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Bioorganic & Medicinal Chemistry 12 (2004) 273–279
Bioorganic & Medicinal Chemistry

Synthesis of 4-methyl-thio-phenyl-propylamine and the evaluation of its interaction with different amine oxidases

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Received 16 April 2003; accepted 1 October 2003

Abstract—A new molecule, the 4-methyl-thio-phenyl-propylamine ($PrNH_2$) was synthesized and its biological interaction with different amine oxidases such as semicarbazide sensitive amine oxidase (SSAO) [E.C.1.4.3.6], and monoamine oxidase [E.C.1.4.3.4] under its two isoforms, MAO A and MAO B, has been assessed. The substrate specifities of MAO and SSAO overlap to some extent. In this context, the search of new molecules, able to discriminate between these different amine oxidases is very important as it will allow greater elucidation of the SSAO's role in physiological and pathological conditions. We report for the first time, the synthesis and evaluation of a new molecule which has a high affinity towards the SSAO family of enzymes, more so than previously described and furthermore an ability to discriminate between the different amine oxidases.
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1. Introduction

The term semicarbazide sensitive amine oxidase (SSAO) is used to describe a group of enzymes that catalyse the oxidative deamination of aliphatic and aromatic primary amines. They are classificated as E.C.1.4.3.6 (amine: oxygen oxidoreductase (deaminating) (copper-containing), and all of them have 2,4,5-trihydroxyphenylalanine (TPQ) as cofactor. Semicarbazide is frequently used to distinguish the SSAOs from the monoamine oxidase, [amine: oxygen oxidoreductase (deaminating) (flavin-containing), E.C.1.4.3.4; MAO], that contains flavin adenine dinucleotide (FAD) as cofactor and are sensitive to acetylenic inhibitors such as clorgyline and l-deprenyl, but are not affected by semicarbazide. The substrate specificities of MAO-A and SSAO overlap to some extent but, whereas MAO catalyses the oxidative deamination of primary, secondary and tertiary amines, SSAO activity appears to be restricted to primary amines. At present methylamine, which arises from the metabolism of adrenaline, lecithin, sarcosine, and creatinine, is the only physiological substrate known that can discriminate between both amine oxidases. Methylamine is exclusively metabolised by SSAO from many sources[1,2] and does not interact with MAO. Both enzymes catalyse the oxidative deamination of amines to ammonia, hydrogen peroxide and the corresponding aldehyde, according to the overall reaction:

$$RCH_2NH_2 + O_2 + H_2O \Rightarrow +RCHO + NH_3 + H_2O_2$$

SSAO is tightly associated with membranes in several mammalian tissues and also occurs as a soluble form in blood plasma.[3,4] The membrane-bound SSAO shows high activity in vascular tissue and it appears to be associated with smooth muscle cells.[5,6] SSAO activity has been also found in other non-vascular cell types such as chondrocytes,[7] bovine eye,[8] in dental pulp,[9] and in adipocytes from rat white and brown fat.[10] The physiological role of SSAO is still far from clear, despite its wide distribution in mammalian tissues.[11] SSAO located in the plasma membrane may act as a scavenger of circulating toxic amines and in this context, the enzyme present in the microsomal fraction of human and bovine lung[12,13] may be important in inhaled volatile amines metabolism.[14] This enzyme could also be involved in the control of cellular activities through the generation of H_2O_2.[15] It has been recently reported that SSAO co-localizes with GLUT4 glucose transporter in endosomal compartment in rat adipocytes and that SSAO substrates cause a marked stimulation of glucose transport, mimicking the effects of insulin.[16]

Keywords: 4-Methyl-thio-phenyl-propylamine; Synthesis; Biological evaluation; Amine oxidases inhibitor; SSAO; MAO.
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doi:10.1016/j.bmc.2003.10.007

The vascular-adhesion protein VAP-1, present in endothelial cells, has been shown to have an identical amino-acid sequence to SSAO.[17] Because VAP-1 is induced in inflammation and it is involved in lymphocyte migration to lymphoid organs, a new physiological role in cellular trafficking has been proposed for SSAO.[18] In this context, design and synthesis of new compounds that behave as specific SSAO substrates or inhibitors could be useful for discriminating between SSAO and MAO. Furthermore the search of new substrates with high affinity towards SSAO may have therapeutic value in diabetes, due to its importance in glucose transport mimicking insulin effects.

