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Development of selective and reversible pyrazoline based MAO-A inhibitors: Synthesis, biological evaluation and docking studies

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ABSTRACT

3,5-Diaryl pyrazolines analogs were synthesized and evaluated for their monoamine oxidase (MAO) inhibitory activity. The compounds were found reversible and selective towards MAO-A with selectivity index in the magnitude of 10^3–10^5. The docking studies were carried out to gain further structural insights of the binding mode and possible interactions with the active site of MAO-A. Interestingly, the theoretical (K_i) values obtained by molecular docking studies were in congruence with their experimental (K_i) values.

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1. Introduction

Monoamine oxidase inhibitors (MAOIs) have shown therapeutic value in variety of diseases.[1] Earlier, MAO inhibitors introduced into clinical practice were abandoned due to adverse side-effects, such as hepatotoxicity, orthostatic hypertension and the so called 'cheese effect' characterized by hypertensive crises.[2] These side-effects were hypothesized to be related to nonselective and irreversible monoamine oxidase inhibition. The new generation inhibitors are warranted to selectively inhibit either of one of the isoforms (MAO-A and MAO-B) to increase the therapeutic efficacy. These two forms of MAO are characterized by their different sensitivities to inhibitors and their different specificities to substrates.[3] MAO-A preferably metabolizes serotonin, adrenaline, and noradrenaline,[4] whereas β-phenylethylamine and benzylamine are predominantly metabolized by MAO-B.[5] Tyramine, dopamine, and some other important amines are common substrates for both the isoenzymes.[6] Nowadays, the therapeutic interest of MAOIs falls into two major categories. MAO-A inhibitors have been used mostly in the treatment of mental disorders, in particular depression and anxiety,[7–9] while MAO-B inhibitors could be used in the treatment of Parkinson's disease and Alzheimer's disease.[10,11] Efforts have been oriented towards the discovery of reversible and selective inhibitors of MAO-A/MAO-B leading to a new generation of compounds.

The classical period of the MAO inhibitors started with hydrazine derivatives. Their prototype, Iproniazid, was originally proposed as a tuberculostatic agent eventually became the first modern antidepressant. 2-Pyrazolines can be considered as a cyclic hydrazine moiety. For this reason, many authors investigated MAO inhibitory and antidepressant activities of 1,3,5-triphenyl-2-pyrazolines.[12–33]

Recently, we have reported[31–33] few *N*,3,5-triaryl-4,5-dihydro-1*H*-pyrazole-1-carbothioamide as MAO inhibitors (Fig. 1). Their SAR analysis revealed some important structural insights like: (a) 4-hydroxy substitution in ring A increases the potency towards MAO-A[31] as well as reduces the cytotoxicity,[33] (b) approximately 10–15 fold increase in potency was observed with five membered hetero aromatic groups as ring B in comparison with phenyl group[31] (c) selectivity towards MAO-A was observed with any substitution in ring C. In the present study molecules with 4-hydroxy substitution in ring A, thiophen-2-yl/furan-2-yl group as ring B and various substitutions in ring C phenyl group were explored for potent MAO-A inhibitory activity.

The structural comparison of human MAO-A and MAO-B[37,38] reveals high sequence similarity of 72% and the overall structure of human MAO-A is quite similar to that of MAO-B (rms deviation of 1.2 Å, 488 equiv C-α atom). The significant conformational difference observed in the cavity-shaping loop of MAO-A from

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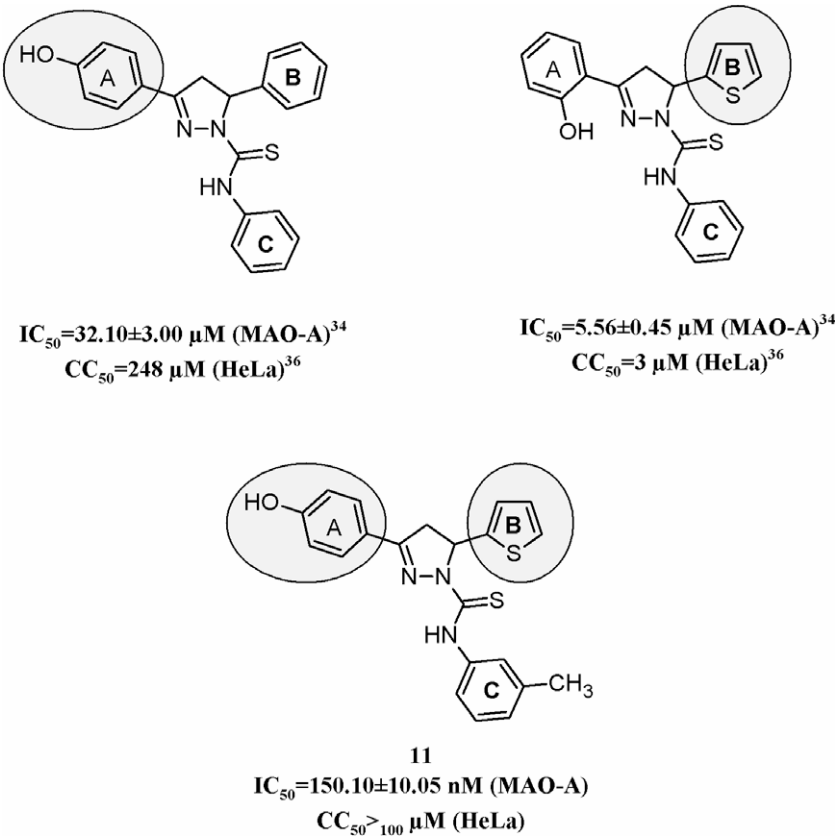


