Title: PRODRUGS FOR THE SELECTIVE INHIBITION OF MONOAMINE OXIDASE–B

Abstract

Prodrugs targeting cells rich in monoamine oxidase (MAO), such as those found in the central nervous system, have been identified. These compounds are chiral drug or cytotoxic products that have been derivatized with 1–methyl–4–(1,2,3,6–tetrohydropyridinyl)group (THP). Experiments have demonstrated that certain compounds within the class exhibit selective inhibition of brain mitochondrial monoamine oxidase–B.
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PRODRUGS FOR THE SELECTIVE INHIBITION OF MONOAMINE OXIDASE-B

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is generally related to pharmaceuticals used in central nervous system applications, and, more particularly to prodrugs targeted to the brain.

Background Description

The excellent monoamine oxidase-B (MAO-B) substrate properties of the Parkinsonian inducing cyclic tertiary allylamine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Identified as 1 in Reaction Scheme 1 below) have led to extensive structure-enzyme substrate activity studies on a variety of related tetrahydropyridine derivatives. The reaction pathway involves initial \( \alpha \)-carbon oxidation to yield the dihydropyridinium species MPDP\(^+\) (2) which, following subsequent oxidation, is converted to the neurotoxic 1-methyl-4-phenylpyridinium species MPP\(^+\) (3) (Scheme 1, path a). The 4-phenoxy analog, 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine (Identified as 4 in Scheme 1 below), proved to be a better MAO-B substrate than MPTP, but was not toxic \textit{in vivo}, presumably because the intermediary dihydropyridinium metabolite (5) undergoes rapid hydrolysis (Scheme 1, path b) to yield the aminoenone (6) and phenol (7) rather than oxidation to form the putative neurotoxic pyridinium species (8).
Scheme 1. MAO-B Catalyzed Oxidation of MPTP(1) and 1-methyl-4-phenoxo-1,2,3,6-tetrahydropyridine (4).

This behavior suggests the possibility of using the tetrahydropyridyl moiety as a "carrier" for prodrugs. For example, since monoamine oxidase-A (MAO-A) and MAO-B, the two well documented forms of this flavoenzyme system, are localized in specific cell types in the central nervous system, this approach offers the possibility of selectively targeting these cells with tetrahydropyridyl derivatives which would be converted, in an MAO catalyzed reaction, to the corresponding dihydropyridinium species that would release the active drug. A similar concept has been explored with prodrugs constructed by attaching an amine containing drug moiety to a 1-methyl-1,4-dihydropyridine carrier via a carbonyl group at C-3 (See, Simpkins et al., Adv. Drug Deliv. Rev. 14:243-249 (1994)). With reference to reaction scheme 2, oxidation of the dihydropyridine 9 leads to the corresponding pyridinium metabolite 10 which, because of the electropositive character at the amide carbonyl functionality, undergoes rapid hydrolysis to give the N-methylpyridinium analog 11 of nicotinic acid and the drug 12 (R, R' = alkyl or aryl groups).
Scheme 2. Bioactivation of a 1,4-Dihydropyridine Prodrug

Reaction scheme 3 shows the bioactivation pathway for tetrahydropyridyl carbamates. In order to adapt the tetrahydropyridine carrier to construct potential amine containing prodrugs, a carbamate linkage (13) is employed to circumvent the hydrolytic instability of the enamine functionality that results from direct attachment of the amino group and the tetrahydropyridyl carrier. The MAO catalyzed oxidation of (13) generates the dihydropyridinium intermediate (14) which, following 1,4-hydrolytic cleavage and decarboxylation of the resulting carbamic acid (15), releases the amine drug (12). Preliminary studies established that model carbamates (13: R, R' = alkyl or aryl groups) were moderate MAO-B substrates but only with small groups attached to the carbamoyl nitrogen atom (see, Zhao et al., J. Med. Chem. 35:4473-4478 (1992)). Subsequent studies, however, showed that larger groups could be accommodated by the A form of the enzyme (see, Kalgutcar et al., J. Med. Chem. 37:944-949 (1994)).
SUMMARY OF THE INVENTION

It is an object of this invention to provide new, chiral prodrugs and methods for targeted activity against MAO-B.

It is another object of this invention to provide a new, chiral prodrugs which are useful in central nervous system applications and anti-tumor applications.