Starting from the 4-methyl-thio-amphetamine (4-MTA), a high selective and reversible MAO-A inhibitor and poor MAO-B inhibitor,[19] we have studied the effect of side-chain enlargement on its amine oxidase inhibitory potency. Here, we report by the first time, the design and synthesis of a new molecule, the 4-methyl-thio-phenylpropylamine ($PrNH_2$), and its biological evaluation as inhibitor or substrate of different amine oxidases.

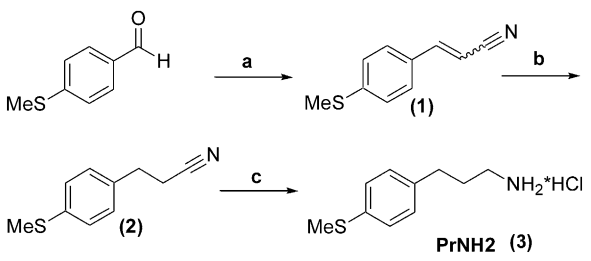
2. Results

2.1. Synthesis

Scheme 1 shows the route used to prepare the novel compound (**3**), starting from 4-methylthiobenzaldehyde, following the method published for the synthesis of *p*-methoxy-cinnamonitrile.[20] The condensation of acetonitrile with the aldehyde, catalyzed by powdered KOH, afforded a 4:1 mixture of (*E*)- and (*Z*)-4-methylthio-cinnamonitrile, which was used without the separation of isomers. Although there are published routes[21,22] for the synthesis of intermediate **1**, this method was chosen for the simplicity and low cost of the route. Direct reduction of compound **1** by $LiAlH_4$ gave none of the desired product, and therefore a two-step sequence using $NaBH_4$ and $LiAlH_4$ successively was used, giving **3** (via **2**) in very good overall yield.

2.2. Chemistry, general procedures

^1H NMR spectra were recorded on a Bruker ARX 300 spectrometer. Chemical shifts are expressed in parts per million downfield from internal Me_4Si as a reference. ^1H NMR data are reported in the order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; number of protons, and approximate coupling constant in Hz).



Scheme 1. (a) KOH, MeCN, reflux; (b) $NaBH_4$, MeOH, py, reflux; (c) (i) $LiAlH_4$, THF, reflux; (ii) HCl, IPA, Et_2O.

Elemental analyses (C, H, N) were carried out using Perkin-Elmer 240 B or 240 C instruments and the results were within ±0.4% of the calculated values. Thin-layer chromatography (TLC) was performed under standard conditions using silica gel 60-F_{254} plates, 0.2 mm thickness (Merck). Abbreviations for the following solvents were used: MeCN, acetonitrile; CH_2Cl_2, dichloromethane; Et_2O, diethyl ether; EtOH, ethanol; IPA, propan-2-ol; MeOH, methanol; py, pyridine; THF, tetrahydrofuran.

2.2.1. 3-(4-Methylthiophenyl)-acrylonitrile (1). To a suspension of KOH (1.5 g, 26.7 mmol; fine powder) in 20 mL of acetonitrile was added 4-methylthiobenzaldehyde (4.0 g, 26.0 mmol). The reaction mixture was heated at reflux for 15 min with magnetic stirring. The mixture was poured onto 50 g of crushed ice and the organic layer separated. After washing with water, the organic layer was removed under reduced pressure resulting in a thick yellow oil, which was purified using bulb to bulb distillation (145–150 °C/0.02 Torr) to give 3.4 g (75% yield) of an *E*/*Z* mixture in a 4:1 ratio (by ^1H NMR analysis). ^1H NMR ($CDCl_3$): δ 7.40 (d, $J = 8.4$ Hz, 2H, Ph-3-H, Ph-5-H), 7.30 (d, $J = 12.6$ Hz, 1H, PhCHCHCN), 7.20 (d, $J = 8.4$ Hz, 2H, Ph-2-H, Ph-6-H), 5.80 (d, $J = 12.6$ Hz, 1H, PhCHCHCN), 2.50 (s, 3H, CH_3S).

