ChemInform Abstract: Synthesis and Inhibitory Activity Against Human Monoamine Oxidase of N1-Thiocarbamoyl-3,5-di(hetero)aryl-4,5-dihydro-(1H)-pyrazole Derivatives

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Short communication

Synthesis and inhibitory activity against human monoamine oxidase of N1-thiocarbamoyl-3,5-di(hetero)aryl-4,5-dihydro-(1*H*)-pyrazole derivatives

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ABSTRACT

A series of N1-thiocarbamoyl-3,5-di(hetero)aryl-4,5-dihydro-(1*H*)-pyrazole derivatives has been synthesized and assayed for their ability to inhibit the activity of the A and B isoforms of human monoamine oxidase (hMAO). Some of these compounds were endowed with a selective inhibitory activity against hMAO-B in the micromolar range. The most active of the series is the compound **13**, N1-thiocarbamoyl-3-(fur-2′-yl)-5-(4′-fluoro-phenyl)-4,5-dihydro-(1*H*)-pyrazole, with IC_{50} 2.75 ± 0.81 μM value and selectivity ratio of 25, which is the best candidate for further investigations.

1. Introduction

Monoamine oxidase (MAO; EC 1.4.3.4) is a flavoprotein located at the outer membrane of mitochondria in neuronal, glial, and other cells. It catalyzes oxidative deamination of monoamine and so is a target enzyme for antidepressant drugs. In addition, it is also responsible for the biotransformation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into 1-methyl-4-phenylpyridinium a Parkinsonian producing neurotoxin [1].

MAO exists in two forms, namely MAO-A and MAO-B. Specific substrates and inhibitors characterize both MAO subtypes. MAO-A oxidises norepinephrine and serotonin [5-hydroxytryptamine, 5-HT], whereas MAO-B preferentially deaminates 2-phenylethylamine (2-PEA) and benzylamine. These properties determine the clinical importance of MAO inhibitors. In fact, interest in selective MAO-B inhibitors has increased in the last years due to their therapeutic potential in aging related neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) and in selective MAO-A inhibitors due to their therapeutic potential in the treatment of neurological disorders such as depression [2–5]. Furthermore, interest in selective inhibitors of hMAO-B has renewed due to the discovery of an age-related increase in hMAO-B expression after the 60th year of life especially in glial cells [6,7].

The recent description of the crystal structure of the two isoforms of human MAO provides to elucidate the mechanism underlying and allows investigation of the selective interactions between these proteins and their ligands, to probe the catalytic mechanism, and to gain a complete understanding of the pharmacophoric requirements necessary for the rational design of new inhibitors [8,9].

Several N1-thiocarbamoyl-3,5-diaryl-4,5-dihydro-(1*H*)-pyrazoles have been synthesized in these last years and assayed as MAO inhibitors against monoamine oxidases isolated and purified from the mitochondrial extracts of rat liver homogenates and human platelets [10]. The results showed a good MAO inhibition and a better selectivity toward isoform A of the enzyme. Further some authors have also evaluated their antidepressant activity by the predictive "forced swimming test" and other neurological tests in mice [11–13]. In addition, other authors recently investigated some *N*-alkyl-3,5-di(hetero)aryl-1-thiocarbamoyl-pyrazolines for their monoamine oxidase inhibition [11,13–15], for their interaction with rat lung semicarbazide-sensitive amine oxidases (SSAOs) [16] and as dual agents (MAO-B inhibitors and anti-inflammatory analgesics) in the treatment of Alzheimer's disease [17].

On the basis of these studies, in the past we evaluated the importance of the substitution at N1 of the pyrazoline nucleus with

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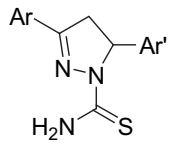


Fig. 1. General formula of derivatives **1**–**20**.

