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Pyrazoline carboxylates as selective MAO-B inhibitors: Synthesis and Biological screening

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Abstract: A series of carboxylates of pyrazolines (**3a-3h**) were synthesized from ethyl 2-benzylidene-3-oxobutanoate esters using hydrazine hydrate and ethanol as solvent. All the compounds (**3a-3h**) screened for their MAO inhibitory activity using rat brain MAO. All the compounds were found to be selective, reversible and competitive inhibitors of MAO-B except compound **3d** and **3h** which was found to be non-selective. These compounds were found to be active against MAO-B at concentration close to 1 μM (**3e** = 0.99 μM, 3a = 0.92 μM and **3b** = 1.11μM). Compound **3a** was potent amongst these while **3b** was more selective towards MAO-B with selectivity index 2.88.

Keywords: Pyrazoline; Rat liver enzyme; MAO isoform; Selectivity; Inhibitors

1. Introduction

Monoamine oxidases (MAOs) are flavoenzymes bound to the outer mitochondrial membrane and are responsible for the oxidative deamination of neurotransmitters, dietary amines and trace of amines.[1][1] The scientist Mary Bernheim reported existence of MAO enzyme first time from the various animal livers.[2, 3] Existence of two isoforms have been identified MAO-A and MAO-B based on their amino acid sequences[4], substrate preference and inhibitor selectivity by Youdim et al.[5, 6] MAO-A has a higher affinity for noradrenaline and serotonin,[7] where as phenyl ethylamine and benzylamine were preferentially deaminated by MAO-B, this leads to the rapid degradation of these molecules and ensure that the proper functioning of synaptic neurotransmission, regulation of emotional behaviours and other brain functions.[8] The lack of selectivity towards specific isoforms (MAO-A and MAO-B) created problems during the clinical use of several MAO inhibitors due their side effects. [9] In the current perspectives, enormous MAO inhibitors with selectivity towards either MAO-A or MAO-B were developed and employed for curing of the psychic disorders like anxiety, depression, Parkinson's and Alzheimer's diseases respectively.[10-12] Pyrazolines are a class of compounds that are extensively studied for their inhibitory activity and a small review of the same is presented in the following paragraph.[13-15]

Pyrazolines are a class of heterocyclic compounds; important five membered nitrogen containing heterocycle and have been reported for their anti-inflammatory,[16] antimicrobial,[17] antitubercular,[18] antiviral,[19] cerebroprotective,[20] analgesic,[21] anticancer,[22] anti tubercular and anticonvulsant etc.[23, 24] Pyrazolines are now becoming a popular pharmacophore for developing the new drug entities against the MAO and as anti depressant agents.[15] Pyrazolines are having the capability of binding to MAO enzyme and its subtypes reversible or irreversible, they could be considered a valid pharmacophore for design, synthesis of selective monoamine oxidase (MAO) inhibitor.[25] Our research team has reported a divergent class of 3,5 diaryl pyrazolines which consists of carbothiamides along with their monoamine oxidase inhibitory activity in rat MAO (either A or B).**[Figure 1]** [26-29] The ethyl and phenyl carbamates of pyrazoline against human MAO A and B isoforms became the promising lead molecules also been reported recently.[30] Stability and possibility for carrying various substitutions on pyrazolines led us to develop further newer agents against monoamine oxidase enzyme (MAO-A & MAO-B).[25] The current work explains the effect of carboxylate group in the 4th position of pyrazoline and without an aryl group at 3rd position.

2. Results and Discussions

In the present context we have synthesized a few different pyrazoline analogues i.e, ethyl 3-methyl-5-phenyl-4,5-dihydro-1H-pyrazole-4-carboxylates **(3a-3h)** and screened for their inhibitory properties against both isoforms of MAO, as a part of continuous effort to develop the suitable lead molecules. Interestingly all the carboxylates of pyrazolines were found to be inhibiting the MAO-B isoform by competitive, reversible and selectively except the compound **3f** which was found to inhibit the MAO-A isoform as others.