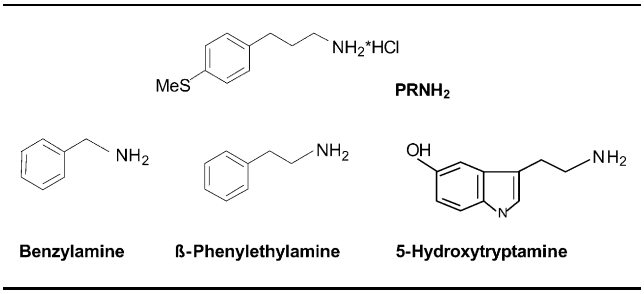
2.2.2. 3-(4-Methylthiophenyl)-propionitrile (2). To a stirred solution of **1** (1.0 g, 5.7 mmol) in 10 mL of pyridine and 3 mL of MeOH was added in portions, $NaBH_4$ (0.22 g, 5.7 mmol). The reaction mixture was heated at reflux for 3 h. After cooling it was poured into 100 mL of 10% v/v HCl in ice water. The solution was extracted with 2 × 50 mL CH_2Cl_2, the organic layer separated and the solvent removed under reduced pressure affording a colorless oil which was purified using bulb to bulb distillation (123–125 °C/0.02 Torr) to give 700 mg (70% yield) of the title compound. ^1H NMR ($CDCl_3$): δ 7.24 (d, $J = 8.4$ Hz, 2H, Ph-3-H, Ph-5-H), 7.15 (d, $J = 8.4$ Hz, 2H, Ph-2-H, Ph-6-H), 2.91 (t, $J = 7.6$ Hz, 2H, $PhCH_2CH_2CN$), 2.60 (t, $J = 7.6$ Hz, 2H, $PhCH_2CH_2CN$), 2.47 (s, 3H, CH_3S). Anal. calcd for $C_{10}H_{11}NS$: C, 67.76; H, 6.25; N, 7.90; S, 18.09. Found: C 68.05; H 6.11; N, 7.84; S, 18.16.

2.2.3. 3-(4-Methylthiophenyl)-propylamine ($PrNH_2$). A solution of 2 (1.0 g, 5.6 mmol) in 5 mL of THF was added dropwise to a suspension of $LiAlH_4$ (0.5 g, 13.0 mmol) in 10 mL of freshly distilled THF. The reaction mixture was heated at reflux for 6 h. The excess $LiAlH_4$ was decomposed by successive addition of 0.5 mL of distilled water, 0.5 mL 15% w/v NaOH and 1.5 mL of distilled water. The cake was filtered and washed with 3 × 10 mL of THF. The solvent was removed under reduced pressure and the residue was purified by bulb to bulb distillation (110–115 °C/0.02 Torr). The resulting oil was crystallized as the hydrochloride in IPA/Et_2O to give 300 mg of white microcrystals (61% yield). ^1H NMR (D_2O): δ 7.29 (d, $J = 8.4$ Hz, 2H, Ph-3-H, Ph-5-H), 7.21 (d, $J = 8.4$ Hz, 2H, Ph-2-H, Ph-6-H), 2.95 (t, $J = 7.6$ Hz, 2H, $PhCH_2CH_2CH_2NH_2$), 2.65 (t, $J = 7.6$ Hz, 2H, $PhCH_2CH_2CH_2NH_2$), 2.45 (s, 3H, CH_3S), 1.88–1.90 (m, 4H, $PhCH_2CH_2CH_2NH_2$). Anal. calcd for $C_{10}H_{15}NS.HCl$: C, 55.16; H, 7.41; N, 6.43; S, 14.73. Found: C 55.15; H 7.39; N, 6.48; S, 14.81.

