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STRUCTURE-ACTIVITY STUDIES ON MONOAMINE OXIDASE INHIBITORS BY CALORIMETRIC AND QUANTUM MECHANICAL CALCULATIONS

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Structure-activity relationship studies were carried out on a new series of hydrazino-thiosemicarbazide derivatives, which inhibit monoamino oxidase (MAO). Fifty-five compounds were synthesized and tested "*in vitro*" for their inhibitory effects on rat liver mitochondrial MAO. The most efficient MAO inhibitors were the benzylidene derivatives (R-CH=N^1-N^2H-C(N^4R_1)(SR_2)) where *R* is the piperonyl radical and ethyl or isopropyl substituents are in R_1 position. Correlation of MAO activity with hydrophobic, electronic and steric properties of tested compounds, evaluated by means of Quantum Mechanical calculations and calorimetric analysis (DSC) suggest that electronic and steric parameters give a better fit than hydrophobicity with the biological activity.

KEY WORDS: Structure-activity relationships, thiosemicarbazide derivatives, monoamine oxidase inhibition

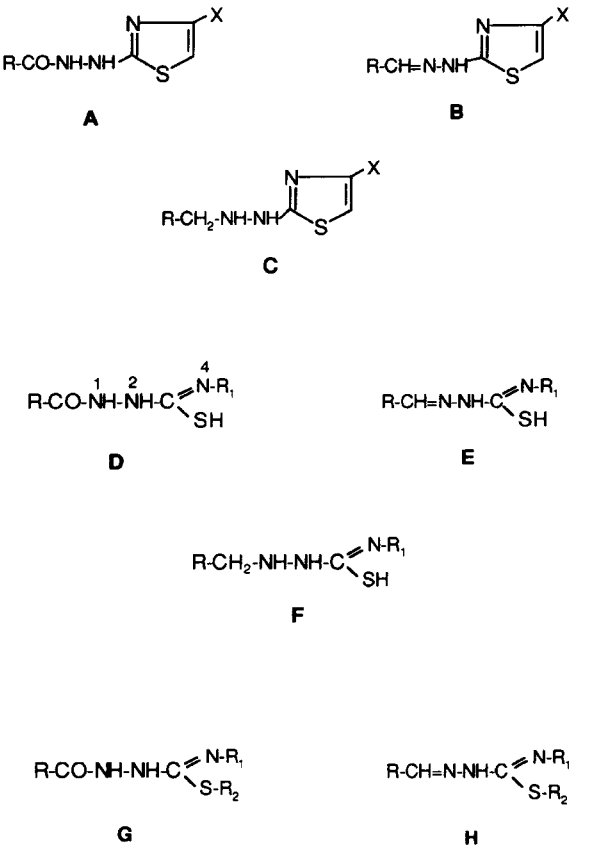
INTRODUCTION

We have previously reported[1] the monoamine oxidase inhibitory (MAOI) activity of some 2-thiazolyl-hydrazine derivatives, corresponding to general formulas **A–C** (Figure 1).

Based on *in vitro* assays, MAOI activity was found to be greater in derivatives containing the N^1-alkoxybenzoyl (**A**) and alkoxybenzylidene groups (**B**).

In the following studies,[2,3] to provide more information on the structure-activity relationship, we have prepared and tested new thiazole derivatives (**A–C**, $X = H$), as well as thiosemicarbazides **D**, **E** and **F** (Figure 1; $R_1 = H$), which represent the 'open' molecular

* Correspondence.



R = 3,4,5-trimethoxyphenyl; 3,4-methylenedioxyphenyl(piperonyl); 3,5-dimethoxy-4-ethoxyphenyl(ethylsyringyl).

X = H; CH_3; C_6H_5; substituted phenyl.

R_1 = H; CH_3; C_2H_5; C_3H_7; i-C_3H_7; C_6H_5; CH_2-C_6H_5.

R_2 = CH_3; C_2H_5.

FIGURE 1 Structures for MAOIs.

structures with respect to the previous compounds containing the thiazole ring. Also in this case, molecules bearing a -CO-NH-NH- (**D**) or a -CH=N-NH- radical (**E**) were the most active, particularly when the aryl substituent *R* was a 3,4,5-trimethoxyphenyl or a 3,4-methylenedioxyphenyl(piperonyl) group (Figure 1).

Here, structure-activity studies were carried out in a new series of thiosemicarbazide derivatives (**D**, **E**, **G**, and **H**; Tables 1–4) which are also 'open models' of the hydrazino-thiazoles (**A**–**C**) but which, because of the presence of the N^4-alkyl chain, better resemble

the thiazole nucleus than thiosemicarbazides **D–F** (R_1 = H). Moreover, to determine the role of -SH group in the mechanism of enzyme inhibition, we also alchylated the sulphur atom, leading to compounds **G** and **H**.

