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Original article

Monoamine oxidase inhibitory activity of 3,5-biaryl-4,5-dihydro-1*H*-pyrazole-1-carboxylate derivatives

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ABSTRACT

Ethyl and phenyl carbamate derivatives of pyrazoline (**3a**–**3h**) were synthesized and tested for their MAO inhibitory activity. All the compounds were found to be selective towards MAO-A. Phenyl carbamates (**3e**–**3h**) were better than ethyl carbamates (**3a**–**3d**) and displayed the best selectivity index. Compound **3f** ($Ki_{MAO\text{-}A}$; 4.96 ± 0.21 nM) was found to be equally potent as that of standard drug, Moclobemide ($Ki_{MAO\text{-}A}$; 5.01 ± 0.13 nM) but with best selectivity index (8.86×10^{-5}). Molecular docking studies with *R* & *S* conformer of 3f revealed *S*-enantiomer is better than *R*-enantiomer as reported earlier by other groups. It is proposed that VdW's radii of the substitution (bulkiness) in ring B determine the potency of phenyl carbamates.

1. Introduction

Monoamine oxidase exists in two different isoform, MAO-A & MAO-B, classified based on their specificity towards their substrate and their selective inhibitors [1,2]. Iproniazide was the first drug approved for the treatment of depressive illness during 1950s and that was followed by imipramine, phenelzine, isocarboxazid and tranylcypromine. All are not selective towards MAO-A and MAO-B isoform as well as not specific towards peripheral & brain MAO. Tyramine-induced hypertensive crisis (Cheese-effect) reported with tranylcypromine during mid 1960s, lead to the establishment of strict dietary restrictions by US-FDA [3,4]. Gradually this factor restricted the use of MAO-inhibitors and practitioner's switched to the newer drugs, acting on different mechanism, that did not required dietary restrictions (tricyclic antidepressants and Selective Serotonin Reuptake Inhibitors) [5]."Cheese effect" was mainly due to the non-specific inhibition of MAO-A in gut (peripheral) that warranted brain-specific MAO inhibitors. While brain specific inhibitor reduces "cheese effect", isoform selective inhibitors are to be developed for their therapeutic potential in treatment of depression (MAO-A selective) and neurodegenerative disorders (MAO-B selective) [6]. Development of new generation of MAO inhibitors that specifically inhibits brain-MAO in isoform selective and reversible mode has renewed the research interest on MAO-inhibitors [7,8].

Chemical class Δ2-pyrazolines have been explored for the development of newer MAO-inhibitor class assuming the structure as a cyclic analog of Iproniazide. Many Δ2-pyrazoline derivatives were synthesized and tested for their antidepressant activity [9–17] and for their MAO-inhibitory activity [18–28]. Our group has also reported few derivatives with MAO-inhibitory activity in rat-MAO [29–31] and in human-MAO [32].

The presented study explores the MAO-inhibitory activity of ethyl and phenyl carbamate derivatives of Δ2-pyrazolines. The compounds (**3a**–**3h**) were synthesized according to the reactions outlined in Scheme 1. Chalcones (**1a**–**1d**) were prepared through Claisen–Schmidt condensation of appropriate benzaldehydes with 2′-hydroxyacetophenone in 60% ethanolic sodium hydroxide

Abbreviations: MAO, monoamine oxidase; hMAO, human MAO; rMAO, rat MAO; SAR, structure–activity-relationship; bb, back-bone; sc, side-chain.