2.1. Chemistry

The Pyrazolines-4-carboxylate derivatives were synthesized through reactions outlined in **Scheme 1.**

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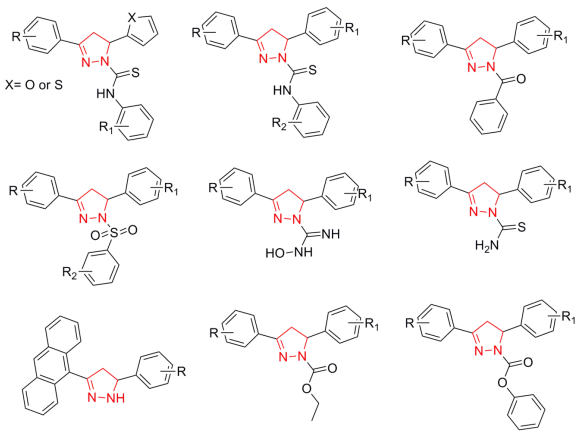
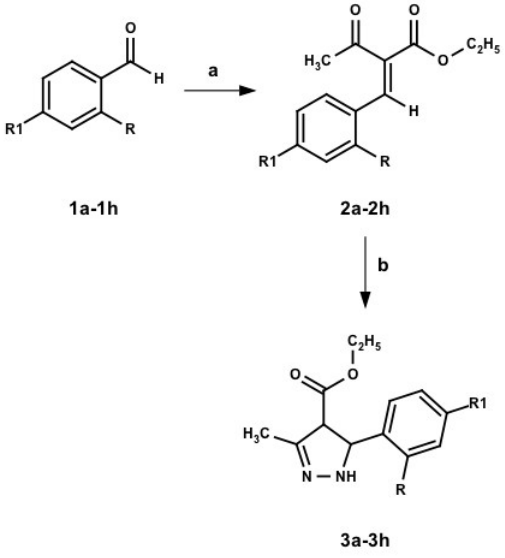


Figure 1. Pyrazolines as MAO inhibitors reported by our group

Knoevenagel condensation of appropriately substituted aromatic aldehyde with ethylacetoacetate in the presence of piperdine and glacial acetic acid provided benzylidene derivatves (**2a-2h**). Benzylidene derivatives (**2a-2h**) upon reflux with hydrazine hydrate (99%) in ethanol for about 5-6 h provided the final pyrazoline carboxylate derivatives (**3a-3h**). The reaction completion was monitored by using TLC plate (Merck) with Iodine chamber/UV chamber. The crude products were recrystallized from suitable solvent and are characterized by FT-IR (Intermediates and final). Compound (**3a-3h**) have been confirmed by the by the difference in the melting point from that of the reactant. The melting point of intermediate **1a** m.p: 68 ºC (Lit. 69-70 ºC),[31] **1b** m.p: 140 ºC (Lit. 142 ºC),[32] **1e** m.p: 48 ºC (Lit. 46-48 ºC),[33] **1g** m.p: 88 ºC (Lit. 87 ºC),[34] **1h** m.p: 60 ºC (Lit. 59.5-61.5 ºC),[35] All the final compounds (**3a-3h**) have been characterized using ^{1}H-NMR and FAB-MS spectra.



Scheme 1. Reagents and conditions: a) R-C_6H_4-CHO, ethylacetoacetate, piperidine, AcOH, rt, 4-5 h; b) $NH_2NH_2.H_2O$ 99%, EtOH, reflux, 5-6 h.