The inhibitory potency of these compounds was correlated with the electron-donating or withdrawing capacity of the substituents, obtained from Molecular Orbital calculations, and with their hydrophobicity parameters. In fact, one of the mechanism proposed for MAO-catalyzed amine oxidation suggests that the first step in the reaction is the electron transfer from the nitrogen lone pair of the amine to the flavine residue inside the catalytic pocket of the enzyme.[4] If so, it could be expected that the most active inhibitors are those which have the lower ionization potential and thus a more elective electron transfer. Moreover, another possible contribution was the steric effect of substituents which could be due to binding at a surface or in a pocket engulfed in the enzyme.[5]

In addition, inhibitor hydrophobicity appears to be a relevant factor in relation to a possible interaction with a complementary hydrophobic site of the enzyme,[6–8] which would generally favours the complex formation with the inhibitor. Therefore, the purpose of this study was to correlate the hydrophobic, electronic and steric parameters of inhibitors with their biological activity.

MATERIALS AND METHODS

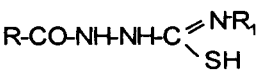
Reagents and solvents were purchased from Sigma Aldrich Chimica, S.r.l. (Milan, Italy). Synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) was obtained from Fluka Chemical Co. (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography (a: $CHCl_3$-MeOH, 3:1; b: $CHCl_3$-MeOH-AcOH-H_2O, 12:60:8:2.5); spots were evidenced by the Dragendorff reagent.

Melting points were obtained with a Büchi mod. 530 apparatus and are uncorrected. Elemental analysis was performed by a Carlo Erba mod. 1106 analyzer. Analytical TLC (silica gel plates; F_{254}, Merck) employed ethyl acetate/cyclohexane mixtures (7:3 to 9:1) as eluent systems, UV light (254 and 306 nm) was used to visualize the spots. IR spectra were obtained in KBr on a Perkin Elmer 1600 series FTIR instrument; ^{1}H-NMR analysis was performed on a Brüker AC 250 MHz spectrophotometer, using DMSO as solvent and tetramethylsilane as the internal standard.

N^1-Alkoxybenzoyl-N^4-alkyl(aryl)-thiosemicarbazides (**1–18**)

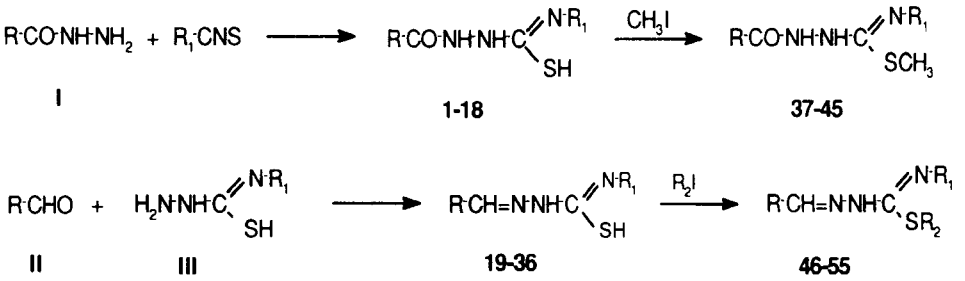
These compounds were prepared[2] from hydrazides **I** by reaction with the appropriate isothiocyanate (Scheme 1). Isopropyl isothiocyanate was prepared according to the procedure of Schmidt *et al.*[9] N^4-methyl-, ethyl- and phenyl derivatives have been previously synthetized and described by Mazzone *et al.*[10] Ethanolic solutions (30 mL) of equimolar quantities (5 mmol) of alkoxybenzoyl hydrazides **I** and the desired isothiocyanate (methyl, ethyl, *n*-propyl, *i*-propyl, phenyl or benzyl) were refluxed for 1 h. After cooling, the white solid formed was filtered off and crystallized from dimethylformamide (DMF)-ethanol, to give compounds **1–18** with a 70–80% yield. Table 1 reports their physico-chemical properties.

TABLE 1
Physico-chemical data of N^1-alkoxybenzoyl-N^4-alkyl(phenyl)-thiosemicarbazides **1–18**.



Compd	*R*	R_1	mp (°C)[a]	Formula	IR (KBr, cm^{-1})
1	3,4,5-trimethoxyphenyl	Me	207[b]	—	—
2	"	Et	193–94[b]	—	—
3	"	*n*-Pr	196–98	$C_{14}H_{21}N_3O_4S$	3300, 1690, 1670, 1585
4	"	*i*-Pr	173–75	$C_{14}H_{21}N_3O_4S$	3295, 1690, 1672, 1590
5	"	Ph	189–90[b]	—	—
6	"	Bz	198	$C_{18}H_{21}N_3O_4S$	3320, 1665, 1635, 1590
7	3,4-methylenedioxyphenyl	Me	211–12[b]	—	—
8	"	Et	199–200[b]	—	—
9	"	*n*-Pr	194–95	$C_{12}H_{15}N_3O_3S$	3330, 3170, 1650, 1590
10	"	*i*-Pr	193–95	$C_{12}H_{15}N_3O_3S$	3360, 3335, 1665, 1605
11	"	Ph	171–72[b]	—	—
12	"	Bz	170	$C_{16}H_{15}N_3O_3S$	3330, 1665, 1640, 1605
13	3,5-dimethoxy-4-ethoxyphenyl	Me	206–7[b]	—	—
14	"	Et	201–2[b]	—	—
15	"	*n*-Pr	198–99	$C_{15}H_{23}N_3O_4S$	3320, 1675, 1640, 1580
16	"	*i*-Pr	181–83	$C_{15}H_{23}N_3O_4S$	3320, 1670, 1642, 1580
17	"	Ph	190–91[b]	—	—
18	"	Bz	197	$C_{19}H_{23}N_3O_4S$	3330, 3240, 1665, 1635

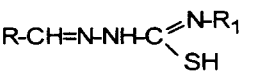
[a] Crystallized from DMF-ethanol; [b] described in Ref. 9.