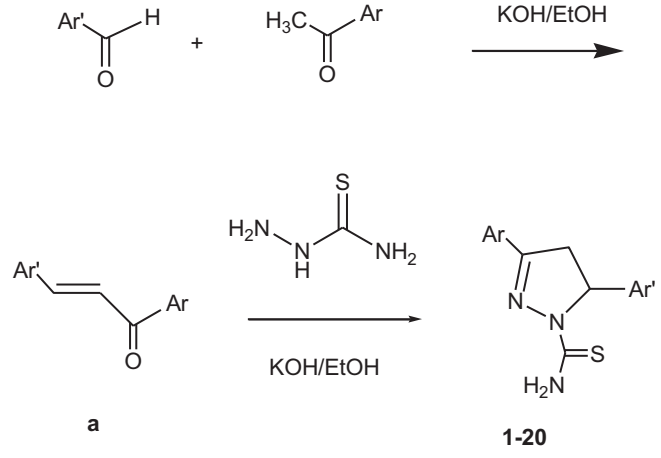
an acetyl or a propanoyl group [18,19], or with a thiocarbamoyl one [20]. This last substitution reduces the steric hindrance in the catalytic site and strengthens the interaction with the isoalloxazine nucleus of the cofactor flavin–adenine dinucleotide (FAD). So, new derivatives with (hetero)aromatic moieties in the 3 and 5 positions of the pharmacophore (Fig. 1) were designed and investigated for their influence in modulating hMAO inhibitory activity.

Further we wished to compare our results obtained by using hMAOs to those evaluated with rMAOs or established animal models. In fact in the literature, human brain, liver and blood platelets have been little used as hMAO sources to screen inhibitors, because of the easy accessibility of rat and bovine brain or liver for in vitro studies. But species-dependent differences (single amino acid substitutions may induce deep changes in the secondary and tertiary structures of these isoforms) have been reported by different authors for several classes of compounds [21,22]. Therefore the divergences found, after extrapolating data on MAO inhibition from different species sources to the human enzyme, could have a deep impact on the development of new and selective hMAO inhibitors. For these reasons we prepared and assayed a new series of N1-thiocarbamoyl-3,5-di(hetero)aryl-4,5-dihydro-(1*H*)-pyrazole derivatives, which all showed suitable physical properties (calculated LogP <5), to verify the effects of structural modifications on inhibition of both human isoforms.

2. Chemistry

The starting 1,3-di(hetero)aryl-2-propen-1-ones (chalcones) (**a**) have been synthesized by Claisen–Schmidt condensation at reflux between substituted aryl or heteroaryl ketones and appropriate substituted aryl or heteroaryl aldehydes according to methods fully optimized in our laboratory (KOH/EtOH) [20]. Then, as shown in Scheme 1, these intermediates were treated with thiosemicarbazide (molar ratio 1:2) in KOH/EtOH to afford the desired products without further purifications (**1**–**20**) in good yields (70–93%).

The structures of the compounds were confirmed by elemental analysis, mass spectrometry, IR and ^{1}H NMR spectroscopy (Tables 1 and 2). In particular, the two methylene protons (H_a and H_b) and methyne proton (H_c), in positions 4 and 5, respectively, of the dihydro-(1*H*)-pyrazole ring, give rise to a well defined system of three double doublets with different *J* values, indicating not only the formation of the pyrazoline, but also the exact position of the C=N double bond.



Scheme 1. General synthetic pathway of derivatives **1**–**20**.

Table 1
Chemical and physical data of derivatives **1**–**20**.

Comp	Ar	Ar′	mp (°C)	ClogP	*m/z*	Yield (%)
1	Ph	Ph	183–185	3.08	281.38	86
2	Ph	4′-CH_3–Ph	164–165	3.56	295.40	82
3	Ph	4′-Cl–Ph	183–189	3.64	315.82	72
4	Ph	fur-2′-yl	203–205	1.69	271.34	83
5	4′-CH_3–Ph	4′-F–Ph	261–265	3.72	313.39	75
6	4′-CH_3–Ph	thiophen-2′-yl	196–200	3.55	301.43	87
7	4′-F–Ph	thiophen-2′-yl	203–205	3.22	305.40	85
8	4′-Cl–Ph	4′-CH_3–Ph	147–149	4.12	329.85	84
9	4′-Cl–Ph	4′-F–Ph	264–267	3.79	333.81	71
10	4′-Cl–Ph	4′-Cl–Ph	238–240	4.19	350.27	80
11	4′-Cl–Ph	pyrrol-2′-yl	230–238	2.18	304.80	89
12	fur-2′-yl	4′-CH_3–Ph	144–156	2.18	285.37	81
13	fur-2′-yl	4′-F–Ph	190–192	1.85	289.33	89
14	fur-2′-yl	thiophen-2′-yl	218–220	1.67	277.37	93
15	thiophen-2′-yl	pyrrol-2′-yl	172–176	1.60	276.38	88
16	pyrrol-2′-yl	Ph	190–210	1.62	270.35	81
17	pyrrol-2′-yl	4′-F–Ph	209–213	1.78	288.34	83
18	pyrrol-2′-yl	4′-Cl–Ph	225–226	2.18	304.80	84
19	pyrrol-2′-yl	fur-2′-yl	123–130	0.24	260.32	70
20	pyrrol-2′-yl	thiophen-2′-yl	220–222	1.60	276.38	75

