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Original article

The stereoselectivity of inhibition of rat liver mitochondrial MAO-A and MAO-B by the enantiomers of 2-phenylpropylamine and their derivatives

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Abstract – As part of a study of the stereoselectivity of inhibition of the different forms of monoamine oxidase (MAO-A and MAO-B), the enantiomers of 2-phenylpropylamine, N-methyl-2-phenylpropylamine and N-methyl-N-propargyl-2-phenylpropylamine have been prepared. The K_i values for each enantiomer when competitively inhibiting both MAO-A and MAO-B are reported. The enantiomers of N-methyl-N-propargyl-2-phenylpropylamine were also evaluated as irreversible inhibitors (first order rate constant [k_2] for formation of the covalent adduct). These compounds represent a series of enantiomers in which asymmetry is due to the presence of a hydrophobic ($-CH_3$) substituent at the carbon atom β to the amino function. The results are discussed in comparison to previous studies of similar enantiomeric compounds in which the asymmetry was present at the carbon atom α to the amino function. © Elsevier, Paris

enantiomeric 2-phenylpropylamines and derivatives / MAO-A and MAO-B / stereoselectivity / competitive inhibition / irreversible inhibition

1. Introduction

The two forms of the enzyme monoamine oxidase (MAO) (monoamine: O_2 oxido-reductase (deaminating) (flavin containing) (E.C. 1.4.3.4.)) within tissue or crude enzyme preparations are detected and differentiated by their response to selective substrates and inhibitors. Thus, MAO-A catalysed oxidative deamination of 5-hydroxytryptamine (5-HT; serotonin) (MAO-A selective substrate) is inhibited by clorgyline (*figure 1*, compound **1**; approximately 10^{-8} M) [1], whereas MAO-B catalysed oxidative deamination of β-phenylethylamine or benzylamine (MAO-B selective substrates) is inhibited by [R]-(-)-deprenyl (Selegiline) (*figure 1*, compound **2**; approximately 10^{-8} M) [2]. Other endogenous amine substrates e.g. tryptamine, tyramine etc. are metabolised by both forms of the enzyme [3].

The selective inhibitors clorgyline and Deprenyl (Selegiline) are both derivatives of N-methylpropargylamine and, although selective towards different forms of the enzyme, exert their inhibition by mechanisms which, from a chemical standpoint, are identical. Both compounds N-alkylate the reduced flavin prosthetic group of the enzyme [4] through formation of an intermediate radical cation [5]; for recent review see [6]. Such a suicide inhibitory mechanism shows time-dependent irreversible kinetics (formation of EI^*) preceded by a competitive reversible phase (formation of the initial EI complex) and represented as:

$$[E] + [I] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI] \xrightarrow{k_2} [EI^*]$$

where $K_i = k_{-1}/k_1$

Since the chemical mechanism of inhibition of the two forms of MAO are identical for the substituted propargylamine inhibitors, selectivity towards a particular form will be largely dependent upon affinity differences (as reflected in K_i values) towards the two forms arising from differences in lipophilicity and/or differences in the steric and stereochemical requirements for inhibition. Quantitative structure activity relationship studies have suggested that, in addition to differences in the steric requirements for optimal inhibition, the two forms of the enzyme also differ in their lipophilicity requirements [7, 8].

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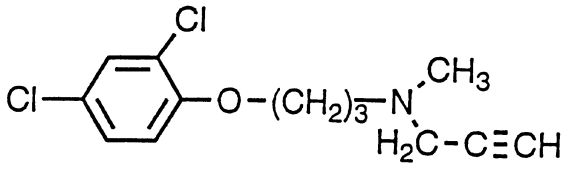
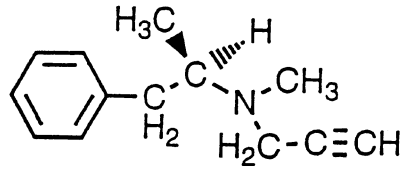


(**1**)



(**2**)

Figure 1. Structures of compounds **1** and **2**.