R = 3,4,5-trimethoxyphenyl; 3,4-methylenedioxyphenyl; 3,5-dimethoxy-4-ethoxyphenyl.
R_1 = CH_3; C_2H_5; n-C_3H_7; i-C_3H_7; C_6H_5; CH_2-C_6H_5.
R_2 = CH_3; C_2H_5.

SCHEME 1 Synthetic routes.

TABLE 2
Physico-chemical data of N^1-alkoxybenzylidene-N^4-alkyl(phenyl)-thiosemicarbazides **19–36**.



Compd	R	R_1	mp (°C)[a]	Formula	IR (KBr, cm^{-1})
19	3,4,5-trimethoxyphenyl	Me	139–40	$C_{12}H_{17}N_3O_3S$	3395, 3330, 2360, 1574
20	"	Et	179	$C_{13}H_{19}N_3O_3S$	3370, 3150, 1610, 1580
21	"	*n*-Pr	159–60	$C_{14}H_{21}N_3O_3S$	3355, 3310, 1607, 1575
22	"	*i*-Pr	145–46	$C_{14}H_{21}N_3O_3S$	3362, 3165, 1615, 1580
23	"	Ph	163–64	$C_{17}H_{19}N_3O_3S$	3315, 3185, 1600, 1580
24	"	Bz	130–32	$C_{18}H_{21}N_3O_3S$	3280, 3150, 1575
25	3,4-methylenedioxyphenyl	Me	212–13	$C_{10}H_{11}N_3O_2S$	3315, 3138, 2360, 1555
26	"	Et	168	$C_{11}H_{13}N_3O_2S$	3280, 3120, 1630, 1590
27	"	*n*-Pr	203–4	$C_{12}H_{15}N_3O_2S$	3300, 1630, 1605
28	"	*i*-Pr	174–76	$C_{12}H_{15}N_3O_2S$	3350, 3130, 1625, 1590
29	"	Ph	193–94	$C_{15}H_{13}N_3O_2S$	3310, 3145, 1600, 1555
30	"	Bz	179–81	$C_{16}H_{15}N_3O_2S$	3275, 3160, 1590
31	3,5-dimethoxy-4-ethoxyphenyl	Me	181–83	$C_{13}H_{19}N_3O_3S$	3355, 3280, 1615, 1580
32	"	Et	133	$C_{14}H_{21}N_3O_3S$	3300, 3220, 1640, 1610
33	"	*n*-Pr	158–59	$C_{15}H_{23}N_3O_3S$	3350, 3160, 1620, 1580
34	"	*i*-Pr	175–76	$C_{15}H_{23}N_3O_3S$	3350, 3160, 1610, 1580
35	"	Ph	174–75	$C_{18}H_{21}N_3O_3S$	3305, 3200, 1600, 1580
36	"	Bz	155–57	$C_{19}H_{23}N_3O_3S$	3260, 3150, 1575

[a] Crystallized from DMF-ethanol.

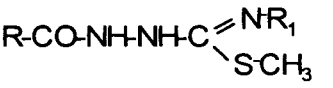
*N^1-Alkoxybenzylidene-N^4-alkyl(aryl)-thiosemicarbazides (**19–36**)*

These compounds were obtained by condensing alkoxybenzaldehydes **II** with N^4-alkyl(aryl)-thiosemicarbazides **III** (Scheme 1); among these latter, 4-propyl-, 4-isopropyl-, and 4-benzyl derivatives are not commercially available, and were prepared as described by the reaction of the corresponding isothiocyanates with hydrazine hydrate.[11] Ethanolic solution (25 mL) of 3,4,5-trimethoxybenzaldehyde, 3,4-dioxymethylenebenzaldehyde (piperonal) or 3,5-dimethoxy-4-ethoxybenzaldehyde **II**[12] (5 mmol) and the opportune N^4-substituted thiosemicarbazide **III** (5 mmol), containing few drops of glacial acetic acid, were reacted under reflux for 1 h. After cooling to room temperature, the yellowish product which precipitated was filtered and crystallized by DMF-ethanol, with 65–80% yields (Table 2).

*Thiosemicarbazide S-alkyl derivatives (**37–55**)*

The required N^4-substituted thiosemicarbazide **1–36** (5 mmol) was dissolved or suspended in ethanol or methanol (40 mL) and warmed for 12 h at about 40°C with a slight excess

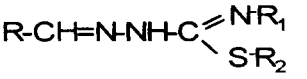
TABLE 3
Physico-chemical data of N^1-alkoxybenzoyl-N^4-alkyl-*S*-methyl-thiosemicarbazides **37–45**.