According to the invention, novel tetrahydropyridyl derivatives of chiral drugs have been synthesized. These drugs fall into two classes. The first class includes tetrahydropyridyl carbamate derivatives where the nitrogen is a primary or secondary amine containing an alkyl moiety with a chiral center. The tetrahydropyridyl carbamate derivative of nordeprenyl has been synthesized. Experiments have demonstrated that under some circumstances the (R)-enantiomer is as effective as (R)-deprenyl in inhibiting brain MAO-B activity with complete retention of the brain MAO-A activity. In vivo experiments demonstrated that the (R)-carbamate provided neuroprotection against MPTP toxicity. The second class includes tetrahydropyridyl phosphorodiamidates.
These compounds were shown to be stable in the presence of MAO-B, but proved to be MAO-A substrates, which makes them useful for the selective release of phosphoramidate mustards in certain applications (e.g., anti-tumor agents).

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:

Figure 1a is a graph showing the linear relationship between the absorbance at 420 nm and the concentration of the synthetic 1-methyl-4-(1-methyl-2-pyrryl)-2,3-dihydropyridinium perchlorate; and

Figure 1b is a graph showing the linear production over time of 1-methyl-4-(1-methyl-2-pyrryl)-2,3-dihydropyridinium metabolite following a thirty minute pre-incubation at 37°C in the presence of mouse brain mitochondrial membranes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

With reference to Formula Set 1, one embodiment of the invention is directed to a novel prodrug which is a tetrahydropyridyl carbamate derivative (R)-(16) of (R)-nordeprynyl ((R)-(17)).
In Formula set 1, THP refers to the 1-methyl-4-(1,2,3,6-tetrahydropyridyl) group. There are known potent and selective MAO-B inactivator properties of (R)-deprenyl ((R)-(18)), a putative neuroprotectant used in the treatment of early stage Parkinson's disease (PD) (see, Calne, N. Engl. J. Med. 14:1021-1027 (1993)). However, concern has been raised recently about the therapeutic advantages of (R)-deprenyl since its use appears to lead to increased mortality in treated PD patients (see, Calne, Brit. Med. Jour. 311:1583-1584 (1995)). The target amine of this invention, (R)-nordeprenyl, also is an effective and selective inactivator of MAO-B which, in the rodent, appears to be about as potent as (R)-deprenyl in vivo (see, Borbe et al. J. Neural Transm. Suppl. 32:131-137 (1990)). The prodrug approach of this invention involving the selective release of the active agent in the central nervous system might help to circumvent mortality problems encountered in PD patients. The (S)-tetrahydropyridyl carbamate derivative (S)-(16) of (S)-nordeprenyl ((S)-17) has also been synthesized and has been used to demonstrate the effect the configuration has on the interactions of the prodrug with MAO-A and MAO-B.

The experiments below demonstrate that the (R) enantiomer of the tetrahydropyridyl carbamate derivative is neuroprotective in vivo. The results
suggests that this invention can be used to produce a wide variety of active
tetrahydropyridyl carbamate derivatives of neurologically active drug
compounds which include chiral centers. Synthesis would proceed in a similar
manner to that described below. The tetrahydropyridyl carbamate derivatives of
this invention would be defined as follows:

\[
\begin{align*}
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{R}_1 \\
\text{R}_2 & \quad \text{N} \\
\text{CH}_3 &
\end{align*}
\]

where the nitrogen can be a primary or secondary amine and where at least one
of \( R_1 \) and \( R_2 \) is an organic moiety containing at least one chiral center. When the
nitrogen is a primary amine, one of \( R_1 \) and \( R_2 \) is a hydrogen. The substitution of
the \( R_1 \) or \( R_2 \) can very widely depending on the drug which is to be delivered.
For example, the drug could include ethers, esters, amino groups, thio groups,
halogens, etc. Furthermore, \( R_1 \) and \( R_2 \) can be connected by an alkyl,
heteroalkyl, aryl, or heteroaryl moiety which encompasses both \( R_1 \) and \( R_2 \) and
the nitrogen atom. Examples of certain drugs within the class contemplated by
this invention include those specified in Formula Set 2.

\text{Formula Set 2}

\[
\begin{align*}
\text{F}_3\text{C} & \quad \text{N}(\text{CH}_3)(\text{THP}) \\
\text{CH}_3 & \quad \text{H} \\
\text{H} & \quad \text{NH}(\text{THP}) \\
\text{Fenfluramine} & \quad \text{Tranylcypromine}
\end{align*}
\]
A second embodiment of this invention contemplates the formation and use of certain chiral tetrahydropyridyl phosphorodiamidates. These compounds are defined by the structure:
where $R_1$ and $R_2$ are amino groups. In particular variations on this embodiment, $R_1$ is $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$ and $R_2$ is either $\text{NH}_2$ or $\text{NHCH}_3\text{C}_6\text{H}_5$.