Aspects of the interaction of monoamine oxidase with substrates and inhibitors, particularly stereochemical aspects of such interactions, have been reviewed [9]. Studies employing the enantiomers of Selegiline and of α-methylpargyline (N-methyl-N-propargyl-1-phenylethylamine) have shown that, at the competitive phase of the inhibition, although each form of the enzyme is sensitive to configurational differences and show a preference towards the [R]-configuration enantiomer, such differences are more pronounced in the MAO-B form of the enzyme [10–12]. At the irreversible phase of the inhibition, although N-alkylation appears to proceed at a slightly faster rate with MAO-A than with MAO-B, the influence of configuration is negligible. Such is consistent with the generally accepted inhibitory mechanism [6] wherein formation of a radical cation intermediate is followed by proton loss from the β-carbon atom leading to a planar (racemic) radical as the alkylating species.

All of the above compounds are MAO inhibitors in which the chiral centre is at the carbon atom α to the basic nitrogen atom. Compounds in which the chiral centre is at the β-carbon have not been extensively studied. The present work therefore reports on the synthesis and testing of the enantiomers of 2-phenylpropylamine (*figure 2*, compounds **3a** and **3b**) and N-methyl-2-phenylpropylamine (*figure 2*, compounds **4a** and **4b**) as competitive inhibitors of both MAO-A and MAO-B. The enantiomers of the N-methyl-N-propargyl derivative (*figure 2*, compounds **5a** and **5b**) have also been prepared and studied under conditions leading to both reversible (competitive) and irreversible inhibition.

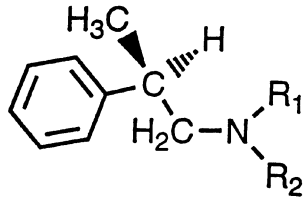
2. Results and discussion.

2.1. Chemistry

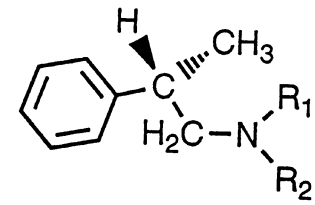
(±)-2-Phenylpropylamine was synthesised by reaction of formamide with (±)-2-phenylpropionaldehyde (Leuckart reaction) and the product resolved into its individual enantiomers using both (+)- and (-)-tartaric acid [13]. The (+)-2-phenylpropylamine enantiomer has been previously established as having the [R] configuration by synthesis from [R](L)-(-)-2-phenylpropionic acid [14] and conversion of [R](D)-(-)-atrolactic acid to [S](D)-(+)-2-phenylpropionic acid [15]. The N-methylation reactions were performed by reaction of dimethylsulphate on the benzilidine derivative of the primary amine and the products further alkylated using propargyl bromide.

2.2. Biological studies

A mitochondrial enzyme preparation of MAO derived from rat liver, and known to contain both MAO-A and MAO-B [16], was employed in the inhibition studies.



[R]



[S]

Figure 2. **3a** (R_1 = R_2 = H)
4a (R_1 = CH_3; R_2 = H)
5a(R_1 = CH_3; R_2 = $CH_2C{\equiv}CH$)

3b (R_1 = R_2 = H)
4b (R_1 = CH_3; R_2 = H)
5b (R_1 = CH_3; R_2 = $Ch_2C{\equiv}CH$)

