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Inhibition of monoamine oxidase by the *R* and *S* enantiomers of N[3-(2,4-dichlorophenoxy)propyl]-N-methyl-3-butyn-2-amine

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Summary — The chemical synthesis of the R-(+)- and S-(–)-enantiomers of N[3-(2,4-dichlorophenoxy)propyl]-*N*-methyl-3-butyn-2-amine is described. These compounds are derivatives of the mechanism-based irreversible monoamine oxidase inhibitor clorgyline in which a methyl group is substituted for a hydrogen atom on the propargyl methylene carbon. The enantiomeric clorgyline derivatives were both found to be reversible, linearly-competitive inhibitors of monoamine oxidase-A and -B. Thus the methyl-substitution does not prevent non-covalent binding of the inhibitors to the active sites, but it does prevent these compounds from reacting within that complex to form covalent adducts with the enzymes. The substitution of the methyl group resulted in a decreased affinity, as compared to clorgyline, for non-covalent binding to monoamine oxidase-A, with the *S*-enantiomer having the lower affinity. In contrast, the *S*-enantiomer showed increased affinity for monoamine oxidase-B. These effects resulted in the high selectivity of the parent compound, clorgyline, towards monoamine oxidase-A being lost. The R-enantiomer showed a small degree of selectivity towards the A-form of the enzyme, whereas the S-enantiomer had a higher affinity for the B-form. These results are discussed in terms of the chemical mechanisms proposed for the interactions of the monoamine oxidases with substrates and inhibitors. .

monoamine oxidase / *R* and *S* N-α-methyl clorgyline derivatives / enantioselective inhibition of MAO

Introduction

The mechanism of inhibition of monoamine oxidase (amine: oxygen oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4, MAO) by mechanism-based propargylamine derivatives has been extensively studied [1–5]. By analogy with the cysteinyl flavocyanine adduct at N(5) isolated from purified MAO-B and *N,N*-dimethylpropargylamine [6], flavocyanine adducts of type **1** (fig 1) have been proposed to account for the irreversible inhibition of MAO by the propargylic MAO-inhibitors pargyline [3], *l*-deprenyl [7], and clorgyline [8].

Three mechanisms have been advanced [6] to rationalize the formation by MAO of flavocyanine adducts from propargylic inhibitors: carbanion formation, radical formation or a complete oxidation. The enzyme-catalyzed removal of an α-proton from the propargyl residue to form an intermediate carbanion which could then add to N(5) of the oxidized coenzyme to give, after protonation, the observed adduct is unlikely. Although the pK value of a propargylic proton is lower than that of an α-proton in alkyl and aralkyl amines, such a mechanism has never been shown to occur with classical substrates of MAO. Weyler [9] has recently demonstrated that on incubation of 2-chloro-2-phenylethanamine with MAO-B, 2-chloro-2-phenylacetaldehyde is almost exclusively formed, suggesting that a carbanion is not involved in the oxidation of this amine by MAO-B. By analogy to the mechanism proposed for the oxidation of amines by MAO [4, 10], the two other mechanisms suggested by Maycock *et al* [6] could be unified as depicted in figure 1. Transfer of one electron from the amine to the oxidized form of the flavin would give rise to a flavin radical ($Fl^{\bullet}$)/radical cation (**2**) pair. Removal of an α-proton from **2** to give radical **3** would be much easier than from the unmodified parent propargylamine derivative. Intermediate **3** would transfer a second electron to the flavin semiquinone radical $F1H^{\bullet}$ to give **4** and/or would be in equilibrium with **5**, which by reacting with $F1H^{\bullet}$ would afford the adduct **6**. Michael addition of the reduced flavin ($F1H^{-}$) to **4** would also produce **6**, which would rapidly convert to the flavocyanine adduct **1**.

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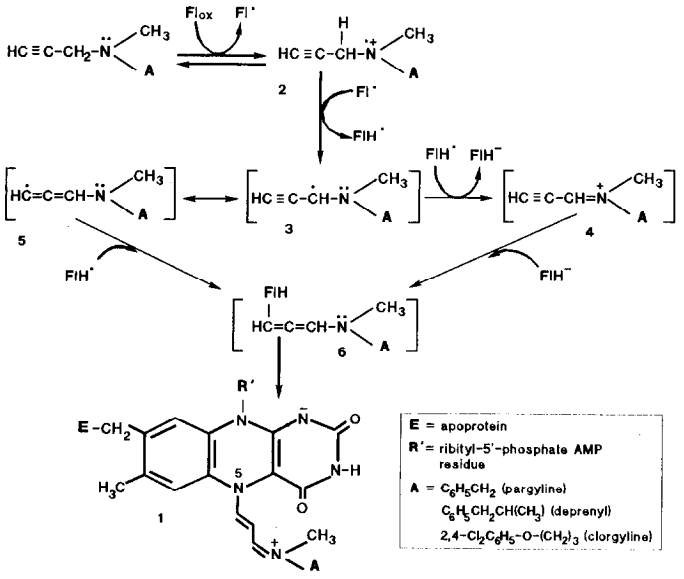


Fig 1. Proposed pathways for the formation of flavocyanine adducts **1** by MAO and propargylamine inhibitors.