The ^{1}H-NMR spectra of compound (**3a-3h**) shows methyl protons present at N3 position of the pyrazoline ring between δ: 1.29-1.97ppm and appeared as singlet. The -$OCH_2C\underline{H}_3$ protons between δ: 1.22-1.31 ppm appeared as triplet. The -$OC\underline{H}_2$- protons between δ: 4.16-4.29ppm and appeared as quartert. The protons present at 4th position of the pyrazoline ring between δ: 2.87-2.97 ppm and appeared as doublet. The protons present at 5th position of the pyrazoline ring between δ: 4.47-4.50ppm and appeared as doublet. The -N$\underline{H}$- proton appeared between δ: 6.01-8.94 ppm. For compound (**3c** and **3e**) 2-$OC\underline{H}_3$ of phenyl ring and 4-$OC\underline{H}_3$ of phenyl ring on 5th position of pyrazoline ring δ: 3.81-3.85ppm appeared as singlet respectively. For compound (**3d** and **3f**) 2-$C\underline{H}_3$ of phenyl ring and 4-$C\underline{H}_3$ of phenyl ring on 5th position of pyrazoline ring at δ: 2.31-2.37ppm appeared as singlet respectively. FAB-MS spectra of all the final compounds displayed the characteristic molecular ion peak (M^+) along with $(M+1)^+$ as base peak and another $[M+2]^+$ as isotopic peak. The Compound **3b** and Compound **3g** have displayed the characteristic $[M+2]^+$ isotopic peak for the presence of chloride.

2.2. Biochemistry

MAO enzyme was purified from rat liver according the procedure reported by Holt.[36] The compounds (**3a-3h)** was screened for their inhibitory activity on MAO isoforms according to the established procedure. The biochemistry results revealed that newer carboxylates of pyrazolines (**3e, 3c, 3h, 3a, 3f** and **3g-3b**) were found to be competitive, reversible and selective towards the MAO-B isoform except compound **3d** which non selective towards the MAO isoform. The compounds **3a** and **3e** were having highest potency (IC_{50}=0.92μM and 0.99μM) among the series with significant selectivity towards MAO-B, having the nitro group and methoxy group substitution at 2nd and 4th position on aromatic ring respectively. Compound **3b** with chloro group substitution on 2nd position of aromatic ring exerted slight less potency and higher selectivity (IC_{50}=1.11μM) than **3a** and **3e.** While other compounds **3g** & **3f, 3h** & **3c** were exhibited the similar potency (IC_{50} = 1.0-2.0 μM) and lesser selectivity than **3b**, **3h** and **3e**; which were having the chloro & methyl, nitro & methoxy groups substitution at 4th and 2nd position on aromatic ring respectively. The only one compound **3d** was found to be having the competitive and reversible inhibitors of MAO-A isoform with weak selectivity having the methyl group at 2nd position on aromatic ring. However, the present activity data may relates with the electronegetivity (EN) effect of compounds; the decline in MAO activity with decreasing EN effect was observed in *ortho* substituted compounds (**3a-3d**). The effect of EN was observed on selectivity index values of compounds **3a-3d**, but the only variation was the chloro group (**3b**) is slightly better than nitro group (**3a**) were noticed. Whereas the effect of EN on the *para* substituted compounds were also studied and given in **Figure 2.** All the experimental results were presented in **Table 1**.

3. Experimental

Materials and methods: Melting points were determined using automated melting point system Optimelt (Stanford research systems) by capillary method. 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. ^{1}HNMR spectra were recorded at 400 MHz in DMSO-d6 using a Bruker Avance 400 instrument (Bruker Instruments Inc., USA). Chemical shifts were measured at d units (ppm) relative to tetramethylsilane

Table 1. Ki values corresponding to the inhibition of rat liver MAO isoforms by compound (**3a-3h)**

Compd No.	R	R1	Ki-Value for (μM)		SI**	Inhibition Type	Reversibility	MAO-Selectivity
			MAO-A*	MAO-B*				
3a	2-NO_2	H	2.45	0.92	2.66	Competitive	Reversible	MAO-B
3b	2-Cl	H	3.20	1.11	2.88	Competitive	Reversible	MAO-B
3c	2-OCH_3	H	2.22	1.41	1.53	Competitive	Reversible	MAO-B
3d	2-CH_3	H	2.16	2.20	0.98	Competitive	Reversible	**Non-Selective**
3e	H	4-OCH_3	2.03	0.99	2.05	Competitive	Reversible	MAO-B
3f	H	4-CH_3	2.09	1.27	1.64	Competitive	Reversible	MAO-B
3g	H	4-Cl	2.61	1.38	1.89	Competitive	Reversible	MAO-B
3h	H	4-NO_2	1.90	1.44	1.31	Competitive	Reversible	**Non-selective**
SEL			105.66	1.35	53.90	Competitive	Reversible	MAO-B
MOC			0.0055	1.08	0.004	Competitive	Reversible	MAO-A

