr) 2250, 1610 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3.1 (t, 2 H), 4.4 (t, 2 H), 5.25 (s, 2 H), 7.3 and 7.95 (2 d, 4 H), 7.5 (br s, 5 H). Anal. ($C_{18}H_{15}N_3O_2S$) C, H, N.

Compounds **13b–h**, shown in Table II, were synthesized by following the same procedure.

5-[4-(Benzyloxy)phenyl]-1,3,4-oxadiazol-2(3*H*)-one (14a). A stirred and cooled solution of **10a** (4.85 g, 20 mmol) in dry dioxane (100 mL) was treated for 30 min with a flux of $COCl_2$. Evaporation in vacuo gave a solid residue which recrystallized from 1-butanol to give **14a** (4.1 g, 76%): mp 117 °C; IR (KBr) 3230, 1760, 1610 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 5.15 (s, 2 H), 7.15 and 7.7 (2 d, 4 H), 7.2–7.5 (m, 5 H), 12.3 (s, 1 H). Anal. ($C_{15}H_{12}N_2O_3$) C, H, N.

5-[4-(Benzyloxy)phenyl]-3-(2-hydroxyethyl)-1,3,4-oxadiazol-2(3*H*)-one (14b). To a solution of **14a** (1.34 g, 5 mmol) in an anhydrous mixture of EtOH/DMF (4:1, 10 mL) was added a solution of NaOH (0.2 g, 5 mmol) in absolute EtOH (4 mL). The mixture was stirred for 16 h at room temperature. The resulting sodium salt was treated dropwise with 2-chloroethanol (0.4 g, 5 mmol) under stirring. The mixture was stirred at 60 °C for 24 h then cooled and poured onto iced water (50 mL). The resulting precipitate was collected, dried, and recrystallized from EtOH to give **14b** (1.05 g, 67%): mp 156 °C; IR (KBr) 3445, 1740, 1615 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3.75 (br s, 4 H), 4.9 (t, 1 H), 5.2 (s, 2 H), 7.2 and 7.75 (2 d, 4 H), 7.45 (br s, 5 H). Anal. ($C_{17}H_{16}N_2O_4$) C, H, N.

Compounds **14c–g**, shown in Table II, were synthesized from **14a** with suitable alkyl chlorides by following the above procedure.

2-[4-(Benzyloxy)phenyl]-4-(2-cyanoethyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (15a). To a solution of **11a** (0.59 g, 2 mmol) in anhydrous $CHCl_3$ (25 mL) was added dropwise at room temperature chloroacetyl chloride (0.23 g, 2 mmol), and the stirred mixture was refluxed for 30 min. After cooling and evaporation of the solvent, the resulting crop was taken up into ethanol (20 mL) and charged with an excess of anhydrous K_2CO_3 (2.1 g). The mixture was stirred to reflux for 2 h and then filtered and evaporated in vacuo. The residue was chromatographed with AcOEt/petroleum ether (1:19) and recrystallized from EtOH to give **15a** (0.43 g, 64%): mp 140.5 °C; IR (KBr) 2240, 1680 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 2.9 (t, 2 H), 4 (t, 2 H), 4.85 (s, 2 H), 5.2 (s, 2 H), 7.15 and 7.85 (2 d, 4 H), 7.5 (br s, 5 H). Anal. ($C_{19}H_{17}N_3O_3$) C, H, N.

Compounds **15b–h**, shown in Table II, were synthesized by following the same procedure.

2-[4-(Benzyloxy)phenyl]-4-(2-cyanoethyl)-4*H*-1,3,4-oxadiazine-5(6*H*)-thione (16). A mixture of **15a** (1.5 g, 4.5 mmol) and an excess of P_2S_5 (2 g, 9 mmol) in dry pyridine (60 mL) was stirred for 2 h at 60 °C. The hot mixture was filtered, cooled, and evaporated under reduced pressure. The resulting solid was recrystallized from 1-propanol to give **16** (1.2 g, 76%): mp 136.5 °C; IR (KBr) 2240, 1595 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3.05 (t, 2 H), 4.45 (t, 2 H), 5.05 (s, 2 H), 5.15 (s, 2 H), 7.1 and 7.85 (2 d, 4 H), 7.4 (br s, 5 H). Anal. ($C_{19}H_{17}N_3O_2S$) C, H, N.