Figure 1. Structures of some potent *N*,3,5-triaryl-4,5-dihhydro-1*H*-pyrazole-1-carbothioamide from earlier work are shown. This forms a basis for synthesizing the current molecules. The most potent analog **11** identified in the current work is also shown.

residues 210–216, which results in Cα movements up to 6 Å in comparison to MAO-B (residues 201–207). This makes the aromatic cage of MAO-A wider than that of MAO-B accommodating bulkier aromatic groups. The previous docking studies[31,32] also revealed the importance of ring C substitution (Fig. 1) which was found to be positioned in the aromatic cage (FAD, TYR407 and TYR444, Pocket1). These observations formed a strong basis for designing selective and potent MAO-A inhibitor.

2. Results and discussion

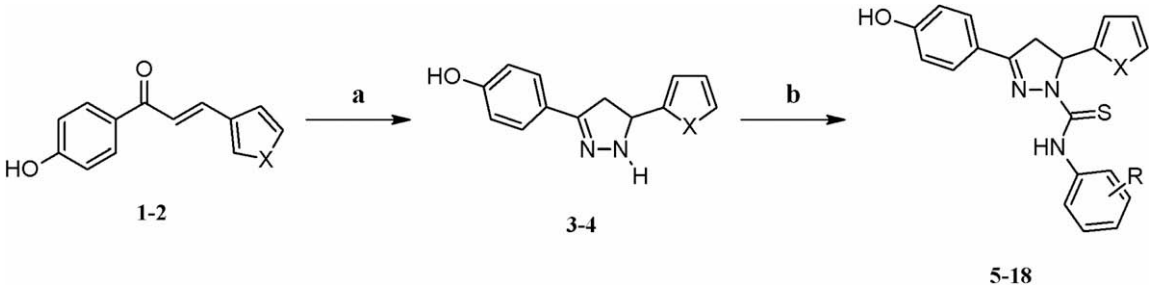
In continuation to our previous work fourteen novel pyrazolines (*N*,3,5-triaryl-4,5-dihydro-1*H*-pyrazole-1-carbothioamide) were synthesized and evaluated for their MAO inhibitory property. The synthesized compounds were found to be competitive, reversible and selective inhibitor of MAO-A. In addition, the molecular docking studies were carried out to reveal the binding mode of synthesized molecules with MAO-A and MAO-B.

2.1. Chemistry

The compounds were synthesized as shown in Scheme 1 according to previously reported method.[31–33] 4-Hydroxy chalcones **1–2** were prepared through Claisen–Schmidt condensation of 4-hydroxy acetophenone with either furfuraldehyde or thiophene-2-carboxaldehyde. Compounds **3–4** were synthesized by the reaction of excess hydrazine hydrate (80%) with **1–2**, respectively in ethanol. Compounds **5–18** were afforded by the reaction of **3–4** with respective substituted phenyl isothiocyanates in dry methanol under reflux condition. The structure, physicochemical, and spectral data of these analogs were given in Table1.

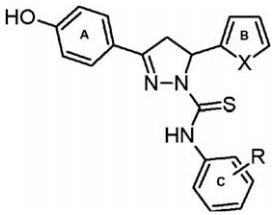
2.2. Biochemistry

The synthesized compounds were subjected to MAO inhibitory assay. MAO was purified from the rat liver and total MAO activity was measured spectrophotometrically according to the method of Holt.[36] Assay mixture contained a chromogenic solution consisted



Scheme 1. Reagents and condition: (a) 80% $NH_2NH_2{\cdot}H_2O$, ethanol, reflux 3–6 h; (b) R-C_6H_4-NCS, dry methanol, 15–30 min.