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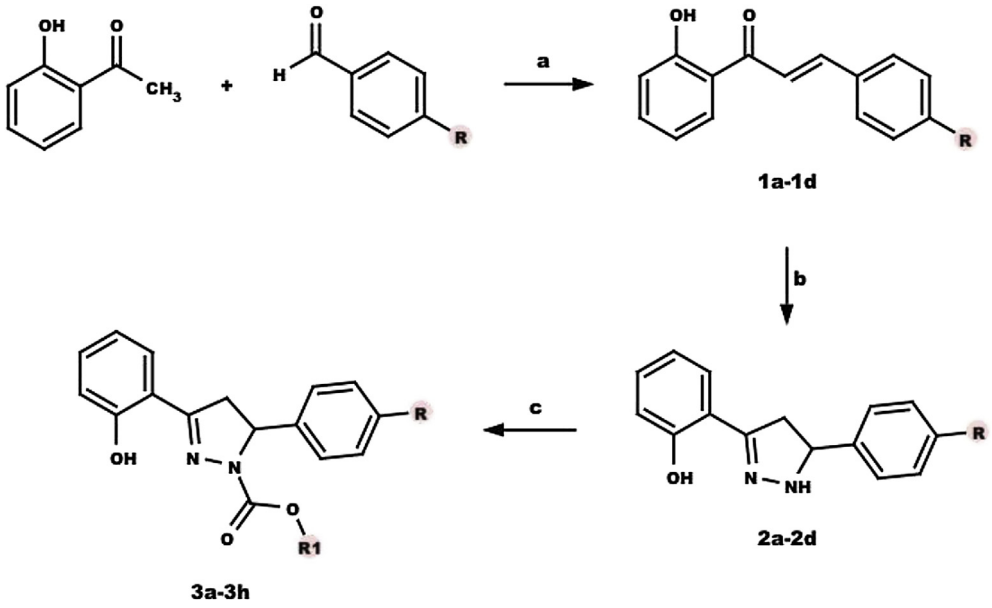


Scheme 1. Reagents & conditions: (a) (i) NaOH (60%), MeOH, 72 h, rt; (ii) HCl (6N) upto pH 2; (b) $NH_2NH_2.H_2O$, reflux, 3–6 h; (c) Cl–CO–OR1 (where R1 = $-C_2H_5$ or $-C_6H_5$), K_2CO_3, stirring, 30 min.

solution [33]. The pyrazoline intermediates (**2a**–**2d**) were obtained by the condensation of (**1a**–**1d**) with excess hydrazine hydrate (99%) in methanol [33]. The final products **3a**–**3h** were obtained by the reaction of **2a**–**2d** with ethylchloroformate/phenylchloroformate with an addition of equimolar quantity of potassium carbonate [34]. All the compounds were tested for their MAO-inhibitory activity using hMAO-A and hMAO-B.

2. Experimental section

2.1. Materials and methods

All the chemicals and solvents for synthesis were purchased from Aldrich. Unless otherwise mentioned the solvents were used without purification. Melting points were determined using Optimelt (Stanford Research Systems, Sunnyvale, CA 94089) by capillary method and are uncorrected. Infrared (IR) spectra were taken on a FT-IR Spectrophotometer IR-Prestige 21 (Shimatzu Corporation, Japan) from 4000 to 400 cm^{-1} using KBr discs. ^1H NMR spectra were recorded at 300 MHz in $CDCl_3$ and ^{13}C NMR at 400 MHz in DMSO using a Bruker Avance 400 instrument (Bruker Instruments Inc., USA) and Varian 400 (Varian Inc., USA), respectively. Chemical shifts were measured at δ units (ppm) relative to tetramethyl silane (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*-nitrobenzylalcohol as matrix, and 10 kV as accelerating voltage at room temperature. Reactions were monitored by thin-layer chromatography on silica gel plates in either iodine or UV chambers.

Monoamine oxidase A human (recombinant, expressed in baculovirus infected BTI insect cells), monoamine oxidase-B human (recombinant, expressed in baculovirus infected BTI insect cells), *R*-(-)-selegiline hydrochloride, resorufin, dimethyl sulfoxide (DMSO) and some other chemicals were purchased from Sigma–Aldrich™ (Germany). The Amplex®-Red MAO Assay Kitcontained benzylamine, *p*-tyramine, Moclobemide (MAO-A inhibitor), Selegiline (MAO-B inhibitor) and horseradish peroxidase.

All computational works were carried out in a single machine with RHEL5 operating system running on 2.93 GHz Pentium4 processor with 2 GB RAM. Maestro-8.4, AutoDock 4.2, MGLTools-1.4.6 and Open Babel were used for simulation studies.

2.2. Chemistry

2.2.1. General procedure for synthesis of pyrazoline-carboxamide (*3a*–*3h*)

To the solution of pyrazoline (**2a**–**2d**, 0.0008 M) in methanol (10 ml), was added an equimolar quantity of ethylchloroformate/phenylchloroformate drop-wise at <10 °C with stirring. An equimolar quantity of potassium carbonate was added and stirring continued for another 15–20 min. The reaction mixture was then filtered and the filtrate upon evaporation provided compound **3a**–**3h** [34].