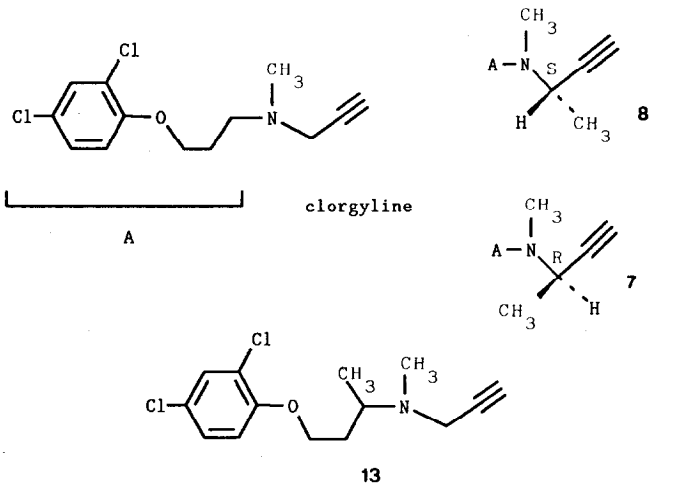


Fig 2. Structures of clorgyline and of compounds **7**, **8** and **13**.

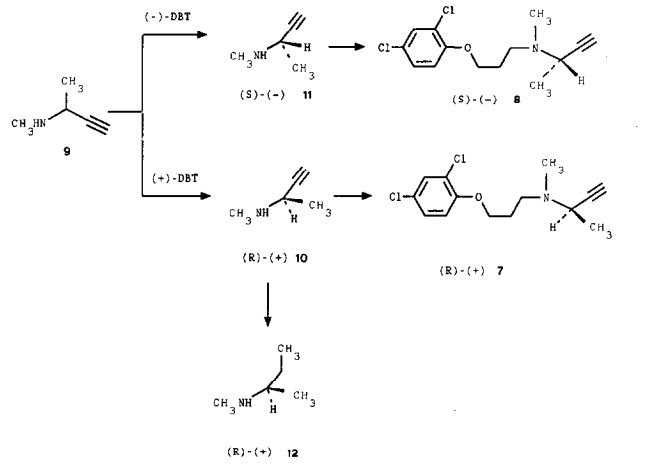
Providing the mechanism-based inhibition of MAO by propargylamine inhibitors occurs according to the general scheme depicted in figure 1, the first two steps would be similar to those proposed for the oxidative deamination of substrates by MAO. Then, it can be asked whether substitution of a methyl group for a hydrogen atom on the propargyl methylene carbon in propargylamine MAO inhibitors would transform these mechanism-based irreversible MAO-inhibitors into reversible inhibitors, similarly to what occurs when amine substrates of MAO are substituted by a methyl group in α-position [11]. In addition, the influence of the spatial disposition of the additional methyl group on the inhibition of MAO, seen with α-methyl aralkylamine derivatives such as (*R*)- and (*S*)-amphetamine [12], could also be studied.

Clorgyline (fig 2) was selected as the parent compound to test this hypothesis. We report here on the synthesis of (*R*)- and (*S*)-*N*[3-(2,4-dichlorophenoxy)propyl]-*N*-methyl-3-butyn-2-amine, **7** and **8** respectively (fig 2), and on the kinetics of inhibition and selectivity of **7** and **8** towards MAO-A and MAO-B as compared with clorgyline.

Chemistry

Racemic *N*-methyl-3-butyn-2-amine **9** (scheme 1) has been previously synthesized [13], in an overall yield of 5%, from commercially available (±) 3-butyn-2-ol according to the following sequence of reactions: 3-butyn-2-amine, prepared from (±) 3-butyn-2-ol [14], was transformed into its *N*-formyl derivative [15], which was reduced with $LiAlH_4$ [13] to give **9**.

We preferred to follow a simpler reaction pathway: (±) 3-butyn-2-ol was transformed into its methylsulfonyl derivative, which, by reaction with methylamine, gave **9** (overall yield: 42%). Separation of the (+) **10** and (–) **11** enantiomers of **9** was achieved by recrystallization of the bibenzoyltartrate salts obtained from (+) and (–) dibenzoyltartaric acid (DBT), respectively (scheme 1). The absolute configuration of **10** and **11** was established by reduction of **10** to (+) *N*-



Scheme 1. Synthesis of compounds **7** and **8**.

methyl-2-butanamine **12**, for which the R configuration had previously been assigned [16]. Reaction of **10** and **11** with 1-bromo-3-(2,4-dichlorophenoxy)-propane [17] gave (*R*)- and (*S*)-*N*[3-(2,4-dichlorophenoxy)propyl]-*N*-methyl-3-butyn-2-amine, **7** and **8**, respectively.