In MS spectra, the fragment peaks which correspond to loss of –SH, $–NH_2$, $–CSNH_2$ from the molecular ion are consistent with the postulated structure. Characteristic M + 2 isotope peaks are observed in the mass spectra of the compounds having a halogen or a sulfur. The IR spectra generally presented C=N stretching bands in the region around 1600 cm^{-1} because of the ring closure.

The syntheses of some compounds (**1**–**4**, **8**, **10**) have been described in previous references [11,13] and were performed with slight modifications; their analytical and spectral data were in full agreement with those reported in the literature.

3. Biochemical assay

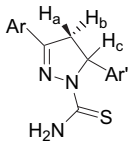
The potential effects of the test drugs on hMAO activity were investigated by measuring their effects on the production of hydrogen peroxide H_2O_2 from *p*-tyramine, using the Amplex® Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and human MAO isoforms in microsomes prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma–Aldrich Química S.A., Alcobendas, Spain). The inhibition of hMAO activity was evaluated using the above method following the general procedure described previously by us [23]. The test drugs (new compounds and reference inhibitors) themselves were unable to directly react with Amplex® Red reagent. In addition, these test drugs had no effects on the resorufin standard fluorescence curve which clearly indicate that these compounds do not react with resorufin and do not quench the fluorescence generated by this product.

The control activity of hMAO-A and hMAO-B (using *p*-tyramine as common substrate for both isoforms) was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min ($n = 20$).

4. Results and discussion

The hMAO-A and hMAO-B inhibition data are reported in Table 3 together with the selectivity index (SI = hMAO-B selectivity ratios

Table 2
^{1}H NMR of new derivatives.