It will be apparent to those of skill in the art that the 1-methyl-4-(1,2,3,6-tetrahydrophyridinyl) group (THP) could be used to derivatize a wide variety of other chiral drug compounds within the practice of the invention. The THP provides for targeted delivery to tissues rich in MAO, including the brain, the spinal chord, tumors, etc. The Examples below describe the synthesis, enzymatic testing, and in vivo neuroprotection afforded by these compounds. The compounds of this invention can be incorporated into sterile solutions or dispersions for delivery by injection or oral routes, or be incorporated into a powder for preparation of sterile solutions. Alternatively, the compounds may be formulated for aerosol delivery as a dry powder or in combination with suitable CFC or HFC propellants. In addition, transdermal and transmucosal and other delivery routes may also be employed. In liquid form, the carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, etc.), suitable mixtures thereof, or oils (e.g., vegetable oils). The prevention of the action of microorganisms can be achieved using various antibacterial and antifungal
agents, for example, parabens, chlorobuntnol, phenol, sorbic acid, thimerosal, and the like. In some cases isotonic agents could be included such as sugars and sodium chloride.

As a general proposition, the compounds of this invention can be combined with any "pharmaceutically acceptable carrier" including any and all solvents, dispersion media, coatings, antibacteria and antifungal agents, buffers, isotonic agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient (e.g., the THP derivative), its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

EXAMPLE 1

EXPERIMENTAL

Chemistry. (R)-Deprenyl and clorgyline were obtained from Research Biochemicals Inc., Natick, MA. All other chemicals were reagent or HPLC grade. Unless otherwise noted, reactions were run under nitrogen (N₂). Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WP200 or 270 spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Gas chromatography electron-ionization mass spectrometry (GC-EIMS) was performed on a Hewlett-Packard (HP) model 5890 gas chromatograph equipped with an HP-1 fused silica capillary column (12 m x 0.2 mm, 0.33 μm film thickness) connected to an HP 5870 mass selective detector. Data were acquired on an HP 5970 ChemStation. Helium was employed as the carrier gas (40 mL/min) and oven parameters were 100°C for one minute followed by 25°C/min to 275°C. High resolution chemical ionization mass spectrometry (HRCIMS) was performed on a VG 7070 HF instrument. Melting points were
determined with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses, performed by Atlantic Microlabs of Norcross, GA, were within 0.4% of the theoretical values calculated for C, H and N.

Reaction Scheme 4 sets forth the synthetic route used to synthesize the tetrahydropyridyl carbamates (R)-(16) and (S)-(16). It should be understood by those skilled in the art that the prodrug (R)-(16) of this invention could be made by other synthetic pathways, and that the quantities of reactants used in the synthesis procedures set forth below are for laboratory purposes, and can vary considerably depending on production scale-up or the like.

Scheme 4. Synthetic Route to the Tetrahydropyridyl Carbamates

(R)-(16) and (S)-(16)

In Reaction Scheme 4 (a) is (ClCO)₂ in CH₂Cl₂; (b) is 4-hydroxypyridine in CH₃CN containing Reillex 402; (c) is CH₃I in Et₂O; and (d) is NaBH₄ in CH₃OH.
(R)-N-(1-Methyl-2-phenylethyl)-N-propargylcarbamoyl Chloride
((R)-(19)).

To a solution of (R)-nordeprenyl ((R)-(17), 1.90 g, 11.0 mmol] (see, MacGregor et al., *J. Labelled Compd. Radiopharm.* 25:1-9 (1988)) and triethylamine (1.67 g, 16.5 mmol) in 40 mL of CH₂Cl₂ was added dropwise triphosgene (1.41 g, 4.75 mmol) in 20 mL of CH₂Cl₂ at 0°C with stirring. After stirring at 0°C for 2.5 hr, the reaction mixture was washed sequentially with cold aqueous 10% HCl, cold aqueous NaHCO₃ and cold saturated NaCl salt solution. The crude product obtained after drying (MgSO₄) and removing solvent was filtered through a column filled with 30 g of Fluosil® with 250 mL of CH₂Cl₂ to give 2.45 g (95%) of the carbamoyl chloride (R)-(19) as an oil. "H NMR (CDCl₃) δ 7.26 (m, 5H, ArH), 4.50 (2q, 1H, N-CH₂), 4.04 (m, 2H, CH₂ of benzyl), 3.95 (2m, 2H, NH-CH₂), 2.30 (t, 1H, alkyne), 1.56 (bs, 1H, NH₂), 1.35 (d, 3H, CH₃). The (S)-enantiomer (S)-(19) was prepared in the same way.