Compd	*R*	R_1	mp (°C)[a]	Formula	IR (KBr, cm^{-1})
37	3,4,5-trimethoxyphenyl	Me	187–88	$C_{13}H_{19}N_3O_4S$	3260, 2936, 1631, 1574
38	"	Et	154–55	$C_{14}H_{21}N_3O_4S$	3180, 2970, 1635, 1567
39	"	Bz	149	$C_{19}H_{23}N_3O_4S$	3450, 1635, 1565, 1550
40	3,4-methylenedioxyphenyl	Me	175–76	$C_{11}H_{13}N_3O_3S$	3280, 3000, 1630, 1580
41	"	Et	137–39	$C_{12}H_{15}N_3O_3S$	3287, 2975, 1632, 1583
42	"	Bz	148–49	$C_{17}H_{17}N_3O_3S$	3450, 3203, 1643, 1555
43	3,5-dimethoxy-4-ethoxyphenyl	Me	197–99	$C_{14}H_{21}N_3O_4S$	3260, 2980, 1628, 1573
44	"	Et	148–49	$C_{15}H_{23}N_3O_4S$	3260, 2970, 1635, 1580
45	"	Bz	158–59	$C_{20}H_{25}N_3O_4S$	3200, 2975, 1633, 1570

[a] Crystallized from ethanol.

TABLE 4
Physico-chemical data of N^1-alkoxybenzylidene-N^4-alkyl-*S*-methyl(ethyl)-thiosemicarbazides **46–55**.



Compd	*R*	R_1	R_2	mp (°C)[a]	Formula	IR (KBr, cm^{-1})
46	3,4,5-trimethoxyphenyl	Me	Me	131	$C_{13}H_{19}N_3O_3S$	3200, 1572, 1505
47	"	Et	Me	117–18	$C_{14}H_{21}N_3O_3S$	3200, 2935, 1570, 1502
48	"	Et	Et	65–6	$C_{15}H_{23}N_3O_3S$	3450, 2965, 1573, 1525
49	"	Bz	Me	83–4	$C_{19}H_{23}N_3O_3S$	3450, 1575, 1500
50	3,4-methylenedioxyphenyl	Me	Me	92–3	$C_{11}H_{13}N_3O_2S$	3470, 1590, 1532, 1490
51	"	Et	Me	90–1	$C_{12}H_{15}N_3O_2S$	3460, 2965, 1890, 1596
52	"	Bz	Me	173–4	$C_{17}H_{17}N_3O_2S$	3252, 3035, 1652, 1597
53	3,5-dimethoxy-4-ethoxyphenyl	Me	Me	144–5	$C_{14}H_{21}N_3O_3S$	3362, 2970, 1580, 1550
54	"	Et	Me	128–9	$C_{15}H_{23}N_3O_3S$	3505, 3200, 1572, 1525
55	"	Bz	Me	179–80[b]	$C_{20}H_{25}N_3O_3S$	3355, 3190, 1605, 1500

[a] Crystallized from ethanol; [b] Isolated as the HI salt.

of ethyl or methyl iodide, respectively. The mixture was then dried *in vacuo* and the crude residue was taken up with distilled water. After filtration, the clear solution was treated dropwise with a 10% aqueous $NaHCO_3$ solution and the resulting white precipitate was filtered, washed with water, and crystallized from ethanol. Yields were in the 50–70% range. Tables 3 and 4 give m.p.'s and the other characteristics of these compounds. In TLC (ethyl acetate-cyclohexane, 8:2), these derivatives appeared as slower-moving spots, with respect to the starting compounds.

Alternatively, these compounds could be obtained by dissolving the 4-alkyl thiosemicarbazide in the equivalent amount of a 10% NaOH solution, the eventual undissolved part was removed and the clear solution was added dropwise to a two-fold excess of the required alkyl iodide. After stirring overnight at room temperature, the resulting white solid was separated and crystallized from ethanol.

The purity of all the synthesized compounds was verified by means of melting point, elemental analysis, analytical TLC, IR and ^{1}H-NMR spectra.

Mitochondrial preparations

Crude mitochondrial fraction was prepared from the livers of male and female albino rats (200–250 g). The animals were decapitated and the organs rapidly removed. The tissue was homogenized in a Potter homogenizer with 9 volumes of ice-cold medium containing 220 mM mannitol, 70 mM sucrose, 0.1 mM EGTA and 5 mM Hepes buffer, pH 7.5. Liver mitochondria were isolated by differential sedimentation at various centrifugal force. The homogenate was centrifuged twice at $800 \times g$ for 10 min at 4°C and the resulting supernatant centrifuged at $12.000 \times g$ for 20 min. The crude mitochondrial pellet was suspended (1:5, w/v) in 100 mM phosphate buffer, pH 7.4, fractionated in 0.5 mL samples and stored at −70°C.