Compound	^{1}H NMR (δ ppm)
5[a]	2.41 (s, 3H, $ArCH_3$), 3.18–3.20 (dd, $J_{ab} = 17.44$ Hz, $J_{ac} = 3.08$ Hz, 1H, H_a), 3.80–3.83 (dd, $J_{ab} = 17.45$ Hz, $J_{bc} = 11.13$ Hz, 1H, H_b), 6.00–6.02 (dd, $J_{ac} = 3.08$ Hz, $J_{bc} = 11.12$ Hz, 1H, H_c), 6.98–7.02 (m, 2H, Ar), 7.20–7.24 (m, 6H, Ar), 7.60 (s, 2H, NH_2, D_2O exch.).
6[b]	2.34 (s, 3H, $ArCH_3$), 3.06–3.08 (dd, $J_{ab} = 17.34$ Hz, $J_{ac} = 3.00$ Hz, 1H, H_a), 3.79–3.86 (dd, $J_{ab} = 17.36$ Hz, $J_{bc} = 11.04$ Hz, 1H, H_b), 6.19–6.22 (dd, $J_{ac} = 3.01$ Hz, $J_{bc} = 11.04$ Hz, 1H, H_c), 6.91–6.98 (m, 2H, thiophene), 7.26–7.28 (m, 1H, thiophene), 7.34–7.36 (m, 2H, Ar), 7.78–7.80 (m, 2H, Ar), 8.03 (s, 2H, NH_2, D_2O exch.).
7[a]	3.34–3.39 (dd, $J_{ab} = 17.48$ Hz, $J_{ac} = 3.08$ Hz, 1H, H_a), 3.76–3.83 (dd, $J_{ab} = 17.48$ Hz, $J_{bc} = 11.12$ Hz, 1H, H_b), 6.36–6.39 (dd, $J_{ac} = 3.09$ Hz, $J_{bc} = 11.12$ Hz, 1H, H_c), 6.94–6.98 (m, 2H, thiophene), 7.05–7.06 (m, 1H, thiophene), 7.12–7.20 (m, 4H, Ar), 7.75 (s, 2H, NH_2, D_2O exch.).
9[b]	3.18–3.20 (dd, $J_{ab} = 17.52$ Hz, $J_{ac} = 3.02$ Hz, 1H, H_a), 3.81–3.84 (dd, $J_{ab} = 17.52$ Hz, $J_{bc} = 11.16$ Hz, 1H, H_b), 6.02–6.04 (dd, $J_{ac} = 3.04$ Hz, $J_{bc} = 11.16$ Hz, 1H, H_c), 7.02–7.04 (m, 3H, Ar), 7.23–7.24 (m, 1H, Ar), 7.41–7.43 (m, 2H, Ar), 7.64–7.66 (m, 2H, Ar), 8.09 (s, 2H, NH_2, D_2O exch.).
11[b]	3.38–3.40 (dd, $J_{ab} = 17.48$ Hz, $J_{ac} = 3.13$ Hz, 1H, H_a), 3.69–3.74 (dd, $J_{ab} = 17.48$ Hz, $J_{bc} = 11.18$ Hz, 1H, H_b), 5.88–5.90 (dd, $J_{ac} = 3.13$ Hz, $J_{bc} = 11.19$ Hz, 1H, H_c), 6.54–6.57 (m, 1H, pyrrole), 7.53–7.55 (m, 2H, pyrrole), 7.86–7.94 (m, 4H, Ar), 7.99 (s, 2H, NH_2, D_2O exch.), 10.64 (bs, 1H, NH, D_2O exch.).
12[b]	2.24 (s, 3H, $ArCH_3$), 2.94–2.98 (dd, $J_{ab} = 17.44$ Hz, $J_{ac} = 3.10$ Hz, 1H, H_a), 3.82–3.86 (dd, $J_{ab} = 17.44$ Hz, $J_{bc} = 11.12$ Hz, 1H, H_b), 5.85–5.88 (dd, $J_{ac} = 3.09$ Hz, $J_{bc} = 11.12$ Hz, 1H, H_c), 6.65–6.67 (m, 1H, furan), 6.97–7.11 (m, 5H, Ar and furan), 7.87–7.89 (m, 1H, Ar), 7.90 (s, 2H, NH_2, D_2O exch.).
13[b]	3.00–3.02 (dd, $J_{ab} = 17.40$ Hz, $J_{ac} = 3.00$ Hz, 1H, H_a), 3.84–3.89 (dd, $J_{ab} = 17.40$ Hz, $J_{bc} = 11.19$ Hz, 1H, H_b), 5.89–5.93 (dd, $J_{ac} = 3.00$ Hz, $J_{bc} = 11.19$ Hz, 1H, H_c), 6.66–6.67 (m, 1H, furyl), 7.03–7.15 (m, 5H, Ar and furan), 7.91 (s, 1H, Ar), 8.01 (s, 2H, NH_2, D_2O exch.).
14[b]	3.28–3.30 (dd, $J_{ab} = 17.38$ Hz, $J_{ac} = 3.03$ Hz, 1H, H_a), 4.01–4.03 (dd, $J_{ab} = 17.38$ Hz, $J_{bc} = 11.29$ Hz, 1H, H_b), 5.49–5.52 (dd, $J_{ac} = 3.03$ Hz, $J_{bc} = 11.29$ Hz, 1H, H_c), 6.12–6.17 (m, 2H, furan), 6.53–7.55 (m, 1H, furan), 6.97–6.99 (m, 3H, thiophene), 7.70 (s, 2H, NH_2, D_2O exch.).
15[b]	3.39–3.43 (dd, $J_{ab} = 17.45$ Hz, $J_{ac} = 3.06$ Hz, 1H, H_a), 3.75–3.81 (dd, $J_{ab} = 17.44$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_b), 6.06–6.09 (dd, $J_{ac} = 3.06$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_c), 6.66 (s, 1H, thiophene), 7.16–7.19 (m, 2H, thiophene), 7.58–7.59 (m, 2H, pyrrole), 7.76–7.78 (m, 1H, pyrrole), 8.00 (bs, 2H, NH_2, D_2O exch.), 10.74 (bs, 1H, NH, D_2O exch.).
16[b]	3.06–3.11 (dd, $J_{ab} = 17.42$ Hz, $J_{ac} = 3.10$ Hz, 1H, H_a), 4.38–4.39 (dd, $J_{ab} = 17.40$ Hz, $J_{bc} = 11.14$ Hz, 1H, H_b), 6.11–6.13 (dd, $J_{ac} = 3.10$ Hz, $J_{bc} = 11.15$ Hz, 1H, H_c), 6.89–7.02 (m, 2H, pyrrole), 7.19–7.27 (m, 4H, Ar and pyrrole), 7.39–7.43 (m, 2H, Ar), 7.75 (bs, 2H, NH_2, D_2O exch.), 11.85 (bs, 1H, NH, D_2O exch.).
17[b]	3.08–3.11 (dd, $J_{ab} = 17.45$ Hz, $J_{ac} = 3.02$ Hz, 1H, H_a), 4.25–4.29 (dd, $J_{ab} = 17.45$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_b), 6.09–6.12 (dd, $J_{ac} = 3.02$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_c), 6.92–7.03 (m, 4H, pyrrole and Ar), 7.40–7.50 (m, 3H, Ar), 7.70 (s, 2H, NH_2, D_2O exch.), 11.69 (bs, 1H, NH, D_2O exch.).
18[b]	3.18–3.20 (dd, $J_{ab} = 17.48$ Hz, $J_{ac} = 3.11$ Hz, 1H, H_a), 4.20–4.24 (dd, $J_{ab} = 17.48$ Hz, $J_{bc} = 11.13$ Hz, 1H, H_b), 6.07–6.10 (dd, $J_{ac} = 3.12$ Hz, $J_{bc} = 11.14$ Hz, 1H, H_c), 6.94–7.00 (m, 2H, pyrrole), 7.30–7.32 (m, 2H, Ar and pyrrole), 7.40–7.44 (m, 3H, Ar), 7.79 (s, 2H, NH_2, D_2O exch.), 11.70 (bs, 1H, NH, D_2O exch.).
19[b]	3.08–3.11 (dd, $J_{ab} = 17.45$ Hz, $J_{ac} = 3.02$ Hz, 1H, H_a), 4.25–4.29 (dd, $J_{ab} = 17.45$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_b), 6.09–6.12 (dd, $J_{ac} = 3.02$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_c), 6.92–7.03 (m, 4H, pyrrole and Ar), 7.40–7.50 (m, 3H, Ar), 7.70 (s, 2H, NH_2, D_2O exch.), 11.69 (bs, 1H, NH, D_2O exch.).
20[b]	3.08–3.11 (dd, $J_{ab} = 17.45$ Hz, $J_{ac} = 3.02$ Hz, 1H, H_a), 4.25–4.29 (dd, $J_{ab} = 17.45$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_b), 6.09–6.12 (dd, $J_{ac} = 3.02$ Hz, $J_{bc} = 11.10$ Hz, 1H, H_c), 6.92–7.03 (m, 4H, pyrrole and Ar), 7.40–7.50 (m, 3H, Ar), 7.70 (s, 2H, NH_2, D_2O exch.), 11.69 (bs, 1H, NH, D_2O exch.).