SEL-selegeline, MOC-moclobemide, *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 preincubated with the homogenates for 60 min at 37 °C. Each value represents the mean ± SEM of three independent experiments. **Selectivity index was calculated as Ki (MAO-A)/Ki(MAO-B).

(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. 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 UV chambers. Final compounds were characterized by 1H NMR and FAB mass spectrometry (MS).

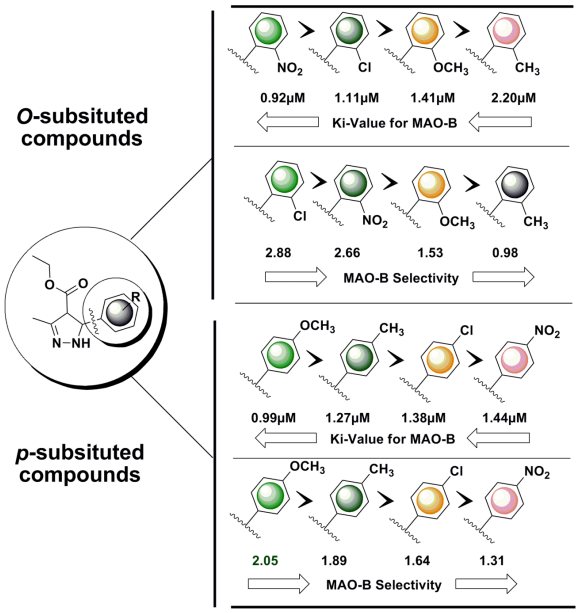


Figure 2. The Ki values and selectivity of compounds (**3a-3d**) against MAO-B

3.1. Chemistry

3.1.1. General procedure for synthesis of (Z)-ethyl 2-benzylidene-3-oxobutanoate (2a-2h)

The equimolar quantities of substituted aromatic benzaldehyde (**1a-1h**, 0.01M) and ethylacetoacetate (0.01M) were taken in RBF. To this 2.96 mL of piperidine and 3.36mL of glacial acetic acid were added. The resulting mixture was stirred for 4-5h in an inert atmosphere at room temperature. The completion of reaction was monitored by thin layer chromatography using 20% ethyl acetate: hexane as mobile phase. After the completion of reaction, the reaction mixture was concentrated under vacuum and without further spectral characterization the resulting crude mixture was utilized for the next step.[37]

3.1.2. General procedure for synthesis of ethyl 3-methyl-5-phenyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3a-3h)

The intermediate **(2a-2h)** 0.01M and 0.015 M (excess) of hydrazine hydrate 99% was taken in RBF. To this 20 mL ethanol was added and mixture was refluxed for 5-6 h. The completion of reaction was monitored by thin layer chromatography using 20% ethyl acetate: hexane as mobile phase. After the reaction completion, it was kept overnight for the crystallization process. The obtained solid product was filter under vacuum and dried.[38]

Ethyl 3-methyl-5-(2-nitrophenyl)-4,5-dihydro-1H-pyrazole-4-carboxylate (3a) Yield: 46%; Rf[a]: 0.33 (n-Hex: EtOAc, 8:2); mp: 167-168 ºC; IR (KBr, cm^{-1}) Vmax: 3213, 2934, 2433, 1589, 1336 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 1.28 (t, 3H, *J* = 5.6Hz, -CH_2-CH_3), 1.90 (s, 3H, Pyr-CH_3), 2.90 (d, 1H, *J*=2.0Hz, Pyr-C4-H), 4.26-4.17 (q, 2H, *J*=2.4Hz, -CH_2-CH_3), 4.49 (d, 1H, *J*=1.2Hz, Pyr-C5-H), 7.01 (d, 1H, -NH), 7.724-7.660 (m, 2H, ArH), 8.06 (d, 1H, ArH), 8.16 (m, 1H, ArH); FAB-MS: 278.1 $(M + 1)^+$