2.3. Biology

2.3.1. Inhibitory behavior of PrNH2 versus different amine oxidases. MAO-A and MAO-B activities from rat liver mitochondria were assayed at the 0-min pre-incubationin at 37 °C, in the presence of different concentration of 4-methyl-thio-phenyl propylamine ($PrNH_2$) [10^{-8}–10^{-3} M], and the activity remaining was measured against [^{14}C]5-HT, (100 μM), serotonin, a specific MAO-A substrate, and against [^{14}C] PEA (22 μM), a specific MAO-B substrate, respectively (see Table 1). In the case of SSAO, bovine lung microsomes were used, and the activity remaining was determined towards (50 μM) [^{14}C] benzylamine as substrate. Figure 1 shows the inhibition curve of MAO A by $PrNH_2$ as representative of the inhibition curves of the rest of the oxidases. The IC_{50} values were determined from the corresponding inhibition curves and this new molecule showed the highest inhibitory potency towards MAO-A ($IC_{50} = 5.8$ μM), followed by SSAO ($IC_{50} = 38.7$ μM) and the lowest inhibition was observed with MAO-B ($IC_{50} = 175$ μM). $PrNH_2$, showed a high inhibitory selectivity towards MAO-A, expressed in terms of IC_{50} MAO-A/IC_{50} MAO-B (0.033) and a high selectivity towards SSAO compared with MAO-B (IC_{50} SSAO/IC_{50} MAO-B = 0.22). However, these enzymes were more potently inhibited by clorgyline (specific MAO A inhibitor, $IC_{50} = 10^{-8}$ M) and l-deprenyl, (specific MAO B inhibitor, $IC_{50} = 3\times10^{-8}$ M) than by $PrNH_2$, whereas in case of SSAO, it showed similar behaviour towards semicarbazide (specific SSAO inhibitor, $IC_{50} = 10^{-4}$ M) and $PrNH_2$. When the assays were repeated with a previous incubation of 30 min, MAO A and MAO B resulted to be no time-dependent inhibited, whereas in case of SSAO a time-dependent inhibition was observed.

Table 1. Chemical structures of β-phenylethylamine (PEA), 5-OH tryptamine (5HT), benzylamine (Bz), and $PrNH_2$



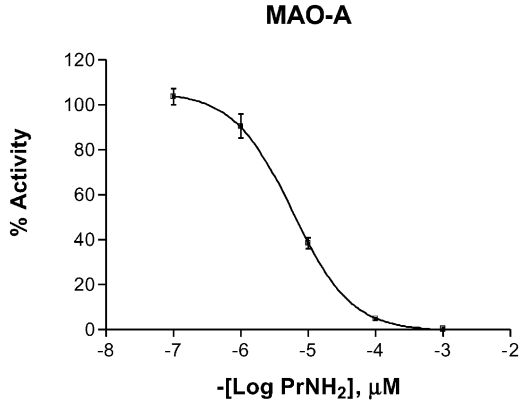


Figure 1. Amine oxidases inhibition by different concentration of $PrNH_2$ by measuring the remaining activity towards 100 μM ^{14}C-5HT (MAO A), 20 μM ^{14}C PEA (MAO B) and 50 μM ^{14}C Benzylamine (SSAO) as substrates. The data are expressed in percentage of control activities determined in absence of $PrNH_2$ as mean ±SD of three independent experiments performed in duplicate with error bar representing the standard deviation.

2.3.2. Kinetic behavior of $PrNH_2$ as substrate of MAO-A, MAO-B and SSAO. Since $PrNH_2$ was an amine, it might potentially behave as a substrate of any amine oxidases. Mitochondria from rat liver that contains both MAO-A and MAO-B, were pretreated by deprenyl 10^{-6} M or clorgyline 10^{-6} M in order to inhibit MAO B and MAO A, respectively, and avoid interferences between them. When $PrNH_2$ (15–500 μM) was assayed as MAO A substrate, identical progress curves were obtained at any $PrNH_2$ concentrations used (data not shown), suggesting that this compound was not a MAO-A substrate. When $PrNH_2$ was assayed as possible MAO-B substrate, the results showed that $PrNH_2$ was oxidized by the enzyme. The kinetic parameters were calculated by non-lineal regression analysis, and they resulted to be $K_m = 91.5$ μM and $V_{max} = 1742$ pmol/min (see Fig. 2). The catalytic efficacy of MAO-B towards $PrNH_2$, expressed as V_{max}/K_m was 19.04. When SSAO bovine lung microsomes[12,13] were assayed, a concentration range of $PrNH_2$ between 1 and 30 μM was used. The kinetic parameters were $K_m = 8.35$ μM $V_{max} = 2669$ pmol/min (see Fig. 3) and the catalytic efficacy was (V_{max}/K_m) 319.6 pmol/min μM. Table 2 shows all the kinetic parameters for $PrNH_2$ in comparison with the common MAO A and MAO B substrates (5HT for MAO A and PEA for MAO B, respectively[23]). Thus, SSAO had the highest activity towards $PrNH_2$ as a substrate, which was oxidised more slowly by MAO B and not at all by MAO A. In this regard, it is worth to remark that $PrNH_2$ resulted to be the best exogenous substrate for SSAO yet discovered, with a $K_m = 8.35$ μM versus benzylamine, the commonly used SSAO substrate ($K_m = 50$ μM). Furthermore, the catalytic efficacy V_{max}/K_m of SSAO versus $PrNH_2$ as substrate, was also higher than for benzylamine as substrate.