Protein concentration was determined according to the method of Lowry *et al.*,[13] in the presence of 0.05% sodium dodecyl sulfate, using bovine serum albumin as standard.

Determination of MAO activity

MAO activity in rat liver mitochondrial fractions was determined by a fluorimetric assay, as described by Morinan and Garratt.[14] Aliquots of 50 μL mitochondrial suspension 800 μg protein), 820 μL of 50 mM phosphate buffer pH 7.4, and 100 μL of solubilizing solution (control) or inhibitor solution at the final concentration of 10^{-4} M, were preincubated at 37°C for 5 min. The tested compounds were dissolved in DMSO/H_2O (3:1, v/v). The reaction was started by addition of 30 μL of 3.07 mM kynuramine (final conc. 92 μM) and the tubes were shaken for 15 min. After addition of 300 μL of 0.4 M $HClO_4$, the tubes were mixed and centrifuged at $12.000 \times g$ for 30 s in a HERMLE microcentrifuge, to remove precipitated protein. The supernatant was transferred to test tubes containing 2 mL 1 M NaOH and the fluorescence was measured against a blank at $\lambda_{ex} = 315$ nm and $\lambda_{em} = 380$ nm, in a Perkin-Elmer LS5 spectro-fluorimeter. The concentration of the product (4-hydroxyquinoline) was calculated from a standard curve (0.25–3.00 nmol). MAO activity was expressed as nmoles of 4-hydroxyquinoline formed/mg protein/h and expressed as % inhibition of the respective control (Table 5). All assays were performed in triplicate.

TABLE 5
In vitro rat liver MAO inhibition values.[a]

Compd	% Inhibition	Compd	% Inhibition	Compd	% Inhibition
1	47.3 (0.75)	**19**	58.4 (3.03)	**37**	24.6 (0.51)
2	60.5 (3.56)	**20**	71.9 (1.21)	**38**	29.7 (2.40)
3	53.3 (0.71)	**21**	48.3 (3.29)	**39**	36.6 (0.80)
4	57.0 (1.10)	**22**	65.2 (0.76)	**40**	34.2 (1.54)
5	56.0 (0.88)	**23**	65.3 (3.20)	**41**	36.8 (0.09)
6	55.5 (0.40)	**24**	61.3 (1.19)	**42**	45.6 (0.36)
7	56.2 (0.11)	**25**	79.0 (1.35)	**43**	22.8 (1.25)
8	64.1 (2.19)	**26**	90.0 (0.23)	**44**	26.8 (2.13)
9	63.9 (2.96)	**27**	87.2 (0.74)	**45**	n.t.
10	63.0 (1.31)	**28**	91.8 (0.63)	**46**	68.9 (0.65)
11	61.4 (3.12)	**29**	86.6 (0.27)	**47**	64.5 (0.58)
12	65.6 (0.10)	**30**	81.4 (3.06)	**48**	65.2 (0.74)
13	34.0 (3.80)	**31**	69.0 (0.33)	**49**	71.2 (1.34)
14	60.8 (0.55)	**32**	61.0 (0.11)	**50**	76.2 (0.63)
15	40.5 (1.60)	**33**	63.3 (1.12)	**51**	73.2 (0.66)
16	59.6 (1.06)	**34**	63.7 (1.46)	**52**	45.7 (1.80)
17	54.8 (0.35)	**35**	66.4 (0.66)	**53**	70.5 (1.52)
18	60.0 (0.21)	**36**	64.0 (0.33)	**54**	62.9 (1.35)
				55	n.t.

[a] Compounds were tested at a concentration of 1×10^{-4} M. Values represent the mean of three measurements (± SE).

Preparation of liposomes

DPPC multilamellar liposomes (MLVs) were prepared in the presence and absence of drugs following this procedure. $CHCl_3$-MeOH (1:1, v/v) stock solutions of lipid and drugs were mixed to obtain the chosen mole fraction of drugs. The solvents were removed under a nitrogen flow and the resulting film was freeze-dried under vacuum to remove the residual solvents.

Liposomes were obtained by adding to the film 50 mM Tris-HCI buffer (pH 7.4), then by heating at a temperature above that of the gel-liquid crystalline phase transition (60°C), and by vortexing three times for 1 min. The samples were then shaken for 1 h in a water bath at 60°C, to homogenize the liposomes.

Differential Scanning Calorimetry (DSC)

Aliquots of 120 μL (5 mg of lipid) were transferred in a 160 μL DSC aluminium pan and submitted to DSC analysis.

DSC was performed by using a Mettler TA 3000 system equipped with a DSC-30 cell and a TC-10 processor. The scan rate employed was 2°C/min in temperature ranging between

10 and 70°C. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. Palmitic acid was employed to calibrate the temperature scale and transition enthalpies (H). Enthalpies were evaluated from the peak areas using the integration program of the TA processor, which permitted a choice of different baselines and ranges of integration. For curves showing an ill-defined baseline, a fix arm planimeter was employed. The areas calculated with these different methods lie within the experimental error (± 5%).