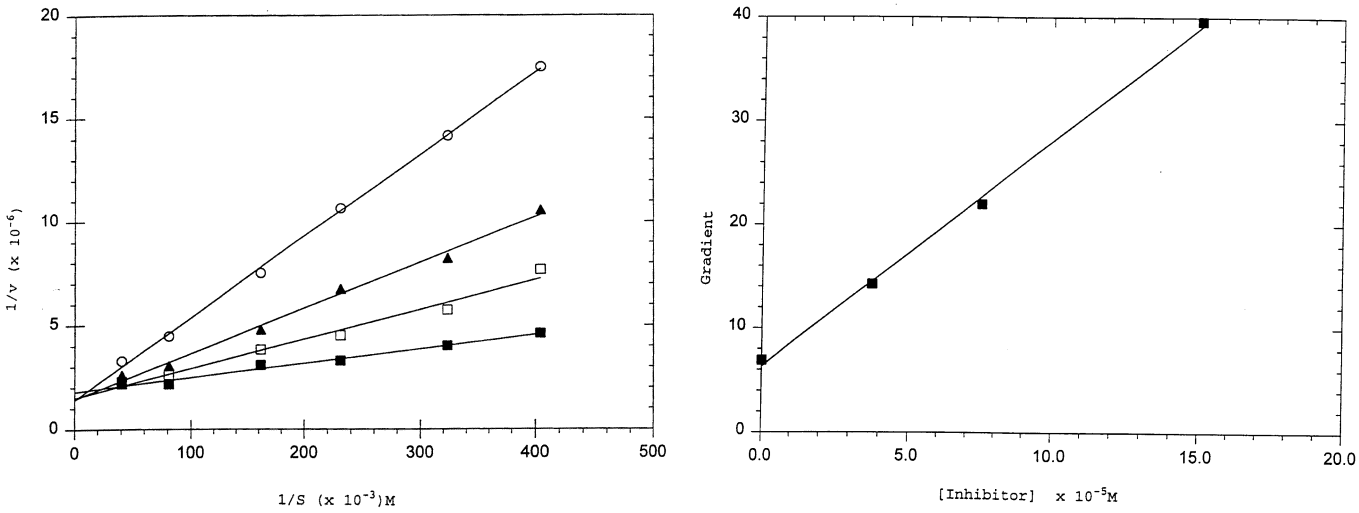


Figure 3. **a.** Double-reciprocal plot of inhibition of oxidative deamination of PEA by [R]-(-)-N-methyl-N-propargyl-2-phenylpropylamine hydrochloride (**5a**). All points are the mean of duplicate determinations. Inhibitor concentrations are 0 μM (■); 37.66 μM (□); 75.32 μM (▲);150.6 μM (○). **b.** Replot of gradient vs. inhibitor concentration, data from *figure 3a*.

Enzyme preparations containing a single active form of the enzyme were prepared from the above by incubation with either Selegiline (MAO-B selective inhibitor) or Clorgyline (MAO-A selective inhibitor) followed by washing and centrifugation to remove the excess inhibitor. Enzyme activity in such preparations was determined at 37 °C and pH 7.4 by a radiochemical method [17, 18] as previously reported [7, 12] using the appropriate specific substrate (^{14}C-labelled 5-HT or PEA). In studies of the competitive phase of the inhibition and in order to prevent any time dependent irreversible inhibition by covalent adduct (EI*) formation, the enzyme catalysed reaction was initiated by addition of enzyme to a solution of substrate and inhibitor i.e. preincubation of the enzyme with the inhibitor was omitted and the enzyme catalysed reaction was allowed to proceed for only 3 min. Employing such conditions, all plots of resulting data were indicative of competitive inhibition kinetics (see *figures 3a* and *3b* for a typical set of data). The computed K_i values are given in *table I*.

In studies of the irreversible phase of the inhibition, aliquots of an enzyme/inhibitor mixture were removed at intervals, diluted with substrate solution and residual enzyme activity determined (amount of product formed during 3 min incubation). Kinetic data was interpreted as previously reported [12] using the method developed by Kitz and Wilson [19]. A typical set of results are presented graphically in *figures 4a* and *4b* and the computed values of k_2, the rate constant for formation of the covalent adduct (EI*), are shown in *table I*.

Table I. Competitive Ki values and kinetic constants (k_2) for the time-dependent inhibition of MAO-A and MAO-B by the enantiomers of 2-Phenylpropylamine and their derivatives.

	Compound	MAO-A		MAO-B	
		Km(5-HT) 28.6 (± 6.2) μM		Km(PEA) 3.46 (± 0.48) μM	
		Ki (μM)	k_2 (min^{-1})	Ki (μM)	k_2 (min^{-1})
3a	[R]-(+)-	133.5 (± 8)	na	14.1 (± 1.3)	na
3b	[S]-(-)-	584 (± 18)	na	156 (± 5.5)	na
4a	[R]-(+)-	169 (± 10)	na	47.8 (± 8.4)	na
4b	[S]-(-)-	614 (± 72)	na	149 (± 33)	na
5a	[R]-(-)-	11.3 (± 3.2)	0.14 (± 0.03)	24.9 (± 2.9)	0.42 (± 0.03)
5b	[S]-(+)-	181.6 (± 40)	0.18 (± 0.04)	44.9 (± 7.8)	0.38 (± 0.005)