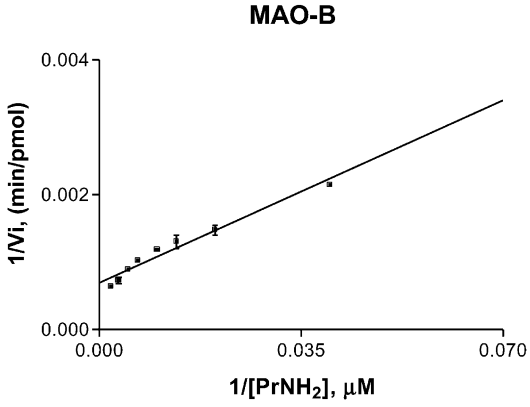


Figure 2. Double reciprocal plot of MAO B activity towards $PrNH_2$ as substrate. The concentration range used was (0–500 μM). MAO B activity was measured as described in the Experimental. Each value is the mean ±SD of two independent experiments performed in triplicate, with error bars representing the standard deviation.

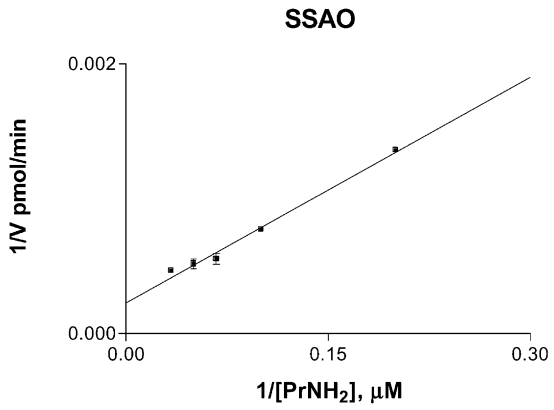


Figure 3. Double reciprocal plot of SSAO activity towards $PrNH_2$ as substrate. The concentration range used was (0–40 μM). SSAO activity was measured as described in the Experimental. Each value is the mean ±SD of two independent experiments performed in triplicate, with error bars representing the standard deviation.

2.3.3. Kinetic behavior of $PrNH_2$ as inhibitor of MAO-A. $PrNH_2$ was a mixed-type inhibitor of rat liver mitochondrial MAO-A, as shown in Figure 4. Plots of $1/v$ versus $PrNH_2$ concentration (Dixon, 1953)[24] (see Fig. 4 insert), confirmed a mixed-type inhibition. The K_{ic} and the K_{iu} values were calculated from the equation $V_{app} = V/1 + i/K_{iu}$ and $K_{mapp} = K_m(1 + i/K_{ic})/1 + i/K_{iu}$. The K_{iu} corresponds to the inhibition constant when the $PrNH_2$ binds to the complex ES, and its value resulted to be 13.35 μM, whereas K_{ic} expresses the inhibition constant of $PrNH_2$ when binds to the free enzyme and showed to be 2.76 μM.

3. Discussion

Several phenylisopropylamines, including amphetamine, have been evaluated as monoamine oxidase inhibitors.[25] Substituents on the aromatic ring of the phenylisoproprylamine molecule (in particular at the *para* position), such as amino,[26] halogens[27] and alkylthio groups,[19] lead to an increase in the potency and selectivity towards MAO-A compared with the parent compound, amphetamine.