*2.2.1.1. Ethyl 3-(2-hydroxyphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3a**).* Yield: 46%; mp: 116–118 °C; ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.25 (appeared as doublet, J = 3.00, 3H, $-CH_3$); 3.28 (dd, J_{AM} = 17.7 Hz, J_{AX} = 5.10 Hz, H_A); 3.79 (S, 3H, $-OCH_3$); 3.84 (dd, J_{MX} = 17.10 Hz, J_{MA} = 10.80 Hz, H_M); 4.19 (appeared as triplet, J = 3.45 Hz, 2H, $-CH_2-$); 5.34 (dd, J_{XA} = 11.85 Hz, J_{XM} = 5.40 Hz, H_X); 6.85–7.36 (m, 8H, Ar–H); 10.70 (s, 1H, –OH); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 14.74 ($-CH_2-\underline{C}H_3$), 43.52 ($-\underline{C}H_2-CH_3$), 55.47 ($O\underline{C}H_3$), 59.74 (pyr-C4), 61.76 (pyr-C5), 114.43–156.17 (Ar–C), 157.34 (–C=O), 159.03 (pyr-C3); FAB-MS: 340 $(M)^+$, 341 $(M+1)^+$

*2.2.1.2. Ethyl 3-(2-hydroxyphenyl)-5-(4-methylphenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3b**).* Yield: 42%; mp: 194–196 °C; ^1H NMR ($CDCl_3$): δ (ppm) 1.24 (appeared as doublet, J = 3.00, 3H, $-CH_3$); 2.33 (s, 3H, $-CH_3$); 3.27 (dd, J_{AM} = 17.55 Hz, J_{AX} = 5.10 Hz, H_A); 3.84 (dd, J_{MX} = 17.55 Hz, J_{MA} = 12.00 Hz, H_M); 4.19 (appeared as doublet, J = 6.90 Hz, 2H, $-CH_2-$); 5.35 (dd, J_{XA} = 11.85 Hz, J_{XM} = 5.4 Hz, H_X); 6.87–7.35 (m, 8H, Ar–H); 10.70 (s, 1H, –OH); FAB-MS: 324 $(M)^+$, 325 $(M+1)^+$

*2.2.1.3. Ethyl 3-(2-hydroxyphenyl)-5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3c**).* Yield: 58%; mp: 180–184 °C; ^1H NMR ($CDCl_3$): δ (ppm) 1.25 (appeared as singlet, 3H, $-CH_3$); 3.25

(dd, J_{AM} = 17.70 Hz, J_{AX} = 5.40 Hz, H_A); 3.87 (dd, J_{MX} = 17.70 Hz, J_{MA} = 12.00 Hz, H_M); 4.21 (appeared as doublet, J = 5.70 Hz, 2H, $-CH_2-$); 5.36 (dd, J_{XA} = 11.70 Hz, J_{XM} = 5.40 Hz, H_X); 6.88–7.37 (m, 8H, Ar–H); 10.61 (s, 1H, –OH); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 14.72 ($-CH_2-\underline{C}H_3$), 43.53 ($-\underline{C}H_2-CH_3$), 59.67 (pyr-C4), 61.91 (pyr-C5), 116.08–152.18 (Ar–C), 156.10 (pyr-C3), 157.34 (–C=O); FAB-MS: 344 $(M)^+$, 345 $(M+1)^+$, 346 $(M+2)^+$

*2.2.1.4. Ethyl 3-(2-hydroxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxylate (**3d**).* Yield: 51%; mp: 159–162 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.23 (appeared as singlet, 3H, $-CH_3$); 3.30 (dd, J_{AM} = 17.70 Hz, J_{AX} = 5.40 Hz, H_A); 3.87 (dd, J_{MX} = 17.55 Hz, J_{MA} = 11.70 Hz, H_M); 4.19 (appeared as triplet, J = 3.30 Hz, 2H, $-CH_2-$); 5.39 (dd, J_{XA} = 11.85 Hz, J_{XM} = 5.40 Hz, H_X); 6.87–7.61 (m, 9H, Ar–H); 10.69 (s, 1H, –OH); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 14.71 ($-CH_2-\underline{C}H_3$), 43.66 ($-\underline{C}H_2-CH_3$), 60.25 (pyr-C4), 61.82 (pyr-C5), 116.10–152.21 (Ar–C), 156.13 (pyr-C3), 157.34 (–C=O); FAB-MS: 310 $(M)^+$, 311 $(M+1)^+$