[a] $CDCl_3$.
[b] DMSO-d_6.

Table 3
IC_{50} values and hMAO-B selectivity ratios ($[IC_{50(hMAO-A)}]/[IC_{50(hMAO-B)}]$) for the inhibitory effects of tested drugs (new compounds and reference inhibitors) on the enzymatic activity of human recombinant MAO isoforms expressed in baculovirus infected BTI insect cells.

Compound	IC_{50} μM hMAO-A	IC_{50} μM hMAO-B	Ratio
1	***	48.31 ± 2.86	>2.1[c]
2	***	32.81 ± 1.43	>3[c]
3	***	33.29 ± 1.25	>3[c]
4	54.65 ± 3.12	32.48 ± 1.64	1.7
5	***	7.18 ± 0.55	>14[c]
6	***	18.26 ± 0.98	>5.5[c]
7	***	23.21 ± 1.07	>4.3[c]
8	***	12.84 ± 0.61	>7.8[c]
9	***	16.33 ± 2.22	>6.1[c]
10	68.87 ± 3.61[b]	39.37 ± 1.65	1.7
11	50.14 ± 4.96[b]	29.38 ± 2.87	1.7
12	65.84 ± 3.14	54.51 ± 4.56	1.2
13	69.45 ± 3.25[a]	2.75 ± 0.11	25
14	**	**	nd
15	69.97 ± 3.78[b]	33.26 ± 1.99	2.1
16	**	**	nd
17	**	**	nd
18	***	**	nd
19	***	69.38 ± 4.23	>1.4[c]
20	**	**	nd
Clorgyline	4.46 ± 0.32 nM[a]	61.35 ± 1.13 μM	0.000073
R-(−)-deprenyl	67.25 ± 1.02 μM[a]	19.60 ± 0.86 nM	3431
Iproniazide	6.56 ± 0.76 μM	7.54 ± 0.36 μM	0.87
Moclobemide	361.38 ± 19.3 μM	*	<0.36[d]
Isatin	***	18.75 ± 1.24 μM	>5.3[c]