In the present work, we have studied the effect of the side-chain rearrangement and elongation of 4-methylthioamphetamine (4-MTA),[19] a high potent MAO-A inhibitor, and highly selective non-neurotoxic serotonin releasing and uptake blocking agent.[28] The IC_{50} values showed that this new molecule, had high potency as an inhibitor of MAO-A. It was less potent as an inhibitor of SSAO and a relatively weak MAO-B inhibitor. The IC_{50} determined at 0 and 30 min pre-incubation time, suggested a reversible inhibitory behaviour in case of both MAO isoforms and a time-dependent inhibition in case of SSAO. $PrNH_2$ had 4 times higher selectivity towards SSAO compared to MAO-B (IC_{50} MAO-B/IC_{50} SSAO). $PrNH_2$ had a 30 times lower inhibitory potency towards MAO-A than 4 MTA ($IC_{50} = 0.2$ μM), whereas the inhibitory potency towards MAO-B[19] was higher. These results suggest that the rearrangement and elongation of the side chain, results in a loss of affinity towards MAO-A, and an increasing affinity towards MAO-B. When $PrNH_2$ was assayed as a possible substrate, it proved to be a very good SSAO substrate followed by MAO-B, however this molecule was not metabolised by MAO-A at all. Considering the kinetic behaviour of $PrNH_2$ as a substrate, SSAO showed 11 times higher activity than MAO-B in terms of K_m values, and 1.5 times higher V_{max} values. Consequently the catalytic efficacy of SSAO towards $PrNH_2$, expressed in terms of V_{max}/K_m was 319.6 versus 19 in the case of MAO-B. When the kinetic behaviour of $PrNH_2$ was assayed as a MAO-A inhibitor, it behaved as a potent and reversible mixed-type inhibitor, indicating that $PrNH_2$ is able to bind whether to the enzyme–substrate complex or to the free enzyme at a different binding site of the active site.

The active sites of different known Cu-amine oxidases are structurally very similar. The TPQ cofactor exhibits different degrees of mobility and it is clearly flexible in all cases. Asp 383 in the active site performs multiple roles in stabilizing the TPQ in an off-copper conformation and assisting substrate binding to TPQ and abstracting the C-H proton from the substrate.[29] Recently, some authors[30] have reported the structure of human MAO-B. The structure of the enzyme shows a flat cavity lined with aromatic and aliphatic aminoacids that constitutes the substrate binding site providing a highly hydrophobic environment. Located between the active site and the protein surface, there is another smaller, hydrophobic cavity. Some residues constitute a loop that separates both cavities and this loop allows the substrate to access to the active site through the small entrance cavity.

Despite these structural differences in the active site of both types of amine oxidases, the two MAO isoforms and SSAO, all of them have in common an ability to recognize hydrophobic structures in their substrates.[31] The present data reported herein on the synthesis and biological evaluation of $PrNH_2$, shows that this mole-

Table 2. Kinetic parameters of MAO A, MAO B and SSAO towards Pr NH_2, PEA, Bz and 5Htas substrates[a]

	Pr NH_2			PEA			5-HT			Bz		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
MAO A							84.2±5.9	5467±249	164			
MAO B	91.5±91	1742±180	19	9.7±0.5	5057±12	522						
SSAO	8.35±76	2669±38	320	312±13	980±99	3.14				40±3	2010±165	50.25

K_m: μM; V_{max}: pmols/min/mg prot.
[a] Each value is the mean ±SD of three independent experiments performed in duplicate.