*2.2.1.5. Phenyl 3-(2-hydroxyphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3e**).* Yield: 71%; mp: 161–165 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 3.39 (dd, J_{AM} = 17.70 Hz, J_{AX} = 4.80 Hz, H_A); 3.80 (S, 3H, $-OCH_3$); 3.93 (dd, J_{MX} = 17.55 Hz, J_{MA} = 12.00 Hz, H_M); 5.47 (appeared as doublet, J_{XM} = 5.40 Hz, H_X); 6.87–7.39 (m, 12H, Ar–H); 10.64 (s, 1H, –OH); FAB-MS: 388 $(M)^+$, 389 $(M+1)^+$

*2.2.1.6. Phenyl 3-(2-hydroxyphenyl)-5-(4-methylphenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3f**).* Yield: 63%; mp: 147–149 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 2.34 (s, 3H, $-CH_3$); 3.38 (dd, J_{AM} = 17.70 Hz, J_{AX} = 4.80 Hz, H_A); 3.94 (dd, J_{MX} = 17.70 Hz, J_{MA} = 11.70 Hz, H_M); 5.47 (appeared as doublet, J_{XM} = 7.50 Hz, H_X); 6.90–7.39 (m, 12H, Ar–H); 10.65 (s, 1H, –OH); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 21.09 ($-CH_3$), (pyr-C4 peak masked by solvent peak); 60.50 (pyr-C5), 116.14–137.32 (Ar–C), 150.47 (pyr-C3), 157.42 (–C=O); FAB-MS: 372 $(M)^+$, 373 $(M+1)^+$

*2.2.1.7. Phenyl 3-(2-hydroxyphenyl)-5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazole-1-carboxylate (**3g**).* Yield: 59%; mp: 135–137 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 3.36 (dd, J_{AM} = 17.70 Hz, J_{AX} = 5.40 Hz, H_A); 3.96 (dd, J_{MX} = 17.70 Hz, J_{MA} = 11.70 Hz, H_M); 5.50 (appeared as triplet, J_{XM} = 5.40 Hz, H_X); 6.91–7.40 (m, 12H, Ar–H); 10.56 (s, 1H, –OH); FAB-MS: 392 $(M)^+$, 393 $(M+1)^+$, 394 $(M+2)^+$

*2.2.1.8. Phenyl 3-(2-hydroxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxylate (**3h**).* Yield: 61%; mp: 151–153 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 3.41 (dd, J_{AM} = 17.25 Hz, J_{AX} = 5.70 Hz, H_A); 3.96 (dd, J_{MX} = 17.55 Hz, J_{MA} = 12.00 Hz, H_M); 5.52 (dd, J_{XA} = 11.55 Hz, J_{XM} = 4.80 Hz, H_X); 6.90–7.38 (m, 13H, Ar–H); 10.63 (s, 1H, –OH); FAB-MS: 358 $(M)^+$, 359 $(M+1)^+$

2.3. Biochemistry

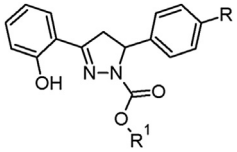
2.3.1. Determination of hMAO-A and -B activities

The activities of hMAO-A and hMAO-B were determined using *p*-tyramine as common substrate and calculated as 180.20 ± 7.33 pmol/mg/min (n = 3) and 149.50 ± 8.11 pmol/mg/min (n = 3), respectively. The interactions of the synthesized compounds with hMAO isoforms were determined by a fluorimetric method described and modified previously [20,35,36]. The production of H_2O_2 catalyzed by MAO isoforms was detected using Amplex®-Red reagent, a non-fluorescent, highly sensitive, and stable probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce the fluorescent product resorufin. The reaction was started by adding (final concentrations) 200 μM Amplex Red reagent, 1 U/mL horseradish peroxidase, and *p*-tyramine (concentration range 0.1–1 mM). Control experiments were carried out by replacing the synthesized compound and reference inhibitors. The possible capacity of novel compounds to modify the fluorescence generated in the reaction mixture due to nonenzymatic inhibition was determined by adding these compounds to solutions containing only the Amplex Red reagent in a sodium phosphate buffer.