Table 1
Physicochemical and spectral characteristics of *N*,3,5-triaryl-4,5-dihydro-1*H*-pyrazole-1-carbothioamides



Compound	R	X	MF	MW	Y (%)	MP (°C)	^{1}H NMR (δ ppm)	FAB-MS (*m*/*z*)
5	–H	S	$C_{20}H_{17}N_3OS_2$	379	66	185–187	3.37 (H_A, appeared as doublet, J_{AM} = 16.5 Hz), 3.87 (H_M, dd, J_{MA} = 17.0 Hz, J_{MX} = 11.1 Hz), 6.32 (H_X, appeared as doublet, J_{MX} = 9.9 Hz), 6.85–7.87 (12H, m, Ar-H), 9.99 (1H, s, Ar-OH), 10.1 (1H, s, Ar-NH–)	379 (M^+, bp) 380 (M+1) 381 (M+2)
6	4-OCH_3	S	$C_{21}H_{19}N_3O_2S_2$	409	47	178–180	3.35 (H_A, appeared as doublet, J_{AM} = 8.9 Hz), 3.84 (3H, s, Ar-OCH_3) 3.89 (H_M, dd, J_{MA} = 17.9 Hz, J_{MX} = 11.1 Hz), 6.31 (H_X, appeared as doublet, J_{MX} = 9.53 Hz), 6.83–7.83 (11H, m, Ar-H), 9.90 (1H, s, Ar-OH), 10.01 (1H, s, Ar-NH-)	409 (M^+) 410 (M+1, bp) 411 (M+2)
7	4-CH_3	S	$C_{21}H_{19}N_3OS_2$	393	85	170–172	3.36 (H_A, appeared as doublet, J_{AM} = 15.3 Hz), 2.29 (3H, s, Ar-CH_3) 3.86 (H_M, dd, J_{MA} = 17.4 Hz, J_{MX} = 11.4 Hz), 6.31 (H_X, appeared as doublet, J_{MX} = 9.9 Hz), 6.84–7.86 (11H, m, Ar-H), 9.91 (1H, s, Ar-OH), 10.09 (1H, s, Ar-NH–)	393 (M^+) 394 (M+1, bp) 395 (M+2)
8	2-OCH_3	S	$C_{21}H_{19}N_3O_2S_2$	409	45	138–139	3.53 (H_A, appeared as doublet, J_{AM} = 15.7 Hz), 3.88 (3H, s, Ar-OCH_3) 3.92 (H_M, appeared as doublet, J_{MA} = 23.4 Hz), 6.31 (H_X, appeared as doublet, J_{MX} = 10.5 Hz), 6.63–8.23 (11H, m, Ar-H), 9.76 (1H, s, Ar-OH), 10.15 (1H, s, Ar-NH–)	409 (M^+) 410 (M+1, bp) 411 (M+2)
9	2-CH_3	S	$C_{21}H_{19}N_3OS_2$	393	49	164–165	3.34 (H_A, appeared as triplet), 2.22 (3H, s, Ar-CH_3) 3.87 (H_M, dd, J_{MA} = 17.55 Hz, J_{MX} = 11.1 Hz), 6.27 (H_X, appeared as doublet, J_{MX} = 9.6 Hz), 6.83–7.84 (11H, m, Ar-H), 9.81 (1H, s, Ar-OH), 10.07 (1H, s, Ar-NH–)	393 (M^+) 394 (M+1, bp) 395 (M+2)
10	3-OCH_3	S	$C_{21}H_{19}N_3O_2S_2$	409	41	139–140	3.38 (H_A, appeared as triplet), 3.74 (3H, s, Ar-OCH_3), 3.87 (H_M, dd, J_{MA} = 16.95 Hz, J_{MX} = 11.4 Hz), 6.32 (H_X, appeared as doublet, J_{MX} = 9.6 Hz), 6.72–7.87 (11H, m, Ar-H), 9.94 (1H, s, Ar-OH), 10.10 (1H, s, Ar-NH–)	409 (M^+) 410 (M+1, bp) 411 (M+2)
11	3-CH_3	S	$C_{21}H_{19}N_3OS_2$	393	47	162–164	3.33 (H_A, merged with solvent peak), 2.29 (3H, s, Ar-CH_3), 3.78 (H_M, dd, J_{MA} = 17.7 Hz, J_{MX} = 11.4 Hz), 6.08 (H_X, appeared as doublet, J_{MX} = 10.2 Hz), 6.33–7.86 (11H, m, Ar-H), 9.90 (1H, s, Ar-OH), 10.08 (1H, s, Ar-OH)	393 (M^+) 394 (M+1, bp) 395 (M+2)