Results and discussion

In order to assess the selectivity and possible time-dependence of inhibition of MAO by compounds **7** and **8**, the extent of inhibition was determined after preincubating enzyme samples with fixed concentrations (range: 10^{-7} to 10^{-4} M) of each inhibitor for either 0 or 30 min at 37°C before the remaining activity was assayed after the addition of either 100 μM 5-HT, for MAO-A, or 10 μM 2-phenylethylamine, for MAO-B. No significant time-dependence inhibition of MAO-A or MAO-B was found with either enantiomer, suggesting that, unlike clorgyline, **7** and **8** do not act as mechanism-based irreversible inhibitors of MAO. The inhibitor concentrations necessary to give 50% inhibition (IC_{50} values) are shown in table I, together with the corresponding values obtained with clorgyline under these conditions. Compounds **7** and **8** were in the forms of their oxalate salts, whereas the clorgyline, which was used for comparison, was obtained as the hydrochloride salt. However, control experiments showed that the presence of oxalate had no effects on the activities of either form of MAO at the concentrations, and under the conditions, used in the present studies.

Time-courses of inhibition were determined by incubating enzyme samples, at 37°C, for times of up to 4 h with sufficient inhibitor to give an initial degree of inhibition within the range 20–36%. The extent of inhibition of MAO-A or -B by either enantiomer did not increase during this time. In some experiments a slight decline in the inhibition given by the *S*-(−)enantiomer was observed when incubation times exceeded 2 h. This was not a result of metabolism of the inhibitor, since control experiments, in which the inhibitor was incubated under identical conditions but in the absence of enzyme before the reaction was initiated by the simultaneous addition of enzyme and substrate (2-phenylethylamine), showed a similar decline in the inhibitory potency of the *S*-(−)-, but not the *R*-(+)-, enantiomer. The magnitude of this effect was found to be variable and it probably reflects precipitation of the *S*-enantiomer.

Table I. IC_{50} values for the inhibition of MAO by clorgyline and by compounds **7** and **8**. Each value represents the mean ± SEM from at least three determinations.

Compound	*Preincubation (min)*	*IC_{50} (μM)*	
		MAO-A	*MAO-B*
Compound **7** [R]	0	8.8 ± 0.7	27.5 ± 1.3
	30	8.8 ± 0.7	27.5 ± 1.3
Compound **8** [S]	0	18.6 ± 1.3	13.0 ± 3.5
	30	18.6 ± 1.3	13.0 ± 3.5
Clorgyline	0	0.016 ± 0.004	21.0 ± 0.6
	30	0.005 ± 0.002	3.6 ± 1.3

During the course of these experiments it was found that lower inhibitor sensitivities were obtained if incubations were performed in 25-ml polypropylene scintillation vials rather than in glass universal vessels. In the former case there was a rapid loss of inhibitory potency with time whichever enantiomer was used. This is illustrated for the inhibition of MAO-A and -B by the *R*-(+)-enantiomer **7** in figure 3. Since there was a similar loss of potency when the inhibitor was incubated in the absence of enzyme, this phenomenon can be attributed to the adsorption of the inhibitor to the polypropylene vessel. Parallel experiments showed that the inhibitory effects of clorgyline were not affected by the nature of the assay vessels. In all experiments reported here, enzyme-inhibitor preincubations were performed in glass vessels. Further experiments (data not shown) showed that stock solutions of these inhibitors could be made up in water at room temperature and stored at − 4°C without any significant decline in inhibitory potency over the course of one day. Neither was there any significant decline in inhibitory potency when samples of the inhibitors were placed in plastic Eppendorf centrifuge tubes at 4°C under the conditions of the experiments to test for reversibility of inhibition.

A procedure involving repetitive dilution and centrifugation of the enzyme sample, after it had been incubated with inhibitor for 30 min at 37°C, was used to test whether inhibition was reversible [18]. Amphetamine, which is a reversible inhibitor with selectivity towards MAO-A [12] was taken through the same procedure with that enzyme. The results shown in figure 4 indicated the inhibition by **7** and **8** proved to be reversible, although a small irreversible component to the inhibition of MAO-A by the *R*-(+)-enantiomer **7** cannot be completely excluded. The reversibility of the inhibition, after enzyme-inhibitor preincubation for 30 min at 37°C was also assessed by dilution, as described by Tipton *et al* [19]. The results obtained (not shown) indicated there to be no significant irreversible inhibition of MAO-A or -B by either enantiomer of the inhibitor.