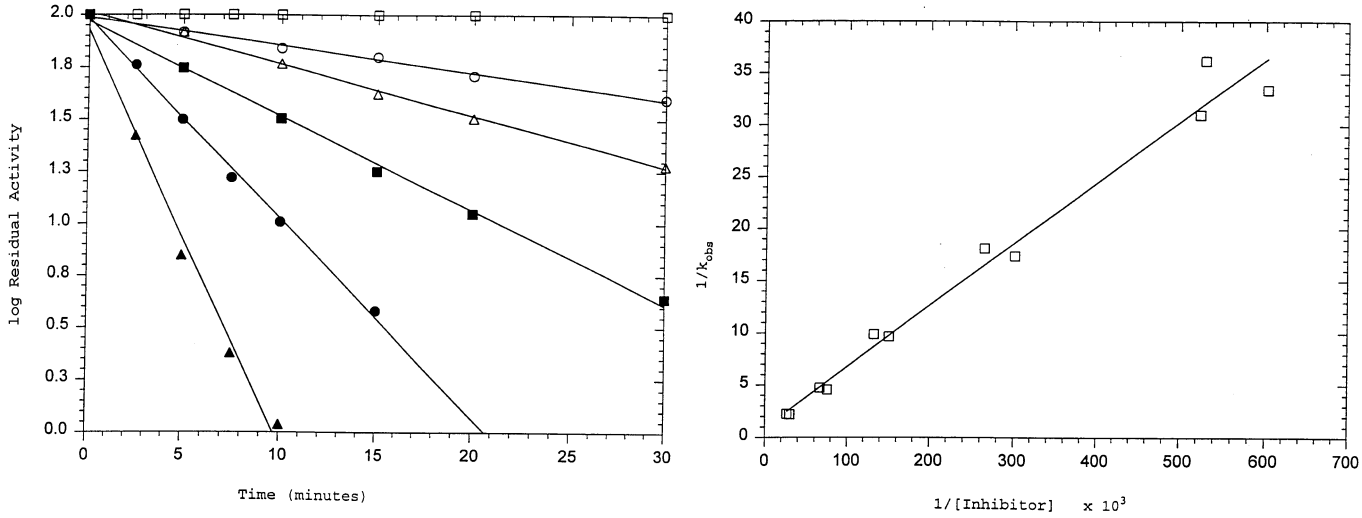


Figure 4. **a.** Time-dependent inhibition of MAO-B by [R]-(-)-N-methyl-N-propargyl-2-phenylpropylamine hydrochloride (**5a**) (typical data from one experiment). Plot of log (% residual activity) against incubation time. All points are the mean of duplicate determinations. Concentrations of **5a** are 0 μM (□); 1.66 μM (○); 3.32 μM (△); 6.64 μM (■); 13.29 μM (●); 33.22 μM (▲). **b.** Plot of $1/k_{obs}$ (data from *figure 4a* plus additional experiments) against 1/[I]. The line is that of best fit by linear regression.

Data regarding the influence of a chiral centre at the carbon atom β to the amino function with respect to either stereoselectivity of inhibition, substrate selectivity or selectivity towards a particular form of the enzyme is sparse. Thus, while the compounds [R]-(-)-noradrenaline and [R]-(-)-adrenaline are selective substrates for MAO-A [20, 21] the opposite enantiomers of the above compounds do not appear to have been studied in detail. The results of studies of the enantiomers of β-phenylethanolamine suggest that the [D]-(-)- enantiomer is a substrate for rat liver mitochondrial MAO-A, whereas both enantiomers are substrates for MAO-B [22]. The racemic form of compounds having a chiral centre at the β-carbon atom and carrying an N-methyl-N-propargyl function have been tested as irreversible inhibitors of MAO-A and MAO-B, but showed only limited ability to differentiate between the two forms of the enzyme [23, 24].

In all the examples quoted above, the substituent at the β-carbon atom has been a polar hydroxyl group. The present work therefore differs from previous studies in that a hydrophobic methyl substituent has been introduced at the β-carbon atom. Further, the results are readily compared with similar studies upon the enantiomers of amphetamine, methamphetamine and selegiline (Deprenyl series of compounds) [10], and of α-methylbenzylamine, N-methyl-α-methylbenzylamine and N-propargyl-N-methyl-α-methylbenzylamine (α-methyl-Pargyline series of compounds) [11].