12	-H	O	$C_{20}H_{17}N_3O_2S$	363	93	197–199	3.39 (H_A, dd, J_{AM} = 7.2 Hz, J_{AX} = 3.3 Hz), 3.79 (H_M, dd, J_{MA} = 17.85 Hz, J_{MX} = 11.9 Hz), 6.09 (H_X, dd, J_{MX} = 11.1 Hz, J_{AX} = 2.7 Hz), 6.33–7.86 (12H, m, Ar-H), 9.97 (1H, s, Ar-OH), 10.08 (1H, s, Ar-NH–)	363 (M^+) 364 (M+1, bp) 365 (M+2)
13	4-OCH_3	O	$C_{21}H_{19}N_3O_3S$	393	43	158–160	3.36 (H_A, appeared as doublet, J_{AM} = 8.85 Hz), 3.82 (3H, s, Ar-OCH_3) 3.87 (H_M, dd, J_{MA} = 17.85 Hz, J_{MX} = 10.8 Hz), 6.31 (H_X, appeared as doublet, J_{MX} = 4.35 Hz), 6.84–7.87 (11H, m, Ar-H), 9.89 (1H, s, Ar-OH), 9.92 (1H, s, Ar-NH–)	393 (M^+) 394 (M+1, bp) 395 (M+2)
14	4-CH_3	O	$C_{21}H_{19}N_3O_2S$	377	52	149–151	3.34 (H_A, dd, J_{AM} = 18.9 Hz, J_{AX} = 3.0 Hz), 2.29 (3H, s, Ar-CH_3) 3.77 (H_M, dd, J_{MA} = 17.7 Hz, J_{MX} = 11.4 Hz), 6.07 (H_X, dd, J_{MX} = 11.4 Hz, J_{AX} = 2.7 Hz), 6.32–7.85 (11H, m, Ar-H), 9.89 (1H, s, Ar-OH), 10.06 (1H, s, Ar-NH–)	377 (M^+) 378 (M+1, bp) 379 (M+2)
15	2-OCH_3	O	$C_{21}H_{19}N_3O_3S$	393	55	121–124	3.37 (H_A, dd, J_{AM} = 13.65 Hz, J_{AX} = 2.4 Hz), 3.87 (3H, s, Ar-OCH_3) 3.82 (H_M, dd, J_{MA} = 19.8 Hz, J_{MX} = 8.7 Hz), 6.08 (H_X, dd, J_{MX} = 11.1 Hz, J_{AX} = 2.4 Hz), 6.35–8.24 (11H, m, Ar-H), 9.75 (1H, s, Ar-OH), 10.15 (1H, s, Ar-NH–)	393 (M^+) 394 (M+1, bp) 395 (M+2)
16	2-CH_3	O	$C_{21}H_{19}N_3O_2S$	377	86	123–125	3.34 (H_A, dd, J_{AM} = 17.7 Hz, J_{AX} = 2.7 Hz), 2.21 (3H, s, Ar-CH_3) 3.79 (H_M, dd, J_{MA} = 17.7 Hz, J_{MX} = 11.7 Hz), 6.05 (H_X, dd, J_{MX} = 11.1 Hz, J_{AX} = 2.4 Hz), 6.30–7.83 (11H, m, Ar-H), 9.79 (1H, s, Ar-OH), 10.07 (1H, s, Ar-NH–)	377 (M^+) 378 (M+1, bp) 379 (M+2)
17	3-OCH_3	O	$C_{21}H_{19}N_3O_3S$	393	78	73–75	3.38 (H_A, dd, J_{AM} = 17.8 Hz, J_{AX} = 2.7 Hz), 3.68 (3H, s, Ar-OCH_3), 3.73 (H_M, dd, J_{MA} = 17.5 Hz, J_{MX} = 11.1 Hz), 6.32 (H_X, dd, J_{MX} = 11.4 Hz, J_{AX} = 2.7 Hz), 6.72–7.87 (11H, m, Ar-H), 9.93 (1H, s, Ar-OH), 10.07 (1H, s, Ar-NH–)	393 (M^+) 394 (M+1, bp) 395 (M+2)
18	3-CH_3	O	$C_{21}H_{19}N_3O_2S$	377	47	168–169	3.34 (H_A, dd, J_{AM} = 18.3 Hz, J_{AX} = 3.3 Hz), 2.29 (3H, s, Ar-CH_3), 3.78 (H_M, dd, J_{MA} = 17.85 Hz, J_{MX} = 11.4 Hz), 6.08 (H_X, dd, J_{MX} = 11.1 Hz, J_{AX} = 3.0 Hz), 6.33–7.86 (11H, m, Ar-H), 9.90 (1H, s, Ar-OH), 10.09 (1H, s, Ar-OH)	377 (M^+) 378 (M+1, bp) 379 (M+2)