Kinetic parameters for the reversible inhibition of the two forms of MAO by compounds **7** and **8** were evaluated from initial-rate studies in the presence of different substrate and inhibitor concentrations. The

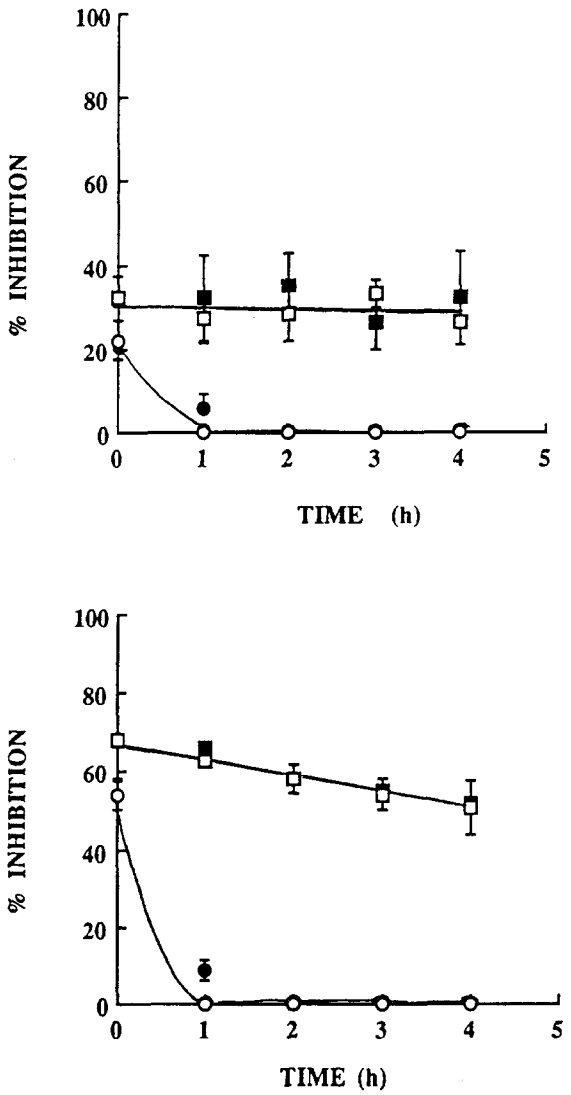


Fig 3. Time-courses of the inhibition of MAO by compound **7** determined in glass (■) and polypropylene (●) reaction vessels. The inhibitor was incubated with enzyme (closed symbols) or with buffer (open symbols) for the times indicated before assay of MAO-A (upper panel) or MAO-B (lower panel) activity. The inhibitor concentration was 5 x 10^{-5} M for the experiments with MAO-B and 6 10^{-6} M for the experiments with MAO-A. Each point represents the mean ± SEM from triplicate determinations.

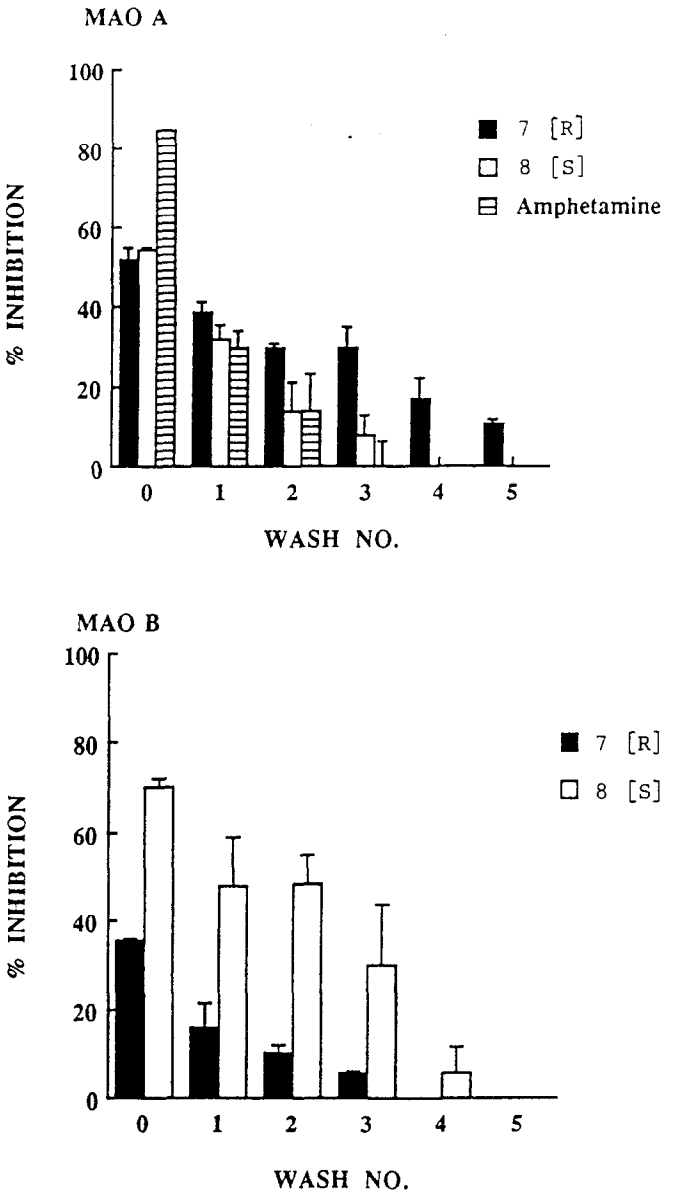


Fig 4. Reversibility of the inhibition of MAO by compounds **7** and **8** and by amphetamine. Preincubation of enzyme and inhibitor followed by centrifugation and resuspension was performed as described in the text. Activities of MAO-A (upper panel) and -B (lower panel) were assayed immediately after preincubation (0) and after each of 5 centrifugation-resuspension cycles (1–5). Each value is the mean ± range from triplicate determinations in two separate experiments.

data were fitted by non-linear regression analysis to allow the type of inhibition and K_i values to be determined. Figure 5 shows representative plots for the inhibition of MAO-A and -B by the *R*-(+)-enantiomer, which are presented as double-reciprocal plots for illustrative purposes.