In considering the competitive inhibition studies, the results reported show very similar trends to those demonstrated in the α-methyl-Pargyline series of compounds. The major difference shown is in affinity, where a higher affinity (greater potency) is shown among the present compounds. However, using the present data as an example, it will be noted that, for any enantiomeric pair of compounds, whether they be primary, secondary or tertiary amines, it is the [R]-configuration enantiomer which is the more potent inhibitor. Further, when comparing the activity of any enantiomeric pair of compounds as inhibitors of MAO-A and MAO-B, the MAO-B form of the enzyme is more sensitive to inhibition (only in the case of compound Va is this observation reversed).

However, within the present series of compounds (and similarly within the Deprenyl and α-methyl-Pargyline series) it should be noted that there are considerable differences in the pK_a values of the tertiary amines relative to the primary and secondary amines. Thus, whereas the pK_a of β-phenylpropylamine is reported as 9.80 at 25 °C [25], and N-methylation will slightly increase the basicity, the introduction of an N-propargyl substituent is known to produce significant base weakening effects of about two pK units [26] (pKa of Deprenyl is reported to be 7.4 at 25 °C [27]. Thus, within the present series, compounds **3** and **4** would be 99% ionised at pH 7.4 whereas compound **5** would be less than 50%

ionised at the same pH value. The active species, either as a substrate or as an inhibitor, of the enzyme MAO is the base form [28–30] and correction of the above K_i values for the concentration of unionised species show that the enantiomers of N-methyl-N-propargyl-β-phenylpropylamine (compounds **5a** and **5b**) have considerably less affinity towards both MAO-A and MAO-B than do the enantiomers of β-phenylpropylamine (compounds **3a** and **3b**) and N-methyl-β-phenylpropylamine (compounds **4a** and **4b**). This is in direct contrast to similar calculations performed on the compounds within the Deprenyl [10] and α-methyl-Pargyline series, in which both [R]-(-)-N-methyl-N-propargyl-1-phenylisopropylamine ([R]-(-)-Deprenyl) and [R]-(+)-N-methyl-N-propargyl-α-methylbenzylamine display greater affinity towards MAO-B than any of the enantiomers of the corresponding primary and secondary amines. Thus, the introduction of a nonpolar asymmetric centre on the carbon atom β to the amino function causes, with respect to competitive inhibition of MAO-B, a considerable loss of affinity and stereoselectivity.

The enantiomeric propargylamine derivatives (compounds **5a** and **5b**) are also capable of irreversible inhibition of both MAO-A and MAO-B and the determined values of k_2 (the first order rate constant for formation of the covalent adduct) are reported in *table I*. As with previous studies, stereoselectivity is negligible or absent while the rate constants are slower than those previously found for the enantiomers of Deprenyl and α-methyl-Pargyline [12].

Thus, while treatment of Parkinson's disease by means of irreversible inhibition of MAO requires the use of inhibitors highly stereoselective towards MAO-B, such stereoselectivity is not attained when employing a nonpolar asymmetric substituent at the carbon atom β to the amino function. This loss of stereoselectivity is accompanied by both a loss of affinity and a reduction in the rate of development of irreversible inhibition. In the absence of significant knowledge of the active site and the surrounding area of MAO-A and MAO-B it is not currently possible to present any logical reason for these findings.

3. Experimental protocols

3.1. Chemistry

Melting points were determined using a Thomas Hoover Unimelt capillary melting point apparatus and are uncorrected. IR spectra were obtained from a Perkin Elmer 1330 Infra-red spectrophotometer and NMR spectra (in CCl_4 solution) from a Varian T60 spectrometer using tetramethylsilane as external standard. Determination of Cl^- was by non-aqueous titration in glacial acetic acid in the presence of mercuric acetate and using perchloric acid in glacial acetic acid as titrant. Analyses of all synthesised compounds were within 0.4% of the theoretical value unless otherwise stated.