of vanillinic acid, 4-aminoantipyrin and peroxidase type II in potassium phosphate buffer (pH 7.6). Assay mixture was pre-incubated with substrate *p*-tyramine before addition of enzyme. The reaction was initiated by the addition of homogenate and increase in absorbance was monitored at 498 nm at 37 °C for 60 min. Results were expressed as nmol h^{-1} mg^{-1}. Newly synthesized compounds were dissolved in DMSO and used in the concentration range of 1–1000 μM. Inhibitors were then incubated with purified MAO at 37 °C for 0–60 min prior to adding to the assay mixture. Reversibility of the inhibition of MAO by these compounds was assessed by dilution.

All the synthesized compounds were found to be selective and reversible inhibitor of MAO-A with outstanding selectivity index in the magnitude of 10^3–10^5. Compounds **5**–**11** with thiophen-2-yl group as ring B were superior in potency in comparison to those with furan-2-yl group as ring B, compounds **12**–**18**. Amongst compounds **5**–**11** methyl substitution (**7**, **9**, and **11**) in ring C was found better in terms of potency than methoxy (**6**, **8**, and **10**). The order of activity for methyl substituted compounds (**7**, **9**, and **11**) is 3-CH_3 (**11**) > 2-CH_3 (**9**) > 4-CH_3 (**7**). Similarly, for methoxy substitution in ring C (**6**, **8**, and **10**) the order of activity is 3-CH_3 (**6**) > 2-CH_3 (**8**) > 4-CH_3 (**10**). Compound (**5**) where ring C is unsubstituted was found least active.