Ethyl 5-(2-chlorophenyl)-3-methyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3b) Yield: 54%; Rf[a]: 0.78 (n-Hex: EtOAc, 8:2); mp: 145-147 ºC; IR (KBr, cm^{-1}) Vmax: 3009, 2484, 1934, 1610, 1446, 1273, 1043 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 2.46 (s, 5H, -CH_2-CH_3), 3.27 (s, 1H, Pyr-CH_3), 7.47 (t, 2H, *J* = 0.8Hz, Pyr-C4 & C5-H), 7.51 – 7.59 (m, 3H, *J* = 2.0Hz, ArH), 8.12 (d, 2H, *J* = 1.6Hz, ArH), 8.94 (s, 1H, Pyr-NH); FAB-MS : 267.7 $(M + 2)^+$

Ethyl 5-(2-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3c) Yield: 59%; Rf[a]: 0.70 (n-Hex: EtOAc, 8:2); mp: 218-220ºC; IR (KBr, cm^{-1}) Vmax: 3061, 2961, 2843, 2033, 1606, 1486, 1031 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 1.29 (t, 3H, *J*=1.8Hz, -CH_2-CH_3), 1.96 (s, 3H, Pyr-CH_3), 1.96 (s, 3H, Pyr-CH_3), 2.96 (d, 1H, *J*=2.0Hz, Pyr-C4-H), 3.85 (s, 3H, Ar-OCH_3), 4.15 (t, 2H, J=3.2Hz, -CH_2-CH_3), 4.50 (d, 1H, *J*=1.6Hz, Pyr-C5-H), 6.85-6.97 (t, 1H, *J*=3.6Hz, ArH), 7.01 (d, 2H, *J*=0.8Hz, Pyr-NH merged with ArH), 7.15 (d, 2H, *J*=2.0Hz, ArH); FAB-MS : 261.0 $(M - 1)^+$

Ethyl 3-methyl-5-o-tolyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3d) Yield: 74%; Rf[a]: 0.85 (n-Hex: EtOAc, 8:2); mp: 203-205 ºC; IR (KBr, cm^{-1}) Vmax: 3001, 1923, 1610, 1460, 1219, 1039 cm^{-1}; ^{1}HNMR (400MHz, DMSO-

d_6): δ (ppm) 1.30 (d, 3H, *J*=5.2Hz, -CH_2-$C\underline{H}_3$), 1.97 (s, 3H, Pyr-$C\underline{H}_3$), 2.96 (d, 1H, *J*=3.5Hz, Ar-$C\underline{H}_3$), 4.14 (m, 2H, *J*=2.0Hz, -$C\underline{H}_2$-CH_3), 4.56 (d,1H, *J*=2.8Hz, Pyr-C4-H), 6.96 (d, 2H, *J*=3.5Hz, ArH and merged with Pyr-NH), 7.12 (d, 1H, *J*=1.6Hz, ArH), 7.24 (m, 1H, *J*=1.6Hz, ArH); 7.43 (m, 1H, *J*=1.6Hz, ArH); FAB-MS : 247.5 $(M + 1)^+$

Ethyl 5-(4-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3e) Yield: 38%; Rf[a]: 0.55 (n-Hex: EtOAc, 8:2); mp: 252ºC (Lit. 250°C)[38]; IR (KBr, cm^{-1}) Vmax: 2926, 2083, 1608, 1249, 1024 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 1.29 (d, 3H, -CH_2-$C\underline{H}_3$), 1.95 (s, 3H, Pyr-$C\underline{H}_3$), 2.96 (d, 1H, *J*=2.0Hz, Pyr-C4-H), 3.81 (s, 3H, Ar-O$C\underline{H}_3$), 4.28 (d, 2H, *J*=3.6Hz, -$C\underline{H}_2$-CH_3), 4.50 (d, 1H, *J*=2.0Hz, Pyr-C5-H), 6.96 (d, 2H, *J*=1.6Hz, ArH), 7.04 (d, 1H, = 2.0Hz, Pyr-NH), 7.15 (d, 2H, *J*=2.0Hz, ArH); FAB-MS : 262.0 $(M)^+$