Inhibition was found to be linearly competitive in all cases. The inhibition constants determined are shown in table II. These values are consistent with the

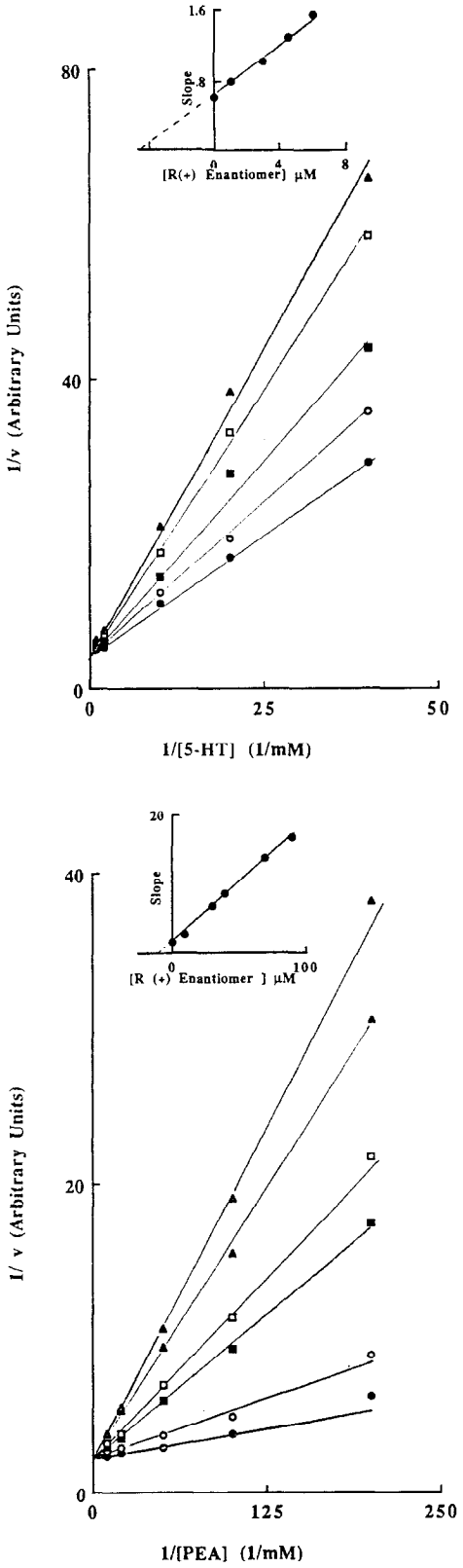


Fig 5. Kinetics of the inhibition of MAO by compound **7**. Upper panel: inhibition of MAO-A by 0 (●), 1 (○), 3 (■), 4.5 (□) and 6 (▲) µM inhibitor. Lower panel: inhibition of MAO-B by 0 (●), 9 (○), 30 (■), 40 (□), 70 (▲) and 90 (△) µM inhibitor. Each point is the mean from triplicate determinations.

Table II. Inhibition constants (K_i) of **7** and **8** toward MAO-A and MAO-B. Values are mean ± SEM from three separate experiments in each of which individual determinations were made in triplicate.

	K_i (µM)	
Compound	*MAO-A*	*MAO-B*
7 [*R*]	4.8 ± 0.7	10.0 ± 0.5
8 [*S*]	6.8 ± 0.7	2.7 ± 0.7

IC_{50} values, given in table I, showing the *S*-(–)-enantiomer (**8**) to be a rather better inhibitor of MAO-B, whereas the *R*-(+)-enantiomer (**7**) inhibits preferentially MAO-A. This behaviour can be compared with the high degree of selectivity towards MAO-A shown by clorgyline. In that case kinetic analysis of the mechanism-based inhibition [20] has shown that the observed selectivity derives from a considerably higher affinity for non-covalent binding to MAO-A, which is further enhanced by a faster rate of reaction within that complex resulting in covalent adduct formation. Thus, the α-methyl substitution not only prevents these compounds from reacting within the initial, non-covalent, enzyme-inhibitor complex to give mechanism-based inhibition but also results in a considerable decrease in the selectivity of non-covalent complex formation. This latter effect is stereospecific with the *R*-(+)- and *S*-(–)-enantiomers showing different preferences.

The effects of the methyl substitution can also be compared with those of amphetamine where the introduction of an α-methyl group in 2-phenylethylamine, a preferred substrate of MAO-B [21, 22], results in the formation of MAO-A selective reversible inhibitors in which the selectivity for that form is much higher with the *S*-(–)-enantiomer [11, 12].