2.3. Molecular docking

The structural and binding mode studies of the compounds with MAO-A and MAO-B were carried out using molecular docking by AutoDock 4.0. The best scoring molecules from the largest cluster were considered for further structural and interaction studies. The calculated inhibition constants (K_i) as well as their experimental values for each enzyme–inhibitor complex are shown in Table 2. All the analogs share a common binding mode and occupy similar position in the active site. Two compounds **11** (ring B = thiophenyl) and **17** (ring B = furyl) in complex with MAO-A, was shown in Figure 2a. Ring A (*p*-hydroxy phenyl) is accommodated well in pocket3 which is formed due to cavity-shaping loop, characterized by GLY74, ARG206, ILE207, PHE208, GLU216, TRP441. Hydrogen bonding was observed between the hydroxyl hydrogen and the peptidyl oxygen of ILE207. Ring B (five membered heteroaromatic ring) is well accommodated in pocket2 (ILE180, ILE335, LEU337, MET350, PHE352) and ring C (substituted phenyl) were positioned in the aromatic cage (FAD, TYR407, and TYR444, Pocket1. As discussed earlier amongst compounds **5–11** methyl substitution (**7**, **9**, and **11**) in ring C was found better in terms of potency than methoxy (**6**, **8**, and **10**) substitution. Also 3-methyl derivative (**11**) was found superior than 2-methyl or 4-methyl derivative (**7**, **9**). The superior potency of the 3-methyl derivative (**11**) can be attributed to the π–π stacking that was observed with TYR444 and ring C (Figure 2b). Whereas, for compounds **7** and **9** (4-methyl and 2-methyl substitution, respectively) the particular interaction (π–π stacking) is not observed (Figure 2c). 3-Methyl substitution in ring C orients the phenyl ring in the aromatic cage in such a way, so as to make the π–π stacking possible. Low selectivity of these compounds towards MAO-B can be attributed to the following: In case of MAO-B pockets 2 and 3 were reduced and merged to narrow cavity primarily due to different orientation of the cavity forming loop.[34,35] Therefore, compounds **5–18** with additional ring C experience a significant steric hindrance. Interestingly, the results obtained computationally are in good agreement with their experimental values. The calculated and experimental pK_i were plotted and presented in Figure 3. The outstanding co-relation between the predicted and the experimental values ensures that the docking and scoring function adopted here, appropriately depicts the molecular recognition process of these compounds with either isoform of MAO.

From the above study we have successfully identified few compounds which are reversible and selective inhibitor of MAO-A. A concise idea about their structure–activity relationship was also drawn and importance of different substitutions especially at ring B and ring C was understood. Presence of five membered heterocyclic ring B and substitution at 1N position of the pyrazoline ring shifts the selectivity spectrum towards MAO-A. Therefore the present finding reveals some significant novel molecules along with structural insights using molecular docking calculation. Further it initiates detail investigation to optimize the molecules and to discover a significant drug candidate as reversible MAO inhibitors.

3. Experimental

3.1. Chemistry

Melting points were determined using Thermonik Melting Point Apparatus (Campbell electronics, India) by capillary method and

Table 2
Calculated and experimental K_i values corresponding to the inhibition of MAO isoforms by the newly synthesized pyrazoline derivatives

Code	Experimental[a] (K_i)		Calculated[b] (K_i)		Inhibition type	Reversibility	MAO inhibitory Selectivity	HeLa cell culture CC_{50} (μM)
	MAO-A (nM)	MAO-B (nM)	MAO-A (nM)	MAO-B (nM)				
5	480.10 ± 33.00	47,000 ± 300	395.12	38,300	Competitive	Reversible	Selective for MAO-A	>100
6	500.55 ± 36.70	71,51,000 ± 40,260	326.52	67,65,900	Competitive	Reversible	Selective for MAO-A	>100
7	350.55 ± 22.00	71,540 ± 4300	260.36	55,570	Competitive	Reversible	Selective for MAO-A	>100
8	300.55 ± 21.00	44,000 ± 2990	277.24	41,190	Competitive	Reversible	Selective for MAO-A	>100
9	280.60 ± 12.22	70,150 ± 4560	150.85	73,060	Competitive	Reversible	Selective for MAO-A	>100
10	180.55 ± 12.09	11,00,000 ± 770,000	124.14	718,200	Competitive	Reversible	Selective for MAO-A	>100
11	150.10 ± 10.05	22,10,000 ± 110,000	155.78	21,60,000	Competitive	Reversible	Selective for MAO-A	>100
12	700.76 ± 38.00	778,000 ± 50,000	414.15	604,000	Competitive	Reversible	Selective for MAO-A	>100
13	950.23 ± 60.07	10,60,000 ± 100,000	402.47	10,64,345	Competitive	Reversible	Selective for MAO-A	>100
14	480.22 ± 29.00	50,10,000 ± 300,000	305.08	30,60,000	Competitive	Reversible	Selective for MAO-A	>100
15	755.22 ± 40.33	173,500 ± 10,040	284.80	160,440	Competitive	Reversible	Selective for MAO-A	>100
16	230.70 ± 17.00	28,840 ± 1200	129.05	212,700	Competitive	Reversible	Selective for MAO-A	>100
17	175.90 ± 11.80	460,450 ± 29,900	152.05	449,380	Competitive	Reversible	Selective for MAO-A	>100
18	591.00 ± 37.50	580,200 ± 36,000	222.38	218,100	Competitive	Reversible	Selective for MAO-A	>100
SEL	105,660 ± 9210	1350 ± 120	—	—	Competitive	Reversible	Selective for MAO-B	
MOC	5.53 ± 0.27	1080 ± 300	—	—	Competitive	Reversible	Selective for MAO-A	