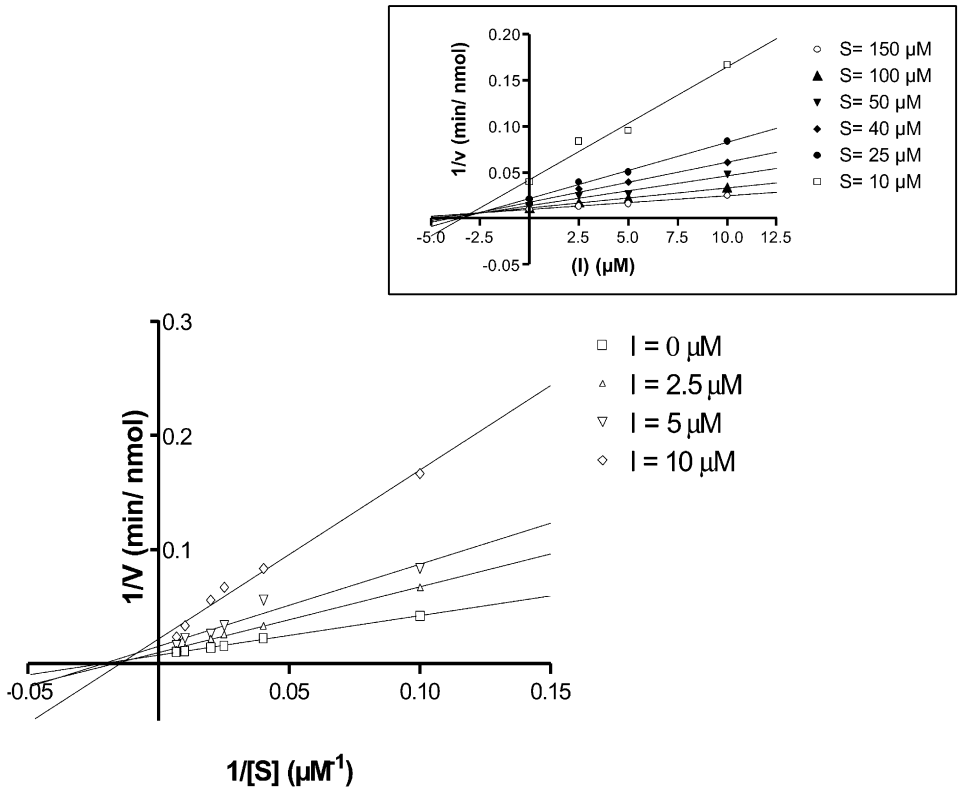


Figure 4. Double reciprocal plot of MAO A inhibition by $PrNH_2$. The $PrNH_2$ concentration range used was (1–10 μM). MAO A activity was measured towards Kynuramine as substrate as stated in the Experimental. Each value is the mean ±SD of two independent experiments performed in triplicate, with error bars representing the standard deviation. Insert: the Dixon plot ($1/v$ versus inhibitor concentration) of MAO A by $PrNH_2$ as inhibitor.

cule is able to interact with different types of amine oxidases. The presence of a *para* methyl-thio group, is responsible for its MAO-A inhibitory behaviour, however the rearrangement of the alpha-methyl group in the linear side chain, results in some loss of its inhibitory potency towards this MAO isoform. On the other hand, its hydrophobicity and the presence of the primary amine group on the side chain, confirm that $PrNH_2$ is a good MAO-B and SSAO substrate. However, this molecule shows better affinity towards SSAO than MAO-B, so it allows discriminating both enzymatic activities, although the amino propynyl side chain probably fits much better in the active site of SSAO than in the MAO-B's. The existence in the human MAO B structure[30] of an entrance cavity, which connects the surface of the protein to the hydrophobic substrate cavity, suggests that $PrNH_2$, could find some structural restrictions in the entry to the first small entrance cavity that could not allow the long side chains to pass to the real active site.

The design and synthesis of new substrates or inhibitors capable to discriminate between different amine oxidases, is very important to elucidate the SSAO role in physiological conditions and to develop new therapies for SSAO related disorders.

4. Experimental

4.1. Materials

The radioactive substrates [7-^{14}C]-benzylamine hydrochloride (specific activity 57 mCi/mmol), (0.2 mCi/mL), the side-chain 2-[^{14}C]-5-hydroxy tryptamine sulphate (serotonin) (55 mCi/mmol) (0.2 mCi/mL) and ethyl-2-[^{14}C phenylethylamine HCl (57 mCi/mmol) (0.2 mCi/mL) were supplied by Amersham International. l-Deprenyl was from Research Biochemical International (R.B.I., USA) and kynuramine dihydrobromide and other common reagents, were from Sigma-Aldrich.

4.2. Preparation of bovine lung microsomes

Bovine lung was obtained from the abattoir after slaughter, packed in ice and transported immediately to the laboratory. After removal of the connective tissue, the lung was weighed, chopped into small pieces with scissors and washed extensively with saline (0.9%, w/v, NaCl) to eliminate blood as a potential source of contaminating plasma amine oxidase. The tissue was then homogenised in 1:10 (w/v) of 20 mM Tris/HCl buffer, pH 7.2, containing 0.25 M sucrose, in a Waring blender, and filtered through two layers of cheesecloth. The homogenate was subjected to differential centrifugation and the microsomal fraction was obtained by adding 10 mM $CaCl_2$ to the post-mitochondrial supernatant and centrifugation as previously described.[32] The final microsomal pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.2, the protein concentration was adjusted to 10 mg/mL and samples were stored in aliquots at −20 °C until required. The resulting preparation, referred to as crude microsomes, contained the SSAO enzyme.

4.3. Preparation of rat liver mitochondria

Rat livers were recovered from male Sprague–Dawley rats (weighing 200–250 g) which had been fasted for 12 h. The liver homogenates were prepared in 10 vol (w/v) of a 50 mM potassium phosphate buffer (pH 7.2) by use of a Dounce homogenizer. The mitochondrial fraction was prepared by a standard differential centrifugation method.[33] The pellets were resuspended in the same buffer and frozen as small aliquots at −20 °C until required.