2.3.2. Kinetic experiments

Synthesized compounds were dissolved in dimethyl sulfoxide, with a maximum concentration of 1%, and used in the concentration range of 1–100 μM. Kinetic data for interaction of the enzyme isoforms with the compounds were determined using the Microsoft Excel package program. The slopes of the Lineweaver–Burk plots were plotted versus the inhibitor concentration and the K_i

Table 1
MAO inhibitory activity of compounds **3a**–**3h**.



Compd	R	R^1	MAO-A K_i (μM) A	MAO-B K_i (μM) B	SI[c]	Inhibition type	Reversibility	MAO selectivity
3a	$-OCH_3$	$-C_2H_5$	0.61 ± 0.03	5.00 ± 0.18	0.121	Competitive	Reversible	MAO-A selective
3b	$-CH_3$	$-C_2H_5$	0.88 ± 0.04	6.00 ± 0.34	0.147	Competitive	Reversible	MAO-A selective
3c	–Cl	$-C_2H_5$	0.76 ± 0.04	15.55 ± 1.03	0.049	Competitive	Reversible	MAO-A selective
3d	–H	$-C_2H_5$	0.81 ± 0.03	11.00 ± 1.05	0.073	Competitive	Reversible	MAO-A selective
3e	$-OCH_3$	$-C_6H_5$	0.31 ± 0.02	305.00 ± 21.55	0.001	Competitive	Reversible	MAO-A selective
3f	$-CH_3$	$-C_6H_5$	$0.005 \pm 2 \times 10^{-4}$	56.00 ± 3.05	8.86×10^{-5}	Competitive	Reversible	MAO-A selective
3g	–Cl	$-C_6H_5$	0.03 ± 0.002	45.00 ± 2.80	6.56×10^{-4}	Competitive	Reversible	MAO-A selective
3h	–H	$-C_6H_5$	0.25 ± 0.002	530.00 + 30.25	4.68×10^{-4}	Competitive	Reversible	MAO-A selective
[a]SEL			9.06 ± 0.44	0.09 ± 0.004	99.92	Competitive	Reversible	MAO-A selective
[b]MOC			$0.005 \pm 2 \times 10^{-4}$	1.08 ± 3.00	0.005	Competitive	Reversible	MAO-A selective

[a] SEL-selegeline.
[b] MOC-moclobemide.
[c] Selectivity index for K_i (MAO-A)/K_i (MAO-B).

values were determined from the *x*-axis intercept as $-K_i$. Each K_i value is the representative of single determination where the correlation coefficient (R^2) of the replot of the slopes versus the inhibitor concentrations was at least 0.98. SI (K_i (MAO-A)/K_i (MAO-B)) was also calculated. The protein was determined according to the Bradford method [37], in which bovine serum albumin was used as a standard.

2.3.3. Reversibility experiments

Reversibility of the MAO inhibition with synthesized compounds was evaluated by a centrifugation–ultrafiltration method [21]. In brief, adequate amounts of the recombinant hMAO-A or B were incubated together with a single concentration of the newly synthesized compounds or the reference inhibitors in a sodium phosphate buffer (0.05 M, pH 7.4) for 1 h at 37 °C. After this incubation period, an aliquot was stored at 4 °C and used for the measurement of MAO-A and -B activity. The remaining incubated sample was placed in an Ultrafree-0.5 centrifugal tube with a 30 kDa Biomax membrane and centrifuged at 9000× *g* for 20 min at 4 °C. The enzyme retained in the 30 kDa membrane was resuspended in a 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 mL) and an aliquot of this suspension was used for MAO-A and -B activity determination. Control experiments were performed simultaneously (to define 100% MAO activity) by replacing the test drugs with appropriate dilutions of the vehicles. The corresponding values of percent (%) MAO isoform inhibition was separately calculated for samples with and without repeated washing.