SEL - Selegeline, MOC - Moclobemide.

[a] Values were determined from the kinetic experiments in which *p*-tyramine (substrate) was used at 500 μM to measure MAO-A and 2.5 mM to measure MAO-B. Pargyline or clorgyline were added at 0.50 μM to determine the isoenzymes A and B. Newly synthesized compounds and the known inhibitors were pre-incubated with the homoganates for 60 min. at 37 °C. Each value represents the mean ± SEM of three independent experiments.

[b] Values obtained through AutoDock program.

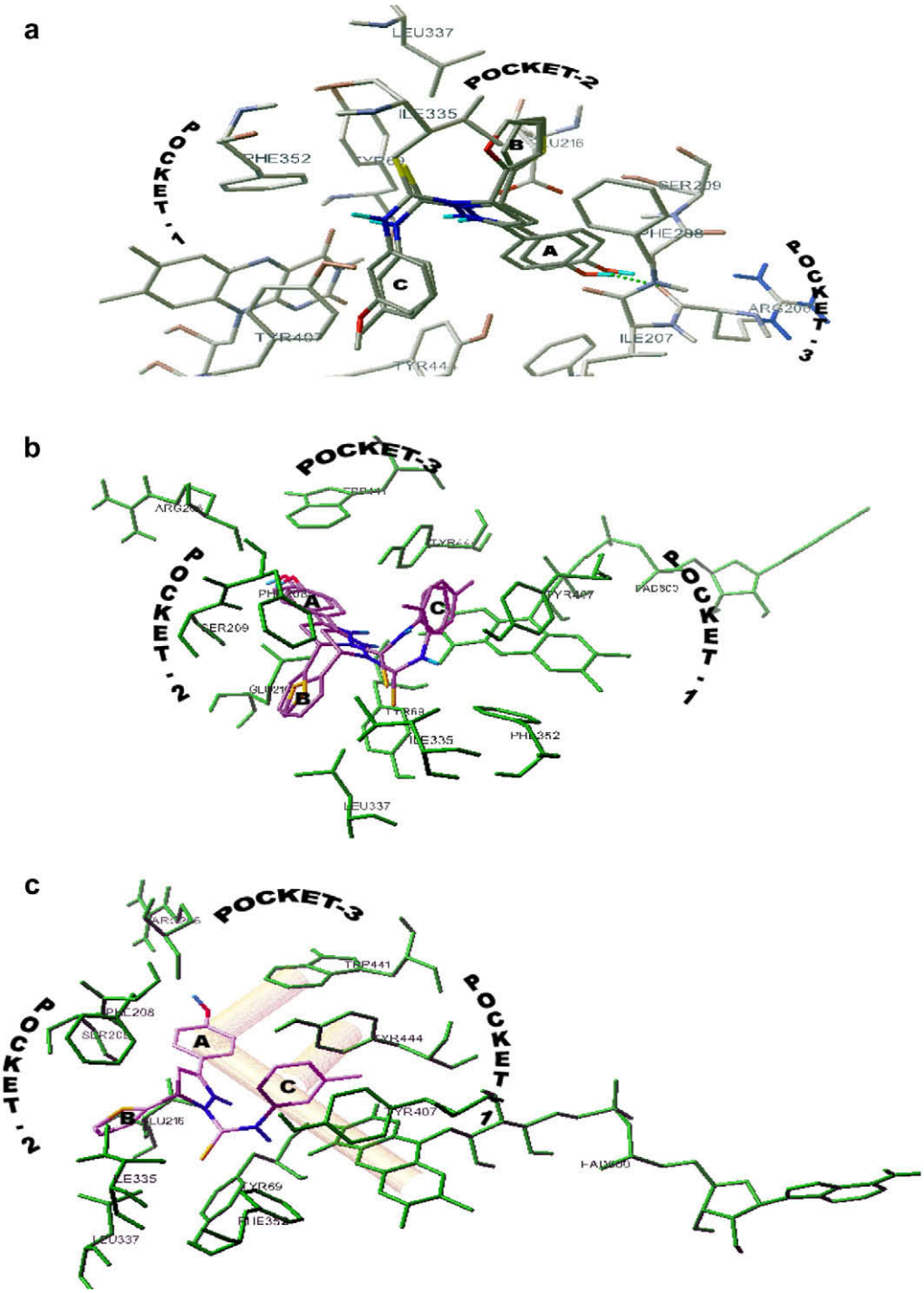


Figure 2. (a) Compounds **11** and **17** in complex with MAO-A, was shown. Ring A (*p*-hydroxy phenyl) is accommodated well in pocket3 which is formed due to cavity-shaping loop, characterized by GLY74, ARG206, ILE207, PHE208, GLU216, TRP441 whereas, ring B (five membered heteroaromatic ring) is well accommodated in pocket2 (ILE180, ILE335, LEU337, MET350, PHE352) and ring C (substituted phenyl) of these compounds were positioned in the aromatic cage (FAD, TYR407, and TYR444, pocket1). (b) Compounds **7** and **9** in complex with MAO-A are shown. (c) Compound **11** in complex with MAO-A are shown. 3-Methyl substitution in ring C orients the phenyl ring in the aromatic cage in such a way, so as to enable the pi–pi stacking.

are uncorrected. Infrared (IR) spectra were taken on a Fourier Transform Infrared Spectrophotometer IR-Prestige 21 (Shimatzu Corporation, Japan) from 4000–400 cm^{-1} using KBr discs. ^1H NMR spectra were recorded at 400 MHz in DMSO-d_6 using a Bruker Avance 400 instrument (Bruker Instruments Inc., USA). Chemical shifts were measured at δ units (ppm) relative to tetramethylsilane (TMS). Fast-atom bombardment (FAB) mass spectra were recorded on a Jeol SX 102/DA-6000 mass spectrometer (Jeol Ltd Akishima, Tokyo, Japan) using argon/xenon (6 kV, 10 mA) as FAB gas, *m*-nitrobenzyl alcohol as matrix, and 10 kV as accelerating voltage at room temperature. Elemental analysis was performed on a Vario EL III Elemental Analyser (Elementar, Germany) using sulfanilamide as standard. All chemicals were purchased from Merck, Spectrochem or CDH, India. Solvents were of reagent grade and were purified and dried by standard procedure. Reactions were monitored by thin-layer chromatography on silica gel plates in either iodine or

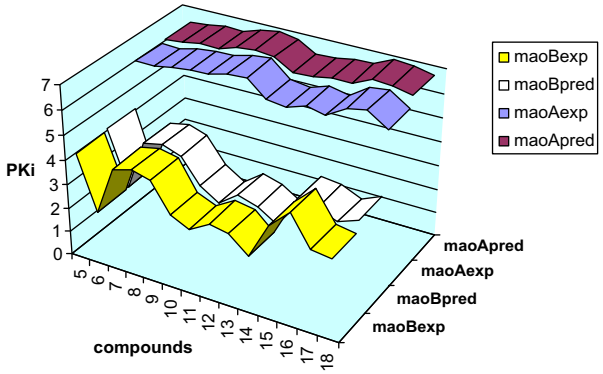


Figure 3. Experimental (exp) and predicted (pred) pK_i values of the synthesized compounds against MAO-A and MAO-B.

UV chambers. Intermediates were characterized by IR spectroscopic analysis and elemental analysis for CHN. In the elemental analysis, the observed values were within ±0.4% of the calculated values. Final compounds were characterized by ^{1}H NMR and FAB mass spectrometry (MS). The final yields and the physicochemical and spectral data of the compounds **1–32** are presented in Table 1.

3.1.1. General procedure for the synthesis of chalcones (1–2)

To a solution of 4-hydroxy acetophenone (0.01 M) and suitably substituted aldehydes (0.01 M) in ethanol (10 ml) was added aqueous solution of potassium hydroxide (60%) drop wise with continuous stirring at 0°C over a period of 15 minutes. The reaction mixture was kept at room temperature for about 48 h with occasional shaking. After 48 h it was poured into ice-cold water, and then neutralized to pH 2 using 6 N hydrochloric acid. The yellow precipitate obtained was filtered, washed, dried, and recrystallized from dry methanol. The intermediates **1–2** were obtained.

3.1.2. General procedure for the synthesis of 3,5-diaryl-4,5-dihydro-1*H*-pyrazole (3–4)

Appropriate chalcone (**1–2)** was treated with 10 times excess of hydrazine hydrate (80%) in dry ethanol and refluxed for 3–6 h. The hot reaction mixture was then poured into ice-cold water. The solid separated out was filtered, washed, dried and recrystallized from ethanol to afford respective pyrazoline (**3–4**).

3.1.3. General procedure for the synthesis of *N*,3,5-triaryl-4,5-dihydro-1*H*-pyrazole-1-carbothioamide (5–18)

An equimolar quantity of appropriate pyrazoline (**3–4**) and suitably substituted phenyl isothiocyanates in dry methanol was refluxed for 15–30 min to afford compounds **5–18.** The structures, physicochemical and spectral characteristics of compounds **5–18** was presented in Table 1.