4.4. Radiochemical assays

SSAO activity in bovine lung microsomes, was determined radiochemically, by the method of Fowler and Tipton,[34] using 25 μL of [^{14}C]-benzylamine (3 mCi/mmol) (50 μM) in the assay mixture. The reaction was carried out at 37 °C in a final volume of 200 μL of 50 mM potassium phosphate buffer, pH 7.2, and the incubation was stopped by the addition of 100 μL of 2 M citric acid. Radioactive-labeled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO) before liquid scintillation counting. In the case of MAO-A and MAO-B, [^{14}C]-serotonin (100 μM) and [^{14}C phenylethylamine (22 μM) were used as specific substrates, respectively.

Selective inhibition of SSAO without affecting MAO-B activity was achieved pre-incubating the enzyme with 1 mM semicarbazide for 30 min at 37 °C before the addition of the substrate. MAO-B activity was selectively inhibited, by preincubating the bovine lung microsomes with 10^{-6} M of l-deprenyl for 30 min at 37 °C, before adding the substrate. Time course assays were used to ensure that initial rates of the reaction were determined and proportionality to enzyme concentration was also established in each case. The IC_{50} values of $PrNH_2$ towards different amine oxidases, were determined after incubating the compound with the enzyme preparation for either 0 or 30 min at 37 °C in the concentration range of 10^{-10}–10^{-3} M. The activities remaining, were then measured against the specific substrates and expressed as percentages of the controls, which have been incubated in the same way but in the absence of $PrNH_2$.

4.5. Coupled-assay method for the assay of $PrNH_2$ as substrate

$PrNH_2$ was studied as MAO-A, MAO-B and SSAO substrates, by the coupled assay method.[36] In the assay, to 808 μL of 0.2 M potassium phosphate buffer, pH 7.6, containing different concentrations of $PrNH_2$, was added 25 μL of the enzyme (final protein concentration 200 μg/mL). After pre-incubation for 5 min at 37 °C, 167 μL of cromogenic mixture containing [1 mL of vanillic acid (10 mM), 0.5 mL of 4-aminoantipyrine (10 mM) and 1 mL of Peroxidase (40 U/mL) in 2.5 mL of potassium phosphate buffer 0.2 M pH 7.6], was added. In this assay, 4-aminoantipyrine is oxidized by the H_2O_2 produced and condensed with vanillic acid to give a red quinoneimine dye. The appearance of product was measured spectrophotometrically at 498 nm and was proportional to the amount of hydrogen peroxide released during the amine oxidase reaction. The extinction coefficient is 4654 M^{-1} cm^{-1}. Kinetic parameters were calculated by linear-regression analysis using the Graph-Pad Prism-3 program. When $PrNH_2$ was assayed as a MAO-B substrate, rat liver mitochondria was previously inhibited by clorgyline (10^{-6} M) in order to leave only MAO-B activity in the assay mixture. In order to assay $PrNH_2$ as a SSAO substrate, MAOB was previously inhibited by preincubation with deprenyl (10^{-6} M).

4.6. Kinetic behavior of $PrNH_2$ as a MAO-A inhibitor

$PrNH_2$ was assayed as a MAO-A inhibitor and the kinetic parameters determined using the spectrophotometric method with kynuramine as substrate in the presence of different $PrNH_2$ concentration (1–10 μM). Spectrophotometric assays for MAO-A activities were performed at 37 °C using kynuramine (40 μM) as substrate, in 50 mM potassium phosphate buffer (pH 7.2) containing 500 μM of mitochondrial preparation in a total volume of 3 mL. The appearance of the product was measured at 324 nm[35] and the absorbance coefficient at this wavelength was 20,000 M^{-1} cm^{-1}. Since kynuramine is a common substrate of both MAO forms, it was necessary to inhibit MAO-B with l-deprenyl (10^{-6} M) to ensure that only MAO-A activity was present.

4.7. Protein quantification

Protein was measured by the method of Bradford,[37] using bovine-serum albumin (BSA) as standard.

Acknowledgements

This work was supported in part by a Grant from the Comisión Interministerial de Ciencia y Tecnología, Ref. SAF 99-0093. The authors would like to thank Mr. Chuck Simmons for revising and correcting the English of this manuscript.

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