3.1.1. (±)-2-phenylpropylamine

(±)-2-Phenylpropionaldehyde (35 g; 0.23 mol) and formamide (75 mL; 1.88 mol) were mixed and the partially immiscible liquids refluxed for 10 h in a flask equipped with a wide necked air condenser topped with a Leibig condenser (ammonium carbonate tends to sublime and to clog the condenser if the air space provided by the air condenser is not available). At the elevated temperature of the reaction the two materials become miscible and the reaction mixture darkens rapidly.

Sodium hydroxide solution (150 mL; 30%) was added and the mixture further refluxed for 15 h after which the solution was steam distilled, the distillate being trapped in HCl solution. The 1 000 mL of distillate was evaporated to dryness under reduced pressure, the residue dissolved in water and the solution made alkaline with ammonia. The mixture was extracted with ether (3×50 mL), the extracts dried (Na_2SO_4), filtered and the ether distilled. The residue was fractionally distilled, the fraction b.p. 106–108 °C at 20 mm (7.2 g) (literature b.p. = 97–98 °C at 19 mm; [31]) being collected as a colourless liquid. I.R. (liquid film) 3 380 and 3 300 cm^{-1} (-NH_2; H-bonding); 1 600, 1 560, 770, 705 cm^{-1} (monosubstituted aromatic) n.m.r. (CCl_4 solution) δ 7.1 (s, 5H, aromatic); 2.66–3.0 (m, 3H, benzylic -CH- and -CH_2-N), 1.86 (broad, 2H, -NH_2, 1.3 (d, J = 6 cycles s^{-1}, C-CH_3).

3.1.2. [R]-(+)-2-Phenylpropylamine (**3a**)

(±)-2-Phenylpropylamine (18 g) was resolved using (+)-tartaric acid using a literature method [13]. After three recrystallisations from methanol, the salt (8.7 g) had m.p. 137–139 °C and $[\alpha]_D^{20}$ = +30.13° (c,4.3, H_2O) (literature $[\alpha]_D^{20}$ = +31.7° (c,4, H_2O) after 23 crystallisations [32]). From the crystalline tartrate salt m.p. 137–139 °C, the base was isolated by dissolving in water, making the solution alkaline with ammonia and extracting with ether. The extracts were dried (Na_2SO_4), filtered and the ether evaporated. The residual oil was distilled, the fraction b.p. 82–84 °C at 8 mm being collected. $[\alpha]_D^{20}$ = +31.9° (c,0.985, EtOH) (literature reports $[\alpha]_D^{22}$ = +35.4° (c,2, EtOH) [32], suggesting a 90% enantiomeric excess in the above product). The hydrochloride salt was prepared by passing dry HCl gas through an

ether solution of the base. The solid product was filtered and recrystallised from ethyl propyl ketone and had m.p. 138.5–139.5 °C. Anal. $C_9H_{14}NCl$ (Cl^-).

3.1.3. [S]-(-)-2-Phenylpropylamine (**3b**)

2-Phenylpropylamine enriched in the [S]-(-)-enantiomer was isolated from the mother liquors from the above reaction. The material was further resolved using (-)-tartaric acid as the resolving acid [13]. Three recrystallisations of the salt from methanol yielded product, m.p. 138–139 °C (8 g) from which the base was isolated, b.p. 75–77 °C at 3 mm, $[\alpha]_D^{20}$ = –33.75° (c,3.5, EtOH) (95% enantiomeric excess based on data of Brode & Raasch [32]). The hydrochloride salt, prepared as reported above for the (+)-enantiomer had m.p. 145–146 °C. Anal. $C_9H_{14}NCl$ (N, Cl^-).

3.1.4. [R]-(+)-N-Methyl-2-Phenylpropylamine (**4a**)