4-Pyridyl (R)-N-(1-Methyl-2-phenylethyl)-N-propargylcarbamate
((R)-(20)).

The above crude carbamoyl chloride (2.42 g, 10.27 mmol) in 25 mL of acetonitrile was added dropwise to a mixture of 4-hydroxypyridine (0.92 g, 9.76 mmol) and Reillex 402 (9.2 g, 8.1 meq) in 50 mL of refluxing acetonitrile with vigorous stirring. After an additional 6.5 hr, the mixture was filtered, the solvent removed under reduced pressure and the resulting oil was chromatographed (silica gel, 40 g, eluent: ethyl acetate) to yield (R)-(20) (2.01 g, 70.0%): GC-EIMS m/z (%) 294 (M⁺, 1), 203 (100), 91 (65), 78 (26), tᵣ = 7.52 min; "H NMR (CDCl₃) δ 8.55 (dd, 2H, C2 and C6), 7.21-7.29 (m, 5H, Ph-H), 6.90 and 7.08 (dd, 2H, C3 and C5), 4.43 (m, 1H, N-CH₂), 4.00 (m, 2H, Ph-CH₂), 2.93 (m, 2H, N-CH₂), 2.04 (s, 1H, Propargyl-H), 1.35 and 1.44 (dd, 3H, CH₃) HRCIMS: calcd. for (C₁₈H₁₄N₂O₂)H⁺ 295.144653, found 295.1444684. The (S)-enantiomer, (S)-(20) was prepared in the same way.
(R)-1-Methyl-4-[N-(1-methyl-2-phenylethyl)-N-propargylcarbamoyloxy] pyridinium Iodide (R)-(21).

Iodomethane (2.70 g, 1.90 mmol) was added dropwise to a solution of (R)-(20) (1.40 g, 4.75 mmol) in 50 mL of dry diethylether (Et₂O). The reaction mixture was heated under reflux for 3 days and then the supernatant was decanted off to yield the pyridinium methiodide (R)-(21) (1.59 g, 76.8%) as a light yellow, solid which was too hygroscopic to obtain a melting point: ¹H NMR (DMSO-d₆): δ 8.89 and 8.94 (dd, 2H, C2 and C6), 7.58 and 7.79 (dd, 2H, C3 and C5), 7.23-7.29 (m, 5H, Ph-H), 4.25 (d, 3H, N'-CH₃), 3.28-3.37 (m, 3H, Ph-CH₂ and N-CH), 2.91 (m, 2H, N-CH₂), 2.49, (s, 1H, Propargyl-H), 1.28 and 1.36 (dd, 3H, CH₃). HRCIMS: calcd. For (C₁₉H₂₁N₂O₄I - CH₃)⁺ 295.14468, found 295.145599. The (S)-enantiomer, (S)-(21), was prepared in the same way.

Oxalate Salt of 4-(1,2,3,6-Tetrahydropyridyl)-(R)-N-(1-Methyl-2-phenyl) ethyl-N-propargylcarbamate (R)-(16).

The above pyridinium methiodide (1.59 g, 3.65 mmol) in 45 mL of dry MeOH was treated portionwise with stirring at 0°C with NaBH₄ (0.21g, 5.47 mmol). The reaction mixture was stirred for an additional 25 min at 0°C, the solvent was removed and the residue was treated with 15 mL of cold H₂O and extracted twice with ethyl acetate to obtain 1.4 g of crude (R)-(16): GC-EIMS m/z (%) 312 (M⁺, 2), 119 (46), 112 (27), 91 (100), tᵣ = 7.65 min. The corresponding oxalate salt, prepared in Et₂O, was recrystallized from acetonitrile/ether to yield 1.14 g (77.6%) of a hygroscopic solid: mp 114-115°C; ¹H NMR (DMSO-d₆) δ 7.18-7.30 (m, 5H, Ph-H), 5.16 and 5.33 (unresolved, 1H, C5), 4.24 (m, 1H, N-CH), 4.00 and 4.07 (d, 2H, Ph-CH₂), 3.61 (unresolved, 2H, C6), 3.18 (unresolved, 2H, C2), 2.81 (m, 2H, N-CH₂), 2.70 (s, 3H, N'-CH₃), 2.33 (unresolved, 2H, C3), 2.15 (d, 1H, propargyl-H), 1.19 and 1.25 (dd, 3H, CH₃). Anal. (C₂₁H₂₉N₂O₄) C, H, N. The (S)-enantiomer, (S)-(16), was prepared in the same way: mp 112-113°C. Anal. (C₂₁H₂₉N₂O₄) C, H, N.
The elemental analysis for the synthesized experimental test compounds is as follows:
Oxalate Salt of 4-(1,2,3,6-Tetrahydropyridyl)-(R)-N-(1-Methyl-2-phenyl) ethyl-N-propargylcarbamate (R)-(16).