Ethyl 3-methyl-5-p-tolyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3f) Yield: 56%; Rf[a]: 0.76 (n-Hex: EtOAc, 8:2); mp: 149-151 ºC; IR (KBr, cm^{-1}) Vmax: 2929, 1936, 1622, 1307, 1089 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm)1.29 (d, 3H, *J*=2.4Hz, -CH_2-$C\underline{H}_3$), 1.95 (s, 3H, Pyr-$C\underline{H}_3$), 2.31 (s, 1H, Ar-$C\underline{H}_3$), 2.96 (d, 1H, *J*=3.6Hz, Pyr-C4-H), 4.15 (d, 2H, *J*=4.8Hz, -CH_2-CH3), 4.50 (d, 1H, *J*=2.0Hz, Pyr-C5-H), 7.02 (d, 2H, Pyr-NH merged with ArH), 7.15 (d, 2H, ArH); FAB-MS : 245.1 (M-1), 247.5 $(M+1)^+$

Ethyl 5-(4-chlorophenyl)-3-methyl-4,5-dihydro-1H-pyrazole-4-carboxylate (3g) Yield: 44%; Rf[a]: 0.72 (n-Hex: EtOAc, 8:2); mp: 209-211 ºC; IR (KBr, cm^{-1}) Vmax: 2933, 1917, 1612, 1483, 1305, 1082 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 2.49 (s, 5H, -$C_2\underline{H}_5$), 3.29 (s, 3H, Pyr-$C\underline{H}_3$), 7.57 (d, 2H, *J* = 4.8Hz, ArH), 7.89 (d, 2H, *J* = 3.5Hz, ArH); FAB-MS : 289.7 $(M + Na)^+$

Ethyl 3-methyl-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazole-4-carboxylate (3h) Yield: 37%; Rf[a]: 0.83 (n-Hex: EtOAc, 8:2); mp: 217-219 ºC(Lit. 220°C)[38]; IR (KBr, cm^{-1}) Vmax: 3088, 2866, 1624, 1529, 1344, 1087 cm^{-1}; ^{1}HNMR (400MHz, DMSO-d_6): δ (ppm) 1.28 (d, 3H, *J*=5.6Hz, -CH_2-$C\underline{H}_3$), 1.90 (s, 3H, Pyr-$C\underline{H}_3$), 2.89 (d, 1H, *J*=2.0Hz, Pyr-C4-H), 4.21 (m, 2H, *J*=3.2Hz, -$C\underline{H}_2$-CH_3), 4.55 (d, 1H, *J*=6.8Hz, Pyr-C5-H), 7.49 (m, 2H, ArH and merged Pyr-NH), 8.31 (d, 2H, *J*=5.6Hz, Ar-H), ; FAB-MS : 278.2 $(M + 1)^+$

3.2. Biochemistry

MAO was extracted and purified from the rat liver according to established method reported by Holt.[29, 36, 39] In brief, the assay mixture contained a chromogen solution consisted of vanillic acid, 4-aminoantipyrin and peroxidise 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 ºC for 60 min. Results obtained expressed as nmol h^{-1} mg^{-1}. All the experimental compounds were dissolved DMSO and used in the concentration range of 1-1000 µM. Inhibitors were then incubated with purified MAO at 37 ºC for 0-60 min prior to adding to the assay mixture. Reversibility of the inhibition of MAO by these compounds was assessed by dilution. Kinetic data for interaction of the enzyme with these compounds were determined using Microsoft Excel package program. 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 inhibitors, versus inhibitor [I] concentration.

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