The samples were cooled and heated four times to achieve the reproducibility of results. All samples, after calorimetric scans, were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay.[15]

Quantum Mechanical calculations

PM 3 package is a recent algorithm[16–18] which allows the calculation of Molecular Orbitals (MO) of medium-sized organic molecules following the classical Hartree-Fock procedure. Computational times with this package are greatly reduced but retain a very good accuracy of the results. Short computational times allow for fully optimization of molecular geometry, interatomic distances and bond angles, together with the possibility to analyze all the existing internal conformations other than the minimum energy one. Useful output parameters of PM 3 are: molecular geometries, relative energy for different molecular conformations, energy barriers among molecular conformers, charge density on each atom, dipole moments, energy of Molecular Orbitals, localization and delocalization of MOs.

The key Quantum Mechanical parameter utilized here is the ionization potential. It measures the work required to extract an electron from a given k-th Molecular Orbital. According to Koopman's theorem,[19] the ionization potential $(\text{I.P.})_k$ is related to the MO energy through the relationship: $(\text{I.P.})_k = (-\text{MO})_k^i$ where $(-\text{MO})_k^i$ is the MO energy of a generic k-th orbital for a molecule lying in the i-th conformation.

The values reported in Table 6 are the mean I.P. (properly normalized) averaged over all molecular conformations. The average has been calculated by the equation:

$$\text{MEAN } (\text{I.P.})_k = \frac{\sum_{i=1}^{N} (-\text{MO})_k^i \exp\left(-\frac{E_i}{k_B T}\right)}{\sum_{i=1}^{N} \exp\left(-\frac{E_i}{k_B T}\right)} \tag{1}$$

where k_B is the Boltzman constant, T the absolute temperature, E_i is the energy of a generic i-th conformation, $(-\text{MO})_k^i$ is the ionization potential $(\text{I.P.})_k$ of a k-th Molecular Orbital for a molecule lying in the i-th conformation. The calculated ionization potential for a molecule closely related to the studied ones has been found to be in good agreement with the experimental values obtained by photoelectron spectroscopy measurements, which supports the reliability of our calculations.[20]

For each conformation, the molar volume was also calculated following the procedure developed by Pascual-Ahuir *et al.*[21] The values reported in the next section are the average over the more stable conformations.

TABLE 6
Ionization Potential (I.P.) of the lowest Molecular Orbitals (MO) of some classes of MAO inhibitors.

Compounds[a]	I.P.[b]	Description of the MO	I.P.	Description of the MO
A	9.23	N^2 + thiazole ring contributions	10.22	N^1 partially delocalized
B	8.92	N^2 + thiazole ring contributions	10.00	Mainly on S
D	9.51	N^2 + N^4 and S contributions	9.75	N^1 partially delocalized
E	9.14	N^2 + N^4 and S contributions	9.58	Mainly on S
G	9.21	Mainly on S	9.62	N^2 + N^4 and S contributions
H	8.98	Mainly on S	9.43	N^2 + N^4 and S contributions

[a] Compds. **A** and **B** were described in Ref. 1; structure **D** refers to compds. **1–18** of the present work, **E** to compds. **19–36**, **G** to compds. **37–44** and **H** to compds. **45–55**. [b] Ionization potentials are expressed in electron volts (eV) (1 eV = 23.06 kcal/mole).

RESULTS AND DISCUSSION

Structure-activity

Biochemical evaluation indicated that all the synthetized compounds possess a MAOI activity (Table 5). Benzylidene derivatives (**19–36**, Table 2) showed a higher activity than the corresponding compounds of the alkoxyaroyl series (**1–18**, Table 1). As described previously,[1,2] the piperonyl (3,4-dioxymethylenephenyl) radical exerted a greater positive influence on MAOI activity than the other alkoxyphenyl substituents. Very high values of MAO inhibition by comparison were observed for molecules bearing an unsubstituted sulphur atom and an ethyl or a benzyl group in the N^4 position. Such derivatives could be respectively considered as the corresponding 'open' molecular models of the 4-methyl and 4-phenyl substituted hydrazinothiazoles (**A** and **B**), i.e., the compounds which, within their series, display the highest MAOI activity.[1]

The opening of the thiazole ring in the structure of inhibitors did not modify their MAOI activity. Moreover, alkylation on the sulfur atom of thiosemicarbazide derivatives caused a clear drop in the inhibitory activity, which was similar in both benzoyl (**37–45**, Table 3) and benzylidene (**46–55**, Table 4) series. This suggests the influence of the free thiol group on the mechanism of enzyme catalysis, as discussed below. In fact, by comparing the inhibition values of benzylidene derivatives bearing an unsubstituted sulphur atom, a piperonyl radical in N^1 and a methyl, ethyl or benzyl group in N^4 (compounds **25**, **26** and **30** with activity ranging between 79 and 90% of inhibition), with the corresponding *S*-methyl derivatives (**50–52**), the latter always retain lower activity (46–76% inhibition). A similar detrimental effect on the activity induced by sulphur alkylation was observed for the alkoxyaroyl series (56–65% of inhibition for *S*-unsubstituted compounds **7**, **8**, and **12** vs. 34–45% for the *S*-methyl entries **40–42**).