Calculated for C_{21}H_{26}N_{2}O_{6}:  C 62.67, H 6.51, N 6.96. Found:  C 62.66, H 6.55, N 6.93.

Oxalate Salt of 4-(1,2,3,6-Tetrahydropyridyl)-(S)-N-(1-Methyl-2-phenyl) ethyl-N-propargylcarbamate (S)-(16).

Calculated for C_{21}H_{26}N_{2}O_{6}.0.2H_{2}O:  C 62.10, H 6.50, N 6.90. Found:  C 61.97, H 6.46, N 6.87

**Enzymology.** MAO-A and MAO-B were prepared from human placenta and bovine liver, respectively, according to the method of Salach (Salach, *Meth. in Enzymol.*, 142:627-637 (1987)) with the following variations.

The phospholipase A was obtained commercially (Sigma, St. Louis, MO) rather than from the crude venom. The MAO-A preparation was not subjected to the sephadex purification step and the MAO-B preparation was not subjected to the gradient purification step. The activity of the MAO-B isozyme was determined by measuring the initial rate of formation of MPDP* (2-Scheme 1, λ_{max} 343, ε=16,000 M^{-1} cm^{-1}) from 5 mM MPTP (1-Scheme 1) at 30°C and was based on the reported k_{cat} (204 min^{-1}) for this reaction (see, Kalgutkar, *J. Med. Chem.* 37:944-949 (1994)). The final enzyme concentration was 9 nmol/mL and the preparation was stable when stored at -15°C over the period of this study.

The specific MAO-A activity was estimated at 37°C by measuring the initial rate of oxidation of 1 mM 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine (4-Scheme 1) to its dihydropyridinium metabolite (5-Scheme 1), (k_{cat} 130 min^{-1}; see Wang et al., *J. Med. Chem.* 38:1904-1910 (1995)), by monitoring the aminoenone hydrolysis product (6-Scheme 1) (324 nm, ε = 15300 M^{-1}·cm^{-1}; see, Rimoldi, *Chem. Res. Toxicol.* 8:703-710 (1995)). This MAO-A enzyme
preparation was not stable, therefore, the activity was monitored on a bimonthly basis and the appropriate estimated activities (17 to 8 nmol/mL) were used in the calculations. Due to the viscosity of the MAO-A preparation, it was diluted with 3 volumes of phosphate buffer just prior to analysis.

Enzyme studies on the tetrahydropyridyl carbamates (R)-(16) and (S)-(16) and (R)- and (S)-nordepeneryl were carried out at 37°C. The MAO-B inactivation properties of the nordepeneryl enantiomers were examined as follows: Aliquots (50 μL) of stock solutions in 100 mM phosphate buffer, pH 7.4, were mixed with 50 μL of the 9.0 μM MAO-B preparation to yield inhibitor concentrations ranging from 10 to 100 μM. The resulting mixtures were incubated with gentle agitation in a water bath. At specific time points 10 μL aliquots of each incubation mixture were added to a 1 mL quartz cuvette containing 490 μL of 5 mM MPTP (pre-equilibrated to 37°C in 100 mM sodium phosphate buffer, pH 7.4). The initial rates of MPTP oxidation were determined by monitoring the absorbance of MPDP+ for 120 seconds (see. Rimoldi, Chem. Res. Toxicol. 8:703-710 (1995)). The MAO-A inactivation properties of the nordepeneryl enantiomers were examined in a similar manner except that the rates of oxidation of the 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine (4-Scheme 1) were used to monitor remaining enzyme activity.