(+)-2-Phenylpropylamine (4.4 g; 0.033 mol) was dissolved in benzene (10 mL) and benzaldehyde (10 mL; 10.4 g; 0.1 mol) was added and the mixture allowed to stand over a Molecular Sieve, type 4A overnight. To the filtered solution was added dimethyl sulphate (10 mL) and the mixture warmed on a steam bath overnight. Water (20 mL) was then added and the mixture refluxed for 4 h. The aqueous phase was separated, the organic phase washed with dilute HCl and the extracts added to the separated aqueous phase. The aqueous solution was made alkaline with ammonia, extracted with ether (3 × 40 mL) and the combined ether extracts dried (Na_2SO_4), filtered and the ether evaporated. The residue was fractionally distilled, the fraction b.p. 80–83 °C at 5 mm (3.3 g) being collected. $[\alpha]_D^{20}$ = +26.5° (c, 4.02, EtOH). Hydrochloride salt m.p. = 143–144 °C after recrystallisation from methyl propyl ketone. I.R. (liquid film) 3 300 cm^{-1} (broad; bonded N-H); 1 600, 1 560, 770, 705 cm^{-1} (aromatic stretching and bending) n.m.r. (CCl_4 solution) δ 6.9 (s, 5H, aromatic); 2.4–3.0, (m, 3H, benzylic -CH- and $-CH_2$-N); 2.1 (s, 3H, N-CH_3); 1.1 (d, *J* = 6 cycles s-1, 3H, C-CH_3).

3.1.5. [S]-(-)-N-Methyl-2-Phenylpropylamine (**4b**)

Similarly prepared but using [S]-(-)-2-Phenylpropylamine (2.5 g). The product amine (1.9 g) had b.p. 81–84 °C at 3.5 mm; $[\alpha]_D^{20}$ = –26.6° (c, 2.4, EtOH). I.R. spectrum identical with that of the [R]-(+)-enantiomer. Hydrochloride salt (recrystallised from methyl propyl ketone) m.p. 146–147 °C. Anal. $C_{10}H_{16}NCl$ (N, Cl^-).

3.1.6. [R]-(-)-N-Methyl-N-propargyl-2-phenylpropylamine (**5a**)

[R]-(+)-N-Methyl-2-phenylpropylamine (2.9 g; 0.019 mol) was dissolved in ethanol (15 mL), K_2CO_3 (3.5 g) added, followed by propargyl bromide solution (2.2 mL of 80% solution in toluene; 2.26 g; 0.02 mol of bromo compound). The solution was refluxed on a steam bath for 20 h.

The mixture was carefully acidified with dilute acetic acid, extracted with ether and the extracts discarded. The solution was made alkaline with ammonia, extracted with ether (3 × 30 mL), the extracts dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The residue was distilled, the fraction b.p. 112–114 °C at 3.5 mm being collected; $[\alpha]^D_{20}$ = –20.32° (c, 3.11, EtOH). Yield 1.5 g. I.R. (liquid film) 3 300 cm^{-1} (C≡C stretch); n.m.r. (CCl_4) δ 6.7 (s, 5H, aromatic); 3.07 (d, *J* = 2 cycles s^{-1}, 2H, -CH_2-C(C); 2.9–2.2 (m, 3H, benzylic -CH- and -CH_2-N); 2.1 (s, 3H, N-CH_3); 1.9 (t, *J* = 2 cycles s^{-1}, 1H, C(CH); 1.15 (d, J = 6 cycles s^{-1}, 3H, C-CH_3). The hydrochloride salt after recrystallisation from methyl propyl ketone had m.p. 128–129 °C. Anal. $C_{13}H_{18}NCl$ (N, Cl^-).

3.1.7. [S]-(+)-N-Methyl-N-propargyl-2-phenylpropylamine (**5b**)

Similarly prepared, but using [S]-(-)-N-methyl-2-phenylpropylamine (1.5 g). The product amine had b.p. 107–112 °C at 3 mm (0.7g) $[\alpha]_D^{20}$ = +15.7° (c, 2.73, EtOH). I.R. (liquid film) identical to that of the R-configuration enantiomer. The hydrochloride salt after recrystallisation from methyl propyl ketone had m.p. 108–113 °C. Anal.: Found N, 5.96; Cl^- 16.51%, 16.49%. $C_{13}H_{18}NCl$ requires N, 6.25; Cl^- 15.84%. [N.B. The above analytical data (N, Cl^- and specific rotation are consistent with the presence of approximately 10% of the starting secondary amine within this product].

3.2. Enzyme studies

Livers of male Sprague-Dawley rats were used as the enzyme source, and preparations of MAO-A and MAO-B were prepared by incubation of the crude mitochondrial enzyme preparation with (-)-Deprenyl and clorgyline respectively [7, 33]. The washed suspension of each form of the enzyme was stored at –20 °C until required. Enzyme activity was determined by radiochemical methods [17, 18]. [^{14}C]-labelled 2-phenylethylamine hydrochloride (50 mCi $mmol^{-1}$) and 5-hydroxytryptamine binoxalate (50 mCi $mmol^{-1}$) were purchased from New England Nuclear, Boston, MA.