No substantial difference in the activity was observed in relation to the length of the alkyl substituent (methyl or ethyl, compounds **47** and **48**, respectively) on the sulphur atom.

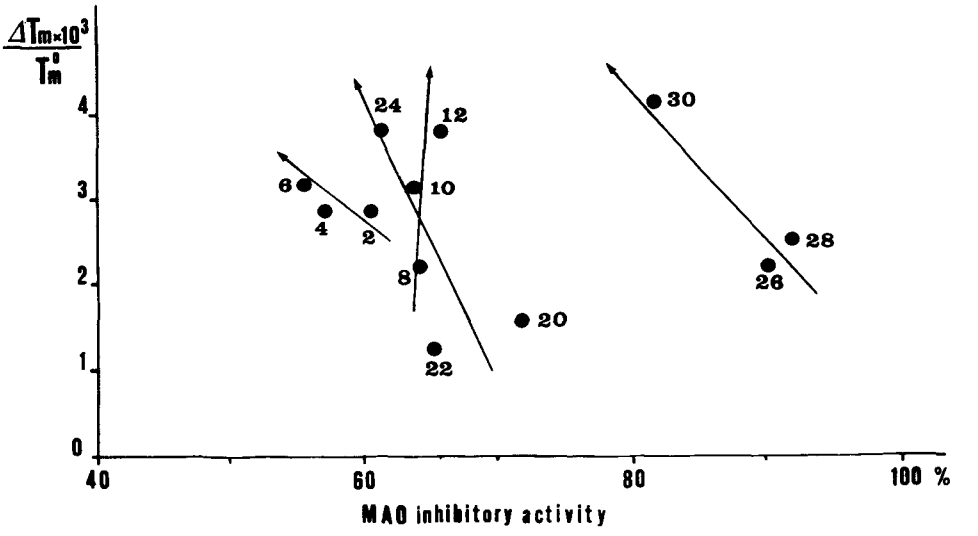


FIGURE 2 Relative variation of transition ($\Delta T/T$) of MAOI-containing DPPC liposomes *vs.* biological activity (% MAO inhibition at a drug concentration of 10^{-4} M). The arrows at the end of the lines indicate increasing hydrophobicity of substituent R_1 (C_2H_5, i-C_3H_7 and $CH_2C_6H_5$).

Calorimetric evaluation of relationship between lipophilicity and MAOI activity

Results of DSC analysis are reported in Figure 2, where the relative shift of transition temperature $\Delta T/T$ of some inhibitor molecules was considered as a measure of drug-membrane interaction.[22] It is known that during the calorimetric scan, the lipid matrix undergoes a phase transition associated with the cooperative melting of their hydrocarbon region and the addition of a foreign molecule causes generally a diminution of transition temperature according to the thermodynamical van't Hoff law. By measuring the melting point depression, it is possible to compare the liposolubility of closely related drugs.

In our opinion, the calorimetric approach is more realistic than the water/organic solvent distribution coefficient for the estimation of the role of hydrophobic forces in structure/activity relationship, because of the close resemblance between the experimental model system and cell enviroment.[23–25] Furthermore, it has been reported that the partitioning of drugs into liposomes is influenced both by hydrophobic forces and other factors such as interfacial phenomena, lipid chain packing and bilayer hydration state which are peculiar to the bilayers but are not present in the apolar phase of a classical water/organic solvent system.[26]

In Figure 2 arrows indicate the increasing hydrophobicity of substituents R_1 (ethyl, isopropyl and benzyl) and each arrow refers to a different class of thiosemicarbazides, with free thiol groups (see Tables 1 and 2 for numbering). It is evident that the hydrophobicity has a rather weak effect on biological activity in all compounds. These findings agree with those previously obtained for some aralkylhydrazine derivatives,[6] where the hydrophobic effect

of substituents is not as important as the electronic and steric effects, suggesting a specific mode of interaction between inhibitors and enzyme, dependent on substituent positions. Thus, great care should be taken in extrapolating the effect of hydrophobic substituents from one class of molecules to another.

The *S*-methyl derivatives **37–55** were not included in the calorimetric experiment because they undergo an hydrolytic cleavage of the *S*-alkyl moiety as a consequence of the temperature rise during thermal analysis (10–70°C).

Quantum Mechanical analysis of the prepared thiosemicarbazides

As opposed to the substrate used (kynuramine), which contain only one aliphatic nitrogen atoms, the MAOIs here investigated contain three unequivalent nitrogen atoms, which could be involved in the electron transfer process. Hence, the first goal of our work was to determine whether or not a prescribed nitrogen atom is involved in this process. The interest in this point is not speculative since it is known from which nitrogen atom the electron is transferred, then the redox properties can be modified by adapting the substituent in close proximity to this specific nitrogen atom.

There is, however, a problem in extrapolating these results which is connected to the noticeable flexibility of the investigated MAOI's, which have several low-lying molecular conformations stabilized by intramolecular hydrogen bonding. During the inhibitor-enzyme binding, MAOI molecules could take conformations different from the one with minimum energy. As redox potentials of nitrogen depend on the molecular conformation, MAOI biological activity will also depend on conformation. This could change the conclusions on the relative basicity of the different nitrogen atoms inferred from the most stable conformation.