To evaluate the MAO-A and B substrate properties of the (S)- and (R)-tetrahydropyridyl carbamates, incubations were carried out in 500 μL of MAO-A (0.17 to 0.08 μM) or MAO-B (0.09 μM) and a range of substrate concentrations (0.25 - 2.0 mM in 100 mM sodium phosphate buffer, pH 7.4) in a 1 mL quartz cuvette. The initial rates of oxidation of these compounds were estimated by monitoring the absorbance of the aminoenone (6-Scheme 1) at 324 nm because the intermediate carbamoyloxydihydropyridinium species were too unstable to be detected. No evidence of pyridinium (λ_max=227nm) was observed. The k_cat and K_M values were calculated from plots of 1/initial velocity vs 1/[Substrate].
Preparation of Mouse Brain Mitochondrial Membranes. ICR white mice (control animals or animals treated as described below) were sacrificed by cervical dislocation, decapitated and the whole brains were removed. Each brain was homogenized in 10 volumes of a solution 250 mM in sucrose and 10 mM in phosphate buffer, pH = 7.4. Cellular debris was pelleted by centrifugation for 10 minutes at 600g. The mitochondria were pelleted from the decanted supernatant at 6,500g for 20 minutes. This pellet was resuspended in 5 mL of sucrose buffer and again centrifuged at 6,500g for 20 minutes. After removal of the supernatant fraction, the mitochondria were lysed in 5 mL of ice-cold water using a glass and teflon homogenizer. This homogenate was centrifuged at 105,000g for 30 minutes and the supernatant was removed. The resulting pellet was resuspended in 5 mL of ice-cold water, homogenized and centrifuged at 105,000g for 30 minutes. The supernatant was again removed and the remaining pellet was homogenized in 1 mL of 10 mM phosphate buffer, pH = 7.4, to yield a preparation with a protein concentration of approximately 2 mg/mL. Protein concentrations were determined on the lysed mitochondrial homogenate by the Coomassie Brilliant Blue dye binding method (see, Bradford, *Analyt. Biochem.* 72:247-254 (1976)). All points in the standard curve were determined in triplicate and sample assays were performed in duplicate.

**Determination of Mitochondrial MAO Activity.** Aliquots of the above preparation (150 µL) containing either no inhibitor (control), $3 \times 10^{-8}$ M clorgyline, $3 \times 10^{-7}$ M deprenyl or clorgyline plus deprenyl were preincubated for 30 minutes following which 100 µL of a 5 mM solution of 4-((1-methyl-2-pyrrol)-1-methyl-1,2,3,6-tetrahydropyridinium hydrochloride (22-Scheme 5; see, Bai, Synthetic and Metabolic Studies on 1-Methyl-4-(1-methylpyrrol-2-yl) -1,2,3,6-tetrahydropyridine, a Neurotoxic Analog of the Parkinsonian Inducing Agent MPTP, M.S. Thesis, 1991, Blacksburg, VA) in phosphate buffer was added.
Scheme 5. Reaction of (22) in MAO Assay

The incubation was continued for an additional 30 minutes and then the reaction was quenched by the addition of 250 μL of 5% acetic acid in acetonitrile. The denatured protein was removed by centrifugation at 16,000g for 10 minutes. The absorption of the supernatant fraction containing the dihydropyridinium metabolite (23-Scheme 5) was read at 420 nm with a Beckman DU-50 spectrophotometer against a blank consisting of homogenate in phosphate buffer denatured with 5% acetic acid in acetonitrile (1/1 v/v). Assays were performed in duplicate.

Animal Studies. ICR male mice (25-35 g, Harlan, Dublin, VA) were housed 1-6 per cage in the Laboratory Animal Resource facility at 21-23°C with free access to standard laboratory chow and tap water on a 12 hour day/night cycle. All compounds were dissolved in sterile saline and injections were administered intraperitoneally in a volume of 100 μL.

(S)-Tetrahydropyridyl Carbamate Studies. Three protocols (each with n = 3) were employed: (1) Untreated negative control, (2) (R)-deprenyl treated positive control [saline on day 1 and 2 and 11 μmoles (R)-(18)/kg on day 3] and (3) drug treated [25 μmoles (S)-(16)/kg-day for three days]. All mice
were sacrificed on day 4, 24 hr after the last injection.