Conclusions

Our investigation has clearly demonstrated that, in keeping with the effect of α-substitution in amine substrates of MAO, substitution of the propargyl residue of clorgyline by a *N*-α-methyl group transforms this mechanism-based irreversible MAO inhibitor into a fully reversible inhibitor. In the case of amine

substrates, however, it is notable that α-methylbenzylamine was found to be deaminated by MAO-B, although at a very low rate compared with benzylamine [23].

The reason why substitution of the α-carbon involved in the oxidation of amine substrates and propargylamine inhibitors by MAO results in a complete, or almost complete, inhibition of the oxidative process remains unknown. It is known that as a carbon atom becomes more alkyl substituted, the pKa of a proton attached to the central carbon atom generally increases. Consequently, the rate of propargyl methylene proton transfer (step **2** → **3**, fig 1) should be decreased by the presence of a methyl group. Electron transfer has been suggested to be the rate-determining step in the electrochemical oxidation of amines [24], although α-methyl substitution in tertiary amines does not appear to greatly influence their oxidation potential [25].

On the basis of the oxidation mechanism suggested in figure 1 for the propargylamine inhibitors of MAO, the resistance of compounds **7** and **8** to oxidation by MAO might result from either their inability to approach the FAD cofactor closely enough for electron transfer or the difficulty of the enzyme to remove the α-proton as a result of steric hindrance by the methyl group (or both). The importance of steric factors in the kinetics of inhibition of MAO by compounds **7** and **8** is questionable, at least for MAO-B. In fact, while the K_i values of both **7** and **8** for MAO-A were found to be about 100 times higher than that reported for clorgyline (0.054 μM) under conditions of thermodynamic equilibrium [20], the K_i values of **7** and **8** towards MAO-B (table II) were about 6 and 20 times lower, respectively, than that of clorgyline (58 μM) [20]. In addition, it is worth noting that the introduction of a *N*-α-methyl group in the propyl residue of clorgyline, compound **13** (fig 2), was shown to result in an increase in potency and selectivity of **13** as an irreversible MAO-A inhibitor as compared with clorgyline [26].

Experimental protocols

Chemistry

Melting points (Büchi 512 apparatus) and boiling points are uncorrected. Proton NMR spectra were recorded on a Varian VXR 200. CHemical shifts are reported as δ values in parts per million relative to tetramethylsilane as an internal standard. Gas chromatographic analyses were obtained using a Fractovap G1 Carlo Erba gas chromatograph equipped with a flame-ionization detector and a 2 m x 4 mm Carbowax 20M column.

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter. Column chromatography was accomplished with Carlo Erba RS silica gel 60 (40–63 μm), and the solvent mixture reported within parentheses was used as eluant. Common reagent-grade chemicals and starting materials were purchased from commercial sources and were used as received. Drying of solvents was performed by storage on 3 Å molecular sieves. Elemental analyses were performed by the analytical laboratory of Farmitalia Carlo Erba and agreed with theorical values within ± 0.4%.

*(±)-N-methyl-3-butyn-2-amine **9***

To a solution of 100 g (1.47 mol) of (±) 3-butyn-2-ol and 298 ml (2.15 mol) of triethylamine in 2.5 l of methylene chloride cooled at – 50°C, 144 ml (1.85 mol) of mesyl chloride was added within 1.5 h under stirring. The solution was allowed to warm to room temperature and stirred for 1 h. The solution was washed twice with water, and then evaporated to dryness to give a quantitative yield of 3-mesyloxy-1-butyne (GC analysis: ≈ 99% chemical purity), which was used for the next step without further purification.

To the solution of 211 g (1.43 mol) of 3-mesyloxy-1-butyne in 600 ml DMF, 1 l 35% methylamine was added and the solution was stirred at room temperature for 16 h. The mixture was distilled using a 40-cm Vigreux column, collecting the distillate up to 95°C. The distillate was poured into 1.5 l Et_2O, carefully dried (Na_2SO_4) and fractionated through a helix-packed column, affording 50.3 g (43%) of **9**, bp 85–89°C ([8] bp = 87–89°C). GC analysis indicated *ca* 98% chemical purity.

*S-(–)-N-methyl-3-butyn-2-amine **11***

To a solution of 67.7 g (0.18 mol) of (–)-dibenzoyl-l-tartaric acid in 800 ml of absolute ethanol, 30 g (0.36 mol) of **9** in 200 ml of absolute ethanol was added. After standing for 24 h at room temperature, the solution was filtered giving 32.4 g of a solid, mp = 145–147°C, $[\alpha]_D^{22}$ – 91.2° (c 1, MeOH). Four recrystallizations from acetone afforded 10 g (20%) of the hemi-(–)-dibenzoyltartrate salt of **11**, mp 151–153°C, $[\alpha]_D^{22}$ – 102.8° (c 1, MeOH), NMR ($(CD_3)_2CO$) δ 1.32 (d, J = 8 Hz, 3H), 2.51 (s, 3H), 2.84 (d, J = 2 Hz, 1H), 3.65 (dq, J = 8 and 2 Hz, H), 5.9 (s, 1H), 7.4–8.2 (m, 5H).