We have therefore calculated the Molecular Orbital energies (proportional to redox potentials) for all possible conformations of each MAOI. Differences among MO energies are slight and depend on the geometrical arrangement of the molecule so that deviations are of the order $\pm$ 0.02 eV for most of the low-lying conformations (1 eV = 23.06 kcal/mole). Thus, the conclusions are not affected by the details of the molecular conformation assumed by MAO inhibitor bound to the enzyme.

For all studied molecules, we found that the I.P. of the orbitals located on nitrogen lone-pairs are arranged as: $N^2 > N^1 > N^4$ (see compound **D**, Figure 1 for atom numbering), which is independent of conformation.

The following results are noteworthy:

(a) the ionization potential of all the investigated MAOIs is consistently lower than that of the kynuramine substrate. Its I.P. relative to the aliphatic nitrogen lone pair is about 9.55 eV.

(b) The lowest energy MOs of MAOI's were centered on N^2 nitrogen (the nitrogen atom nearest to the thiazole ring, see Figure 1). The energies of N^1 and N^4 centered MOs were always higher. As a consequence, the substituents *R* (trimethoxyphenyl, piperonyl and ethylsyringyl) linked to the hydrazine residue had no significant effect on the redox properties of the investigated classes of MAOIs (variations less than 0.1 eV have been calculated by changing *R*), as suggested for other classes of MAO

inhibitors.[27–30] These sustituents, however, may greatly affect the hydrophobic and/or steric properties of MAOIs, thus modulating their biological response.

(c) Since hydrophobic properties of the substituent *R* had a weak effect on inhibitor activity, the other possible contribution was their steric effect. This effect appears to be due to the hindrance of the substituent or to the different types of interaction on the complementary surface of the enzyme[5] and is dependent on the van der Waals radius of the substituent. Indeed, the molar volume of the substituents, calculated as described in the experimental section, are the following: $V_{\text{piperonyl}} = 140.6$ Å^3, $V_{\text{trimethoxyphenyl}} = 205.1$ Å^3 and $V_{\text{ethylsyringil}} = 230.0$ Å^3. The highest activity was shown by inhibitors of every series containing piperonyl substituent, which have the lowest molar volume, suggest that their steric effects play a role in modulating the MAOI activity; the smaller the volume the greater the activity.

(d) The structure of the hydrazinic residue has a relevant effect on the lowest MOs energies. In particular, those containing the carbonyl group (Figure 1, compounds **A**, **D**, and **G**) always exhibited a shift of their MOs energies toward higher values, which disfavours the electron transfer as compared to the compounds containing iminic residues. This fact is in excellent agreement with the activity data of MAOI compounds, as discussed previously.

(e) It is important to study the electron structure in relation to the opening of the thiazole ring. MO energies of the orbital centered on N^2 become higher as a result of ring opening. Namely, the electron transfer is easier when the ring is intact because of the partial conjugation of the electrons belonging to the lone pair with the orbital centered on the thiazole ring (Figure 1, compounds **A** and **B**).

However, a deeper analysis shows that the electron density on N^2 is considerably lower (about 15%) when the thiazole ring is intact. Thus, the electron transfer is modulated by two opposite effects: on one hand, lower MOs energies provide a more effective electron transfer, on the other hand, as far as compounds containing a thiazole ring are concerned, there is less electron density that could be transferred during the redox process. In addition, a reduced electron density results into a greater donor-acceptor distance and weaker electrostatic forces during the transfer, all these factors contributing to reduce the inhibition rate.

(f) Methylation on the sulphur atom (Figure 1, compounds **G** and **H**) causes an (apparent) decrease of MAOI redox potential. However, a deeper analysis of the structure of the different MO's reveals that the lowest energy orbital is now localized on the sulphur atom. By contrast, in all the non-methylated compounds the lowest energy orbital is localized on the N^2 nitrogen (with a partial delocalization over N^4 and *S*), the energy for the sulphur lone pair being at higher figures. Thus, if we compare the energy of the N^2 lone pairs, we find that the electron transfer is easier in non-methylated compounds (see Table 6), providing that the electron transfer from the sulphur atom is not the reactive one, as compared with the electron transfer involving the nitrogen atoms.

In conclusion, Quantum Mechanical analysis and ionization potential calculations showed a close interplay between hydrophobic, electronic and steric effects in determining

the biological activity of the investigated compounds. The results indicate the role of thiazole ring integrity, sulphur atom methylation and hydrazinic residue chemical structure on the activity of MAO inhibitors. Moreover, the molar volume calculation of substituents linked to the hydrazinic residue suggest that steric effects play a role in modulating MAOI activity. On the other hand, calorimetric measurements give information about the hydrophobic properties of inhibitors, allowing an estimate of the amount of drug dissolved in the phospholipid bilayer and their possible interaction with hydrophobic site of enzyme molecule. When compared with *in vitro* MAOI activity, these data show a very satisfactory correlation between chemical structure and biological activity.

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