2.4. Molecular docking simulation

In order to understand the interaction of the potent molecule (6) with MAO-A and MAO-B molecular docking studies were carried out using AutoDock4.2. Docking protocol reported earlier by our group has been followed with X-ray crystal structure of hMAO-A (2BXR) and hMAO-B (2BYB). Protein preparation: Protein Preparation Wizard of Maestro-8.4 (Schrodinger LLC) has been used to prepare protein. Crystallographic models 2BXR (hMAO-A) and 2BYB (hMAO-B) were downloaded from www.rcsb.org. Water and covalently linked ligands were deleted and bond order was corrected for FAD. After assigning charge and protonation state finally energy minimization was done using OPLS2005 force field. PDB written by Maestro for prepared proteins were rewritten by Open Babel for AutoDock compatible atom type. Ligand preparation: Ligand structures were drawn using build panel & prepared using Ligprep module implemented Maestro-8.4 (Schrodinger LLC). Energy minimization is carried out using MMFF force field.

Table 2
Reversibility of hMAO inhibition with the compound **3f** and moclobemide.

Compound	hMAO-A inhibition (%)	
	Before washing	After washing
Moclobemide (300 nM)	89.00 ± 4.55	9.56 ± 0.44
Compound **3f** (300 nM)	94.80 ± 0.45	8.00 ± 0.39

Each value represents the mean ± S.E.M. of three experiments.

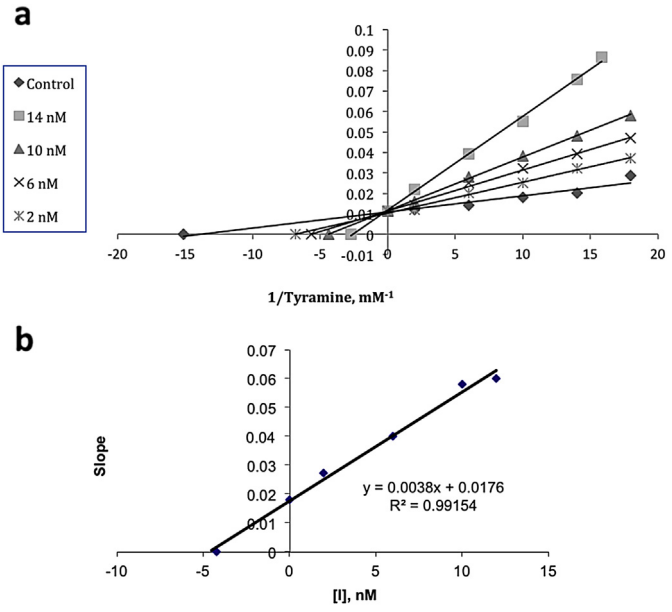


Fig. 1. a. Inhibition of hMAO-A with compound **3f** in the presence of various concentrations of the inhibitor. b. Graph for K_i value was calculation. [S] = p-tyramine (mM), v = velocity (nmol/h/mg), K_i = 0.004 μM.