10 g of **11** hemi-(–)-dibenzoyltartrate salt were dissolved in 80 ml of methanol and 10 ml of a 10% HCl solution in ethanol was added. The solution was evaporated to dryness, then taken up in ethanol and evaporated to dryness (twice). The residue was suspended in anhydrous Et_2O, filtered, washed twice with Et_2O to give 4.4 g (100%) of **11** hydrochloride, mp = 180–183°C, $[\alpha]_D^{22}$– 32.6° (c 1, MeOH), NMR ($CDCl_3$) δ 1.75 (d, J = 7 Hz, 3H), 2.61 (d, J = 2 Hz, H), 2.76 (s, 3H), 4.02 (dq, J = 7 and 2 Hz, H). Anal $C_5H_{10}ClN$ (C, H, Cl, N).

The optical purity was ascertained by complexing **9**, HCl and **11**, HCl with 2,2,2-trifluoro-1-(9-anthryl)ethanol (1:10). **9**, HCl gave decoupling of the following signals: HC-CH_3 (δ 1.51 and 1.53), N-CH_3 (δ 2.63 and 2.64), C≡CH (δ 2.46 and 2.47), whereas **11**, HCl gave signals at δ 1.51, 2.64 and 2.47 only. The optical purity of **11**, HCl was superior to 95%.

*R-(+)-N-methyl-3-butyn-2-amine **10***

The mother liquors obtained during the preparation of the hemi-(–)-dibenzoyltartrate salt of **11** were mixed. Treatment with HCl, as described above for the preparation of **11** hydrochloride from the hemidibenzoyltartrate salt, afforded 29 g of a mixture of the hydrochloride salts **10** and **11** enriched in **10**.

These 29 g were dissolved in 250 ml of anhydrous ethanol, treated with the stoichiometric amount of sodium ethylate in 100 ml of ethanol, and the sodium chloride formed was filtered out. Then, the solution of 45.62 g of (+)-dibenzoyl-D-tartaric acid in 500 ml of absolute ethanol was added. After addition of 500 ml of Et_2O, the solution was allowed to stand at room

temperature for 24 h, and then was filtered to give 33 g of a solid, mp = 143–147°C, $[\alpha]_D^{22}$ + 87.4° (c 1, MeOH). Four recrystallizations from acetone afforded 10.85 g (22%) of the hemi-(–)-dibenzoyl-tartrate salt of **10**, mp = 151–153°C, $[\alpha]_D^{22}$ + 104.4° (c 1, MeOH). Treatment with HCl as described above for the *S*-(–)-enantiomer gave 4.72 g of **10** hydrochloride, mp = 183–186°C, $[\alpha]_D^{22}$ + 33.7° (c 1, MeOH). Anal $C_5H_{10}ClN$ (C, H, Cl, N). The optical purity of **10** was determined as described above for the *S*-(–)-enantiomer, and was found to be superior to 95%.

R-(+)-N-methyl-2-butanamine ***12***

A solution of 0.78 g (6.5 mmol) of **10** hydrochloride in 50 ml of absolute ethanol was hydrogenated under 2 atm with 0.15 g of 10% Pd/C as a catalyst. After 2 h, the solution was filtered and evaporated to dryness. The solid residue was tritured with anhydrous Et_2O and filtered to obtain 0.65 g, which after recrystallization from EtOH/Et_2O afforded 0.6 g (75%) of the hydrochloride salt of **12**, mp = 120–124°C (determined in sealed capillary), $[\alpha]_D^{22}$ + 6.2° (c 1.4, EtOH); [11] $[\alpha]_D^{25}$ + 6.4 (c 5, EtOH). Anal $C_5H_{14}ClN$ (C, H, Cl, N).

S-(–)-N[3-(2,4-dichlorophenoxy)propyl]-N-methyl-3-butyn-2-amine ***8***

A mixture of 1.5 g (12.55 mmol) of **11** hydrochloride, 3.57 g (12.55 mmol) of 1-bromo-3-(2,4-dichlorophenoxy)propane [12] and, 4.33 g (31.38 mmol) of K_2CO_3 in 33 ml of DMF was heated at 50°C for 6 h in a stoppered flask and then poured into an excess of water. After extraction with ethyl acetate (3 x 50 ml), the organic solution was dried (Na_2SO_4) and concentrated to dryness. The residue was flash chromatographed (cyclohexane: ethyl acetate 1:1) to afford 1.55 g (43%) of **8** as a colorless oil, which was converted to its oxalate salt. Recrystallization from ethyl acetate gave 1.85 g of the oxalate salt of **8** (1.5 mol of oxalic acid/mol of **8**) as a white solid, mp = 95–57°C, $[\alpha]_D^{22}$ – 13.4° (c 0.5, MeOH), NMR ($(CD_3)_2SO$) δ 1.32 (d, *J* = 7 Hz, 3H), 1.99 (q, *J* = 7.5 Hz, 2H, CH_2-CH_2-CH_2), 2.43 (s, 3H), 2.84 (m, 2H, CH_2-N), 3.49 (d, *J* = 2.2 Hz, H, C≡*CH*), 4.0 (dq, J = 6.9 Hz, *J* = 2.1 Hz, 1H, *C*H-CH_3), 4.10 (t, *J* = 6.0 Hz, 2H, O-CH_2), 7.15, 7.35, 7.55 (3 aromatic H). Anal $C_{17}H_{20}Cl_2NO_7$ (C, H, N, Cl).