[[[4-(Benzyloxy)phenyl](thiocarbonyl)]thio]acetic Acid (17). To a cold and vigorously stirred Grignard solution of [4-(benzyloxy)phenyl]magnesium bromide, prepared from 4-(benzyloxy)phenyl bromide (52.5 g, 200 mmol) and magnesium (6 g, 247 mmol) in dry THF (150 mL), was added dropwise a solution of CS_2 (25 mL, 415 mmol) in dry THF (50 mL). The mixture was stirred for 12 h at room temperature, treated with a solution of chloroacetic acid (25 g, 265 mmol) and Na_2CO_3 (38 g, 360 mmol) in H_2O (250 mL), and stirred for 1 week at 25 °C. The precipitate was filtered and washed with water. The filtrate was extracted with diethyl ether. The aqueous layer containing the sodium salt of the product was acidified with sulfuric acid and then extracted with ether. The organic phases were dried over Na_2SO_4 and evaporated in vacuo. The obtained solid was recrystallized from benzene to give **17** (33.1 g, 52%): mp 130 °C; IR (KBr) 2900, 1680 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 4.25 (s, 2 H), 5.2 (s, 2 H), 7.1 and 8.05 (2 d, 4 H), 7.4 (br s, 5 H), 12.8 (br s, 1 H). Anal. ($C_{16}H_{14}O_3S_2$) C, H.

1-[[4-(Benzyloxy)phenyl](thiocarbonyl)]-2-(2-cyanoethyl)hydrazine (18). To a solution of NaOH (4 g, 100 mmol) in 100 mL of water and 50 mL of EtOH was added acid **17** (15.9 g, 50 mmol). After dissolution, a solution of (2-cyanoethyl)hydrazine[40] (6.4 g, 75 mmol) in EtOH (20 mL) was added dropwise at 0 °C. The reaction mixture was stirred and heated for 20 min at 60–80 °C and then cooled at room temperature and acidified with 6 N HCl (Congo red). Compound **18** precipitated. After filtration, the crop was dissolved in hot EtOH (150 mL) and the solution was filtered. The filtrate was evaporated and the resulting solid was recrystallized from EtOH to give 5.9 g (38%): mp 135 °C; IR (KBr) 3260, 3205, 3140, 2240 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 2.75 (t, 2 H), 3.25 (t, 2 H), 5.15 (s, 2 H), 6.85 (br s, 1 H), 7.05 and 7.75 (2 d, 4 H), 7.25–7.55 (m, 5 H), 11.8 (s, 1 H). Anal. ($C_{17}H_{17}N_3OS$) C, H, N.

5-[4-(Benzyloxy)phenyl]-3-(2-cyanoethyl)-1,3,4-thiadiazol-2(3*H*)-one (19). Reaction of **18** (3.11 g, 10 mmol) with phosgene using the procedure described for **12a** gave **19** (2.1 g, 62%): mp 143 °C; IR (KBr) 2250, 1650, 1600 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3 (t, 2 H), 4.15 (t, 2 H), 5.15 (s, 2 H), 7.15 and 7.65 (2 d, 4 H), 7.4 (br s, 5 H). Anal. ($C_{18}H_{15}N_3O_2S$) C, H, N.

5-[(4-Benzyloxy)phenyl]-3-(2-cyanoethyl)-1,3,4-thiadiazole-2(3*H*)-thione (20). Reaction of **15** (0.62 g, 2 mmol) with thiophosgene (0.23 g, 2 mmol) using the procedure described for **13a** gave **20** (0.36 g, 51%): mp 138 °C; IR (KBr) 2250, 1605 cm^{-1}; ^1H NMR (90 MHz, DMSO-d_6) δ 3.15 (t, 2 H), 4.55 (t, 2 H), 5.2 (s, 2 H), 7.2 and 7.75 (2 d, 4 H), 7.4 (br s, 5 H). Anal. ($C_{18}H_{15}N_3OS_2$) C, H, N.

Biochemistry. [β-^{14}C]-5-Hydroxytryptamine–creatinine sulfate (5-HT, 54 mCi/mmol) and [1-*ethyl*-^{14}C]-β-phenylethylamine hydrochloride (PEA, 55 mCi/mmol) were purchased from Amersham Laboratories, Amersham, England. Clorgyline (**21**) hydrochloride was obtained from May and Baker, Dagenham, England. L-Deprenyl (**1**) hydrochloride was kindly supplied by "Centre de Recherche Delalande", Rueil-Malmaison, France. Drugs to be tested were dissolved in DMSO to 100 mM. The weak inhibitors were diluted in DMSO/water (1:1, v/v). The

more potent inhibitors were diluted in 10% DMSO/water (v/v), which did not affect MAO activity. L-Deprenyl (1) and clorgyline (21) were dissolved in water. Prior to use, isotopes were diluted with corresponding unlabeled amines to give working solutions of known specific radioactivity.