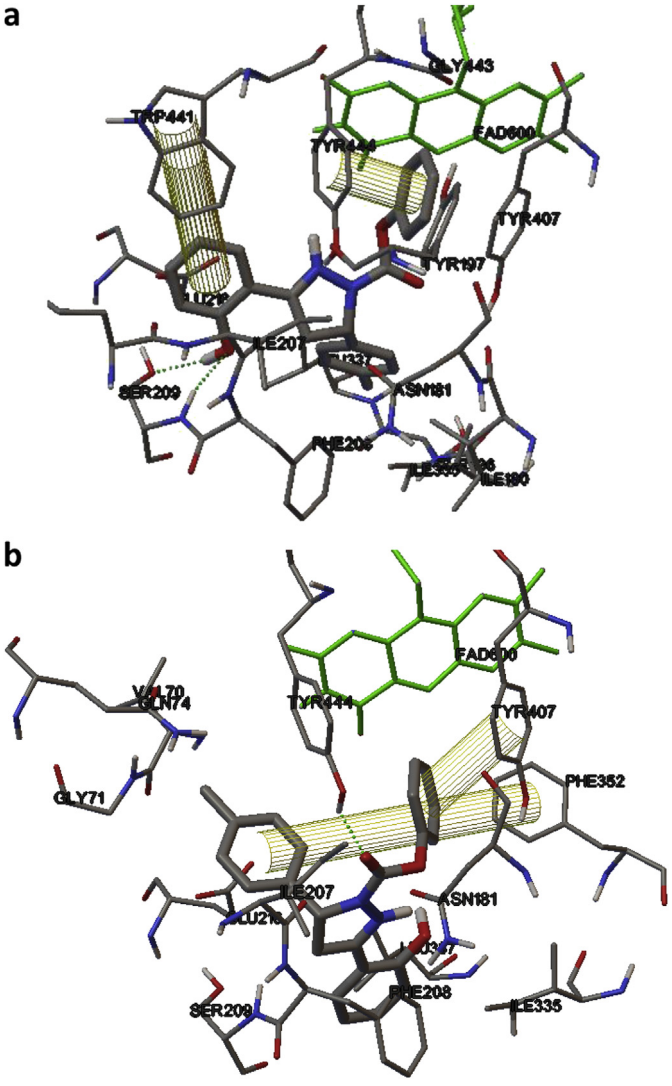


Fig. 2. a. *R*-enantiomer of **3f** in hMAO-A (PDB: 2BXR) active site; b. *S*-enantiomer of **3f** in hMAO-A (PDB: 2BXR) active site. H-bonds are shown as green dots and pi–pi interaction as yellow cylinders.

Table 3
Component energies of *R*- and *S*-enantiomers of **3f** with hMAO-A (2BXR).

Enantiomer	Estimated binding free energy (Kcal/mole)	Estimated K_i value (nM)	Component energies			
			Electrostatic	H-bond	VdW	Desolvation
R–	−8.47	620.30	−0.1406	−0.4693	−13.0428	2.2931
S–	−9.38	133.22	−0.1439	−0.9407	−13.1134	2.1125

Structures were saved in .pdb format and rewritten using openbable for AutoDock compatible atom type. Docking: Grid parameter file (.gpf) and Docking parameter files (.dpf) were written using MGLTools-1.4.6. Receptor grids were generated using 60×60×60 grid points in xyz with grid spacing of 0.375 Å. Grid box was centered on N5 atom of FAD. Map types were generated using autogrid4. Docking was carried out with following parameters with number of runs: 50, population size: 150, number of evaluations: 2,500,000 and number of generations: 27,000 numbers of generations, using autodock4. Analysis: Analysis of docking results was done using MGLTools-1.4.6. Top scoring molecule in the largest cluster was analyzed for its interaction with the protein.

3. Results & discussion

3.1. Chemistry

The intermediate chalcones (**1a**–**1d**) and pyrazolines (**2a**–**2d**) were reported earlier and confirmed by their melting point. The final compounds were confirmed by the ^{1}H NMR and FAB-MS. ^{1}H NMR spectra displayed the characteristic AMX pattern for pyrazolines as three double-doublets (dd) in the range δ 3.252–3.406 ppm (J_{AM}: 17.25–17.70 Hz & J_{AX} 4.80–5.70 Hz), δ 3.837–3.963 ppm (J_{MX}: 17.10–17.70 Hz & J_{MA} 10.80–12.00 Hz) and δ 5.343–5.498 ppm (**3a**–**3d** & **3h**; J_{AM}: 11.55–11.85 Hz & J_{AX}: 4.80–5.40 Hz) for H_A, H_M and H_X, respectively. Only in case of compounds **3e**–**3g**, H_X signal appeared either as doublet or triplet. The phenolic-OH proton appeared in the range of δ 10.559–10.701 ppm as a singlet. Methyl & methylene proton of ethyl group in compounds **3a**–**3d** appeared in the range of δ 1.226–1.252 ppm & δ 4.191–4.207, respectively. Methoxy group in ring B of **3a** & **3e** appeared in the range of δ 3.789–3.799, while methyl group in ring B of **3b** & **3f** in the range of δ 2.328–2.340 ppm. In ^{13}C NMR spectra the peaks for pyr-C3, C4 and C5 are in the range of δ 150.47–159.03 ppm, δ 59.67–60.25 ppm and δ 60.50–61.91 ppm, respectively. The peaks for –C=O, –CH2-CH3, –CH2–CH3, Ar–OCH3 and Ar–CH3 are in the range of δ 157.34–157.42 ppm (**3a**, **3c**, **3d** & **3f**), δ 43.52–43.66 ppm (**3a**, **3c** & **3d**), δ 14.71–14.73 ppm (**3a**, **3c** & **3d**), δ 55.47 ppm (**3a**) and δ 21.09 ppm (**3f**), respectively. FAB-MS of all the compounds displayed $[M]^+$ and $[M+1]^+$ peaks.