3.3. Competitive inhibition studies

Immediately before use, the MAO-B enzyme preparation (5–7 mg protein per mL) were diluted 1 to 10 with phosphate buffer (10 mM; pH 7.4). The MAO-A enzyme

preparation was used undiluted. Within these studies, the inhibitor (50 μL) was incubated at 35 °C for 3 min in the presence of substrate in phosphate buffer (10 mM; pH 7.4) and the enzyme catalysed reaction then initiated by the addition of the enzyme preparation (50 μL). The final volume of the incubation mixture was 300 μL. The reaction was allowed to proceed for 3 min and was stopped by the addition of HCl (200 μL; 2 M). A ten-fold range of substrate concentration was employed and enzyme activity determined in the presence and absence of at least a three fold concentration range of inhibitor. All determinations were carried out in duplicate.

For studies of MAO-B activity and inhibition (PEA as substrate), deaminated products were extracted by addition of toluene (6 mL), the mixture extracted by shaking in a vortex mixer and then centrifuged. A portion (4 mL) of the toluene layer was removed and added to scintillation fluid (5 mL) (prepared from Liquifluor concentrate, New England Nuclear), and containing 0.4% w/v of PPO (2,5-diphenyloxazole) and 0.005% w/v of POPOP (1,4-bis-[2-(4-methyl-5-phenyl-oxazolyl]-benzene in the final toluene solution) and counted in a Beckman LS7500 liquid scintillation counter. All counts were corrected for quenching (counting efficiency 96%).

For studies of MAO-A activity and inhibition (5-HT as substrate), deaminated products were extracted into benzene/ethyl acetate (1:1) (6 mL) and after vortex mixing and centrifuging, the mixture was refrigerated for 4 h at –10 °C. A sample (4 mL) of the organic layer was added to scintillation cocktail (5 mL) and counted as described above.

The data obtained were plotted as Lineweaver-Burk double reciprocal plots, the best straight line being determined by a least squares regression analysis *(figure 3a)*. A re-plot of the gradient of the Lineweaver-Burk plot against inhibitor concentration allowed determination of the K_i from the values of the slope and intercept (y-axis). All such plots were linear, indicating the absence of detectable non-competitive kinetics under the employed conditions (*figures 3a* and *3b*).

3.4. Irreversible inhibition studies

To solutions of the inhibitor prepared in phosphate buffer (10 mM, pH 7.4) (2 mL) was added MAO-B enzyme preparation (500 μL) and the mixture incubated at 35 °C. Aliquots (50 μL) were removed at fixed time intervals up to 30 min (up to 15 min in the case of the more concentrated inhibitor solutions) and immediately added to ^{14}C-labelled phenylethylamine hydrochloride solution (350 μL) (approximately 20 μM in phosphate buffer, 10 mM, pH 7.4) and incubated at 35 °C for 3 min. The enzyme catalysed reaction was stopped by addition of HCl (200 μL, 2 M) and the metabolites extracted and counted as reported above.

Similar studies employing the MAO-A enzyme preparation used inhibitor solution (1 mL) and enzyme preparation (1 mL) and the residual enzyme activity was determined using ^{14}C-labelled 5-hydroxytryptamine as substrate (350 μL; approximately 100 μM).

All determinations were performed in duplicate and at least two independent experiments were performed, each employing 4–5 different concentrations of inhibitor.

Kinetic data derived from the studies of the irreversible phase of the inhibition were interpreted using the method developed by Kitz and Wilson [19]. The apparent first order rate constant for loss of enzyme activity (k_{obs}) was obtained by linear regression of a plot of log (% residual activity) against incubation time (gradient = $-k_{obs}/2.303$) and linear regression of a plot of $1/k_{obs}$ against 1/[I] yielded K_i/k_2 (gradient) [12]. A typical set of experimental data is shown in *figures 4a* and *4b* and the computed results for all inhibition studies are presented in *table I*.

References

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