Preparation of Mitochondrial Homogenate. Male Sprague–Dawley rats (Iffa Credo, L'Arbresle, France) weighing 200–250 g were used. A crude brain mitochondrial fraction (7.8 mg of protein/mL) was prepared as previously described[25] and used as an enzyme source.

Protein Determination. Protein concentrations were measured by the method of Lowry,[41] using bovine serum albumin as a standard.

Determination of MAO Activity in Vitro. MAO activity was determined radiochemically at 37 °C and pH 7.4 with [^{14}C]-5-HT (4.8 μM/μCi, for MAO A) or [^{14}C]PEA (26.6 μM/μCi, for MAO B) as substrates.[25] Unless otherwise specified, final concentrations in the standard assay were set to 0.195 mg of protein/mL for mitochondrial fraction, 10 μM 5-HT, and 5 μM PEA for MAO A and B assays, respectively, and test drugs were preincubated for 20 min with the enzyme, the final concentration of DMSO being 1% in the inhibition kinetic studies. In all studies, care was taken so that MAO activity was linear with the enzyme concentration up to the time period of the assay, and substrate deamination did not exceed 10% (in the absence of drug). MAO activities were expressed either as percent of the control or as nmol/mg of protein per min where appropriate. All the assays were routinely duplicated.

Ex Vivo MAO B Inhibition. Strolin Benedetti's procedure[42] was followed with adjunction of slight modifications. Compounds to be tested were suspended in 50 μL of Tween 80 and then in 0.5 mL of aqueous solution of methylcellulose 0.5% (v/v). Male Sprague–Dawley rats (150 g) fasted for about 16 h received an oral dose of drug (a single or a daily dose) or vehicle (control) and groups of four animals were sacrificed at different times after treatment. Brains were rapidly removed, frozen in liquid nitrogen, and stored at −80 °C. The tissues were homogenized in 0.1 M phosphate buffer, pH 7.4 (1 g/16 mL), using an Ultra-Turrax, and aliquots of 100 μL were used to assay MAO activity in a final volume of 0.5 mL. The reaction was started by addition of the homogenate to a mixture containing 100 μL of [^{14}C]PEA (1 μCi/μM) or [^{14}C]-5-HT (10 μCi/μM) and 300 μL of 0.1 M phosphate buffer, pH 7.4. The final concentration of PEA and 5-HT was 8 and 125 μM, respectively. The reaction was carried out at 37 °C using an incubation time of 1 and 5 min for PEA and 5-HT, respectively. After incubation time, the reaction was acidified with 200 μL of 4 N HCl and cooled on ice. Deaminated products were extracted in 7 mL of toluene/ethyl acetate (1:1, v/v) and radioactivity was counted in 10 mL of toluene containing 0.4% PPO (w/v). The results were expressed as percent inhibition vs control. Mean values and standard errors were calculated from determinations in four brain homogenates (Table IV).

Reversibility Test. A 2.4-mL sample of the mitochondrial homogenate (1.94 mg of protein/mL) was preincubated at 37 °C for 1 h in the absence (control) or presence of 6.25 nM **12a**, 12.5 nM **13a**, 12.5 nM **14b**, or 12.5 nM L-deprenyl (1), concentrations which partially inhibit MAO B. Mitochondria were then diluted 200-fold in 100 mM phosphate buffer, pH 7.4, containing 100 μM PEA, incubated at 37 °C for 4 h, and centrifuged at 20000*g* for 10 min at 20 °C. The mitochondrial pellet was suspended in 200 mL of the same phosphate buffer without PEA and incubated at 37 °C for a further period of 1 h. Washed mitochondria were recentrifugated, resuspended in 0.5 mL of buffer, and tested for MAO activity under standard conditions using a reaction time of 1 and 4 min for PEA and 5-HT deamination, respectively. The results were expressed as percent inhibition vs control (Table V).

Acknowledgment. The authors are grateful to Dr. G. Barbier for his advice and help. We thank the "Centre de Recherche Delalande" for partial support of this research.

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