3.2. Enzyme inhibitory assay (hMAO-A & hMAO-B)

All the compounds (**3a**–**3h**) tested were found to inhibit hMAO-A competitively and reversibly (Table 1). A Lineweaver–Burk reciprocal plot (Fig. 1a) showed that the inhibition of hMAO-A with compound **3f** was competitive with an experimentally-derived K_i value of 0.004 μM obtained from *y*-intercept replots (Fig. 1b) exhibiting increase in K_m. It seems that the novel compounds may interact within the catalytic site of hMAO-A.

In the reversibility and irreversibility tests, hMAO-A inhibition was found to be reversible in presence of the compounds as shown by the recovery enzyme activity after repeated washing (Table 2).

Compounds **3e**–**3h** with phenyloxy-carbonyl substitution at N1 were many-fold potent than their counterparts with ethyloxy-carbonyl substitution, **3a**–**3d**. Substitution in ring B of **3a**–**3d** has a little effect on potency towards MAO-A/MAO-B as well as selectivity. While the same in compounds **3e**–**3h** has pronounced effect on potency and selectivity towards MAO-A and MAO-B. One with methyl substitution (**3f**) has shown potent inhibition that is followed by chloro (**3g**), unsubstituted (**3h**) and methoxy (**3e**). It seems rather than electronic property of the substitution, its VdW volume is responsible for increasing potency. For crucial interaction with protein ring B should have substitution with appropriate VdW volume (bulkiness) that increases potency. Compound **3f** (Ki_{MAO-A}; $0.005 \pm 2 \times 10^{-4}$ μM) was found to be equally potent as that of standard drug, Moclobemide (Ki_{MAO-A}; $0.005 \pm 2 \times 10^{-4}$ μM) but with best selectivity index (8.86×10^{-5}).

3.3. Molecular docking simulation

Molecular level interaction was studied through molecular docking simulation carried out with AutoDock4.2. As racemic mixture was screened for its enzyme inhibitory assay we docked individual enantiomer (*R*&*S*) for interaction studies as reported earlier [18,21,38–41]. Docking results suggested that *S*-enantiomer is better than *R*-enantiomer that is in agreement with all the previous studies reported till date [38–40].

Analysis of *S*-enantiomer of **3f** revealed that 2-hydroxyl functional group in the phenyl ring at C3 position of pyrazoline establishes two H-bonding interaction with SER209 [O–––HN (bb)-SER209& H–––OH (sc)-SER209]. This helps in positioning the ring-A in P2 (lined by GLY71, GLN74, ARG206, ILE207, PHE208, GLU216 and TRP441) ring-B in P3 (lined by ILE180, ILE335, LEU337, MET350 and PHE352) and ring-C in P1 (Aromatic cage, FAD, TYR407, TYR444). The position also favors pi–pi interaction of ring-A with TRP441 and ring-C with TYR444 (Fig. 2a). Analysis of *R*-enantiomer revealed H-bonding interaction of carbonyl oxygen of **3f** with Hydrogen of phenolic hydroxyl functional group of TYR444. This interaction favors the positioning of ring-B in P1 but both ring-A and ring-C are not fully accommodated in other two pockets. This conformer also exhibited pi–pi interaction of ring-B with PHE352 and ring-C with TYR407. All these interactions made ring-A to occupy/share the space at the center along with pyrazoline ring and potentially keeping P2 vacant. This reduces the VdW interaction and preventing the H-bond interaction of phenolic hydroxyl group of **3f** leading to many folds decreased activity of *R*-enantiomer compared with *S*-enantiomer (Fig. 2b). The component energies of these two enantiomers are presented in Table 3.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.010.

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