(R)-Tetrahydropyridyl Carbamate Studies. Four protocols (each with \( n = 6 \)) were employed: (1) Untreated negative control, (2) (R)-deprenyl treated positive control [saline on day 1 and 2 and 11 \( \mu \)moles (R)-(18)/kg on day 3], (3) (R)-nordeprenyl treated [120 \( \mu \)moles (R)-(17)/kg-day for 3 days], and (4) drug treated [124 \( \mu \)moles (R)-(16)/kg-day for 3 days]. All mice were sacrificed on day 4, 24 hr after the last injection.

Results

Chemistry. The syntheses of (R)-nordeprenyl, (R)-(17), and (S)-nordeprenyl, (S)-(17) were readily accomplished using established procedures by N-propargylation of (R)- and (S)-amphetamine (see, MacGregor et al., *J. Labelled Compd. Radiopharm.* 25:1-9 (1988)). With reference to Scheme 4 above, treatment of these secondary amines with triphosgene gave the corresponding chloroformates (R)-(19) and (S)-(19) which were converted to the pyridyl carbamates (R)-(20) and (S)-(20) by reaction with 4-hydroxypyridine. The HCl liberated in these reactions was neutralized effectively by the polyvinylpyridine acid sponge Reillex 402. Subsequent treatment of (R)-(20) and (S)-(20) with iodomethane gave the pyridinium methiodides (R)-(21) and (S)-(21) which were converted to the desired tetrahydropyridyl carbamates (R)-(16) and (S)-(16) with NaBH\(_4\) reduction.

Enzymology. Initial studies focused on characterizing the *in vitro* MAO-A and MAO-B inactivating properties of the (R)- and (S)-propargylamines (R)-(17) and (S)-(17). The time and inhibitor concentration dependent loss of MAO-A activity was examined with 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine (4) as substrate. MPTP (1) served as substrate for the MAO-B inactivation studies. (R)-nordeprenyl displayed excellent MAO-B inactivation properties but estimates of \( k_{\text{inact}} \) and \( K_i \) could not be obtained since the rates of inactivation even at low inhibitor concentrations (10 \( \mu \)M) were too fast to measure. These
results are in agreement with other studies which have shown that
(R)-noradrenyl is a potent MAO-B inhibitor (see, Borbe et al., \textit{J. Neural}
Transm. Suppl. 32:131-137(1990)). (S)-Noradrenyl, (S)-(17) also inactivated
MAO-B in a time dependent process, but was considerably less potent than the
(R)-enantiomer. Although linear plots of the natural log of the percent
remaining enzyme activity vs time were obtained at inhibitor concentrations of
10 to 50 \(\mu\)M, the plot of \(1/k_{obs}\) vs \(1/\text{[inhibitor]}\) did not yield Michaelis-Menten
values, possibly because the rates that could be estimated were below \(K_i\). In
contrast to the MAO-B inhibiting properties of these noradrenyl isomers, no
evidence of MAO-A inhibition was observed with either enantiomer even at high
(1 mM) concentrations of (S)-(17) and (R)-(17).

The MAO-A and MAO-B substrate properties of the (R)- and
(S)-carbamates at 1 mM were examined spectrophotometrically by recording
repeated scans (500 to 250 nm) over a 120 minute incubation period. No new
chromophore was detected during the incubations with MAO-B. This behavior
is consistent with previous results showing the lack of MAO-B substrate
properties of related tetrahydropyridyl carbamates (see, Zhao et al., \textit{J. Med.}
Chem. 35:4473-4478(1992)). The spectral analysis of the MAO-A incubation
mixtures documented the time dependent appearance of a chromophore
corresponding to the aminoenone (6-Scheme 1) indicating that both (R)-(16)
and (S)-(16) are substrates for this form of the enzyme. Double reciprocal plots
of \(1/\text{initial rates of oxidation vs } 1/\text{[substrate]}\) provided estimates of \(k_{cat}\) (88 and
36 min\(^{-1}\)) and \(K_M\) (0.3 mM in both cases) for the (R)- and (S)-enantiomers,
respectively. The corresponding \(k_{cat}/K_M\) ratios [295 min\(^{-1}\)mM\(^{-1}\) for (R)-(16) and
120 min\(^{-1}\)mM\(^{-1}\) for (S)-(16)] established that these compounds are moderate
MAO-A substrates.

\textit{In vivo} Studies. Since (R)-noradrenyl displayed potent MAO-B
inhibition properties, we speculated that the MAO-A catalyzed bioactivation of
(R)-(16) would lead to the inhibition of MAO-B \textit{in vivo}. Furthermore, since
MAO-A is not inactivated by (R)-nordepenryl, the inhibition should be selective for the B form of the enzyme. The poorer MAO-B inhibitor properties of (S)-nordepenryl and the poorer substrate properties of (S)-(16) suggested that the (S)-carbamate would be a less effective inhibitor of MAO-B than the (R)-carbamate.