Each IC_{50} value is the mean ± S.E.M. from five experiments ($n = 5$).
*Inactive at 1 mM (highest concentration tested).
**Inactive at 100 μM (highest concentration tested). At higher concentrations the compounds precipitate.
***100 μM inhibits enzymatic activity around (by approximately) 40–45%. At higher concentrations the compounds precipitate.
[a] Level of statistical significance: $P < 0.01$ versus the corresponding IC_{50} values obtained against hMAO-B, as determined by ANOVA/Dunnett's.
[b] Level of statistical significance: $P < 0.05$ versus the corresponding IC_{50} values obtained against hMAO-B, as determined by ANOVA/Dunnett's.
[c] Values obtained under the assumption that the corresponding IC_{50} against hMAO-A or hMAO-B is the highest concentration tested (100 μM).
[d] Value obtained under the assumption that the corresponding IC_{50} against hMAO-B is the highest concentration tested (1 mM).

$[IC_{50(hMAO-A)}]/[IC_{50(hMAO-B)}]$). Enzymatic assays revealed that all tested compounds were weak to moderate hMAO inhibitors at low micromolar concentrations (Table 3).

We can observe that the presence of a fluorine atom in the 4′-position of the 5-phenyl substituent on the pyrazoline ring is important for the activity. In fact, more potent active and B-selective compounds are derivatives **5**, **9**, and **13** with IC_{50} values ranging between 2.75 ± 0.81 μM and 16.33 ± 2.22 μM and B-selectivity ranging between >6.1 and 25. The most active of the series is compound **13** (IC_{50} 2.75 ± 0.81 μM and selectivity ratio 25) with a 4′-fluorophenyl substituent in 5 position and a fur-2′-yl group in 3-position of the pyrazoline ring. The concurrent presence of heteroaromatic substituent in 3- and 5-position of the pyrazoline ring in compounds **15** and **19** leads to a decrease in the potency and selectivity for the hMAO-B activity, while the presence of a 4′-methylphenyl and a 4′-fluorophenyl, in the same positions of the ring, preserves the activity and a discrete B-selectivity (compound **5**).

Furthermore we can observe that for compounds **8**, **9**, **10**, and **11**, which bear a 4′-chlorophenyl substituent in 3 position, the hMAO-B inhibitory activity increase with the presence of small substituent in 5 position of the pyrazoline nucleus (compounds **8** and **9**).

On the basis of these considerations we can indicate compound **13**, N1-thiocarbamoyl-3-(fur-2′-yl)-5-(4′-fluoro-phenyl)-4,5-dihydro-(1*H*)-pyrazole as the best candidate for further investigations.

Furthermore comparing some N1-acetyl or N1-propanoyl derivatives, synthesized in our previous papers [18,19], with the new N1-thiocarbamoyl derivatives bearing the same substituent (H, H and Cl, Cl and F, Cl and CH_3, CH_3 and F, Cl and Cl) on the 3,5-diaryl moiety, it is possible to highlight that this more polarizable group (the N1-thiocarbamoyl) led to a slight decrease in MAO inhibitory activity and to a better selectivity toward hMAO-B isoform.

In the reversibility and irreversibility tests, hMAO-B inhibition was irreversible in presence of the compounds more active (**5** and **13**) as shown by the lack enzyme activity restoration after repeated washing. Similar results were obtained for *R*-(−)-deprenyl (Table 4). However, significant recovery of hMAO-B activity was observed after repeated washing of isatin, indicating that this drug is a reversible inhibitor of this hMAO isoform.

Although we have not investigated in this study the mechanism by which the compounds **5** and **13** act as irreversible inhibitors of hMAO-B activity, it is possible that these compounds, like most MAO irreversible inhibitors, establish a covalent interaction with the active center of the enzyme [24]. Other possibility is that the compounds **5** and **13** are, like some MAO irreversible inhibitors (e.g.: *R*-(−)-deprenyl), suicide inhibitors, i.e., act as a substrate for the target enzyme, which finally generates a new compound that irreversibly inhibits MAO activity [25].