R-(+)-N[3-(2,4-dichlorophenoxy)propyl]-N-methyl-3-butyn-2-amine ***7***

Starting from 1 g (8.37 mmol) of **10** hydrochloride and 2.38 g (8.37 mmol) of 1-bromo-3(2,4-dichlorophenoxy)propane as described above for the preparation of **8**, 1 g (43%) of **7** was obtained as a colorless oil, which was converted to 1.2 g of the oxalate salt of **7** (1.5 mol of oxalic acid/mol of **7**) after recrystallization from ethyl acetate, mp = 95–97°C, $[\alpha]_D^{22}$ + 13.7° (c 0.5, MeOH), anal $C_{17}H_{20}Cl_2NO_7$ (C, H, N, Cl).

Enzyme preparation and assay

Rat liver mitochondria were prepared as previously described [27] and assayed for activity radiochemically [28] at 37°C with either 10 μM 2-phenylethylamine hydrochloride (ethyl-1-^{14}C: PEA), for MAO-B activity, or 100 μM 5-hydroxytryptamine creatine sulphate (side-chain-2-^{14}C: 5-HT), for MAO-A activity, as substrate. Assay mixtures contained, in a total volume of 0.5 ml, 60 mM phosphate buffer, pH 7.2, 0.1 mg mitochondrial protein, substrate and, where appropriate, inhibitor. After fixed incubation times the reaction was stopped by the addition of 0.5 ml 2.0 M citric acid and the radioactive products were extracted into 10 ml toluene-ethyl acetate (1:1 v/v), containing 0.6% (w/v) DPO (2,5-diphenyloxazole) and the radioactivity in the organic extract was determined by liquid scintillation counting. In all cases control experiments were carried out to ensure that the extent of product formation was linear with time for the assay period and that the initial velocity was proportional to the concentration of the enzyme sample. Unless otherwise stated all assays were performed with triplicate samples and data are expressed as mean values ± SEM.

Inhibition studies

Extents of inhibition were determined after preincubation of enzyme and inhibitor in the assay mixture at 37°C in the absence of substrate for 0 or 30 min before the reaction was started by the addition of the appropriate substrate. In some experiments the enzyme-inhibitor preincubation period was extended for up to 4 h. Control enzyme samples were incubated for the same periods in the absence of inhibitor and the degree of inhibition is expressed as a percentage of the activity of the appropriate control samples.

Reversibility studies

Enzyme samples were preincubated at 37°C for 30 min before the reversibility of inhibition was assessed either by dilution [19] or by repeated dilution and centrifugation [18]. In the former procedure the enzyme samples were incubated with different concentrations of the inhibitors or with an equal volume of water. The samples were then diluted 10 times into the assay mixture containing substrate. The assay mixtures for the samples that had been incubated in the absence of inhibitor also contained the appropriate inhibitor at a concentration of one tenth of that used in the corresponding enzyme-inhibitor preincubation mixture. Thus the final concentrations of inhibitor were the same in the parallel assay mixtures incubated in the absence and presence of inhibitor. In the case of freely reversible inhibition curves of extent of inhibition against the final inhibitor concentration in the assay mixture would be identical for the samples that had been preincubated with inhibitor and those that had not. Significant divergence of the curves, reflecting a greater inhibition of the preincubated samples, would be observed for an irreversible inhibitor. The dilution-centrifugation procedure involved incubation of enzyme samples with inhibitor for 30 min at 37°C. After assay the remainder of the samples was centrifuged at approximately 14 000 *g* for 10 min and the sediment was resuspended in buffer to the original volume and assayed. This procedure was repeated a further 4 times. Control samples were preincubated in the absence of inhibitor but otherwise treated in the same way. The degree of inhibition at each stage is expressed as a percentage of the activity of the corresponding control samples. In order to compare the results with those given by an inhibitor known to be reversible, a series of experiments was also performed with *S*-(+)-amphetamine. In this case the activity was assayed with 5-HT as substrate, since amphetamine is a more potent inhibitor of MAO-A [12].

Inhibitor kinetics

Initial rates of reaction were determined at a series of substrate concentrations in the absence and presence of 4 or 5 fixed inhibitor concentrations. The data were analysed in terms of the Michaelis-Menten equation by non-linear regression analysis [29, 30] and the inhibitor constants were determined from

the dependence of the apparent K_m values on the inhibitor concentration. The apparent maximum velocity was shown to be independent of inhibitor concentration in all cases, indicating the inhibition to be strictly competitive. Data are presented in the form of double-reciprocal plots for illustrative purposes only.

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