3.2. Biochemistry

All chemicals used were purchased from Sigma–Aldrich Co. (Germany).

3.2.1. Isolation of MAO from rat liver homogenates

The ethics Committee of Laboratory Animals at Hacettepe University, Turkey (2001/25-4), approved the animal experimentation. MAO was purified from the rat liver according to the Holt method with some modifications.[36] Liver tissue was homogenized 1:40 (w/v) in 0.3 M sucrose. Following centrifugation at 1000*g* for 10 min, the supernatant was centrifuged at 10,000*g* for 30 min to obtain crude mitochondrial pellet. The pellet was incubated with CHAPS of 1% at 37 °C for 60 min and centrifuged at 1000*g* for 15 min. Pellet was resuspended in 0.3 M sucrose and was layered onto 1.2 M sucrose, centrifuged at 53,000*g* for 2 hours and resuspended in potassium phosphate buffer, pH 7.4, kept at −70 °C until used.

3.2.2. Measurement of MAO activity

Total MAO activity was measured spectrophotometrically according to the Holt method.[34] The assay mixture contained a chromogenic solution consisted of 1 mM vanillic acid, 500 μM 4-aminoantipyrine, and 4 U ml^{-1} peroxidase type II in 0.2 M potassium phosphate buffer, pH 7.6. The assay mixture contained 167 μl chromogenic solution, 667 μl substrate solution (500 μM *p*-tyramine) and 133 μl potassium phosphate buffer, pH 7.6. The mixture was pre-incubated at 37 °C for 10 min before the addition of enzyme. The reaction was initiated by adding the homogenate (100 μl), and an increase in absorbance was monitored at 498 nm at 37 °C for 60 min. A molar absorption coefficient of 4654 M^{-1} cm^{-1} was used to calculate the initial velocity of the reaction. Results were expressed as nmol h^{-1} mg^{-1}.

3.2.3. Selective measurement of MAO-A and MAO-B activities

Homogenates were incubated with the substrate *p*-tyramine (500 μM to measure MAO-A and 2.5 mM to measure MAO-B) following the inhibition of one of the MAO isoforms with selective inhibitors. Aqueous solution of clorgyline (50 μM), as selective MAO-A and -B inhibitors were added to homogenates at the ratio of 1:100 (v/v), yielding the final inhibitor concentrations of 0.50 μM. Homogenates were incubated with these inhibitors at 37 °C for 60 min prior to activity measurement. After incubation of homogenates with selective inhibitors, total MAO activity was determined by the method described above.

3.2.4. Analysis of the kinetic data

Newly synthesized compounds were dissolved in dimethyl sulfoxide (DMSO), maximum concentration 1% and used in the concentration range of 1–1000 μM. Inhibitors were incubated with the purified MAO at 37 °C for 0–60 min prior to adding them to the assay mixture. The reversibility of the inhibition of the enzyme by novel compounds was assessed by dialysis performed over 24 h at 37 °C relative to a potassium phosphate buffer, pH 7.6 capable of restoring 98–100% of the enzyme activity. Kinetic data for interaction of the enzyme with the compounds were determined using the Microsoft Excel package program. The inhibitory activities of the novel compounds for MAO-A and MAO-B were determined at 37 °C after incubation of the homogenates (previously treated with clorgyline for MAO-A or -B measurement) with the compounds for 60 min. Lineweaver–Burk plots were used to estimate the inhibition constant (K_i) of the inhibitors. IC_{50} values were determined from plots of residual activity percentage, calculated in relation to a sample of the enzyme treated under the same conditions without inhibitor, versus inhibitor concentration [I]. IC_{50} values were determined using non-linear regression analysis according to the equation for a sigmoid plot. Logarithmic transformation was also used for the determination of IC_{50} values for some compounds which showed their inhibition in a large concentration range.

3.2.5. Protein determination

The protein was determined according to the Bradford method[37] in which bovine serum albumin was used as a standard.

3.3. Molecular docking

3.3.1. Protein preparation

PDB structures (www.rcsb.org) were downloaded and were refined using Schrödinger protein preparation wizard. Protonation states were adjusted and finally a restrained energy minimization using OPLS2005 force field was carried out.

3.3.2. Ligand preparation

Structure of the ligands were sketched using built panel of Maestro and were energy minimized using OPLS force field of Macro Model (MacroModel, version 9.6, Schrödinger, LLC, New York, NY, 2005).

3.3.3. Docking simulations

All the docking experiments were performed with AutoDock 4.0.[38] Lamarckian Genetic Algorithm was employed as the docking algorithm. Virtual screening protocol was automated by a separate script (ViSTA) that was written and validated in house.[39]

Docking parameters: Number of Genetic Algorithm (GA) runs: 10, Population size: 150, Maximum number of evaluation: 2500,000, Maximum number of generation: 27,000.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.043.

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