5. Experimental protocols

5.1. Chemistry

The chemicals, solvents for synthesis and spectral grade solvents were purchased from Aldrich (Italy) without further purification. Melting points (uncorrected) were determined automatically on an FP62 apparatus (Mettler-Toledo). ^{1}H NMR spectra were recorded at 400 MHz on a Bruker spectrometer using DMSO-d_6 or $CDCl_3$ as the solvent. Chemical shifts are expressed as δ units (parts per millions) relative to the solvent peak. Coupling constants *J* are valued in Hertz (Hz). IR spectra were registered on a Perkin Elmer FT-IR Spectrometer Spectrum 1000 in KBr. Elemental analysis for C, H, and N were recorded on a Perkin–Elmer 240 B microanalyzer and the analytical results were within ±0.4% of the theoretical values for all compounds. All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F_{254} Merck). Electron ionization (EI) mass spectra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature of 200 °C). The samples were introduced directly into the ion source. Lipophilicity parameter, CLogP, was calculated for each molecule by using ChemDraw ultra 8.0.

5.2. General procedure for the synthesis of derivatives *1–20*

The appropriate chalcone (**a**) (50 mmol) was dissolved in 100 mL of a mixture of KOH in ethanol and vigorously stirred at reflux with thiosemicarbazide (100 mmol) for 24 h. The desired product precipitated from reaction mixture after cooling, was filtered off under vacuum and crystallized from suitable solvent (ethanol or 2-propanol) and dried.

Table 4
Reversibility and irreversibility of hMAO-B inhibition.

Compound	% hMAO-B inhibition	
	Before washing	After repeated washing
R-(−)-deprenyl (20 nM)	51.45 ± 2.69	52.05 ± 2.88
Isatin (50 μM)	84.36 ± 5.24	16.62 ± 1.18[a]
5 (10 μM)	54.46 ± 3.16	57.58 ± 3.49
13 (3 μM)	53.34 ± 3.42	47.83 ± 3.14

Each value is the mean ± S.E.M. from five experiments ($n = 5$).
[a] Level of statistical significance: $P < 0.01$ versus the corresponding % hMAO-B inhibition before washing, as determined by ANOVA/Dunnett's.

5.3. Determination of MAO isoform activity

The effects of the test compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us [23].

Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity, i.e., to oxidize (in the control group) the same concentration of substrate: 165 pmol of *p*-tyramine/min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein) were incubated for 15 min at 37 °C in a flat-black-bottom 96-well microtest™ plate (BD Biosciences, Franklin Lakes, NJ, USA) placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 μM Amplex® Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800™, Bio-Tek® Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with Amplex® Red reagent) was determined by adding these drugs to solutions containing only the Amplex® Red reagent in a sodium phosphate buffer.

The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution.

5.4. Reversibility and irreversibility experiments

To evaluate whether some of the tested compounds (**5** and **13**) are reversible or irreversible hMAO-B inhibitors, an effective centrifugation–ultrafiltration method (so-called repeated washing) was used [23].

Briefly, adequate amounts of the recombinant hMAO-B were incubated together with a single concentration (see Table 4) of the test drugs or the reference inhibitor *R*-(−)-deprenyl in a sodium phosphate buffer (0.05 M, pH 7.4) for 15 min at 37 °C.

After this incubation period, an aliquot was stored at 4 °C and used for subsequent measurement of hMAO-B activity (see the subsection determination of MAO activity). The remaining incubated sample (300 μl) was placed in an Ultrafree-0.5 centrifugal tube (Millipore, Billerica, USA) with a 30KDa Biomax membrane in the middle of the tube and centrifuged (9000*g*, 20 min, 4 °C) in a centrifuge (J2-MI, Beckman Instruments, Inc., Palo Alto, California, USA). The enzyme retained in the 30 KDa membrane was resuspended in sodium phosphate buffer at 4 °C and centrifuged again two successive times. After the third centrifugation, the enzyme

retained in the membrane was resuspended in sodium phosphate buffer (300 μl) and an aliquot of this suspension was used for subsequent hMAO-B activity determination.

Similar studies were carried out on MAO-A activity in presence of the reference inhibitors isatin and moclobemide under the experimental conditions described above.

Control experiments were performed simultaneously (to define 100% hMAO-B activity) by replacing the test drugs with appropriate dilutions of the vehicles. The corresponding values of percent (%) hMAO-B inhibition were separately calculated for samples with and without repeated washing.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.11.003.

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