Characterization of the in vivo enzyme inhibitor properties of (R)-(16) and (S)-(16) required an assay that would provide estimates of changes in brain MAO-A and MAO-B activities in drug treated animals relative to control animals. Several ex vivo assays for brain MAO activity have been reported in the literature (Clark et al., J. Biol. Chem. 245:4724-4731 (1970), Hall et al., Comm. Biochem. Physiol. 1:107-110 (1982), and Yu et al., J. Med. Chem. 20:3705-3713 (1992)) and the subject has been reviewed (see, Tipton et al., Methods in Biogenic Amine Research 20:441-465(1983)). In this investigation, a spectrophotometric assay was developed that exploited the excellent substrate properties ($k_{cat}/K_M = 1625 \text{ min}^{-1}\text{mM}^{-1}$ for MAO-A and $k_{cat}/K_M = 1800 \text{ min}^{-1}\text{mM}^{-1}$ for MAO-B) of 1-methyl-4-(1-methyl-2-pyrryl)-1,2,3,6-tetrahydropyridine (22-Scheme 5). The dihydropyridinium metabolite (23) formed in this reaction is stable, has a high $\epsilon$ value (25000 M$^{-1}$) and absorbs maximally at 420 nm, a wavelength far removed from biological background absorbances.

Mitochondria were isolated by differential centrifugation from brain homogenates and were lysed in chilled water (see, Yu, "Monamine Oxidase", in Neuromethods 5: Neurotransmitter Enzymes, A.A. Boulton, G. B. Baker; P. H. Yu, Eds. Humana Press, Clifton, New Jersey, 1986, pp. 235-272). The lysed mitochondrial membrane fragments were suspended in 10 mM phosphate buffer, pH = 7.4, and aliquots of this suspension were used to estimate MAO activities. Studies also were carried out in which mitochondrial membranes were subjected to a sucrose gradient purification step. This step did not improve the precision of the assay, and is not discussed further herein.
Preincubation of the homogenate at 37°C for 30 minutes with either 3×10⁻⁷ M (R)-deprenyl or 3×10⁻⁸ M clorgyline followed by incubation for 30 minutes with (22-Scheme 5) provided estimates of the contribution to substrate oxidation from both MAO-A and MAO-B, respectively. Total MAO activity was estimated by preincubating the membranes in phosphate buffer only. Remaining, 'residual enzyme activity', which proved to be from 6 to 13% of the total activity as observed by others (see, Youngster et al., *J. Neurochem.* 53:1837-1842 (1989)), was measured following preincubation with both 3×10⁻⁷ M (R)-deprenyl and 3×10⁻⁸ M clorgyline. Since these membrane suspensions were turbid, it was necessary to treat the postincubation mixtures with 5% acetic acid in acetonitrile and to read the optical density following centrifugation.

The linearity between the concentration of (23-Scheme 5) and the optical density at 420 nm was verified by constructing a standard curve with synthetic (23). Figure 1a shows the linear relationship between the absorbance at 420 nm and the concentration of the synthetic 1-methyl-4-(1-methyl-2-pyrryl)-2,3-dihydropyridinium perchlorate (23·HClO₄).

The linearity of the rate of metabolite formation during the 30 minute incubation period also was verified by assaying the concentration of (23-Scheme 5) vs time over a 40 minute incubation period. Figure 1b shows the linear production over time of 1-methyl-4-(1-methyl-2-pyrryl)-2,3-dihydropyridinium metabolite (23-Scheme 5) following a 30 minute pre-incubation at 37°C in the presence of mouse brain mitochondrial membranes.

With reference to Figures 1a and 1b, it can be understood that the behavior of these membrane preparations gave us confidence that we could estimate rates of enzyme catalysis by measuring the concentration of the dihydropyridinium metabolite (23) at 30 minutes.

Preliminary toxicity studies suggested that an intraperitoneal (i.p.) dose of 25 μmol/kg/day x 3 days of (S)-(16) would be well tolerated. A single 11 μmol/kg i.p dose of (R)-deprenyl, which has been shown to cause 90% depletion
of brain MAO-B activity in the rat (see, Youngster et al., *J. Neurochem.* 53:1837-1842 (1989)), was used as a positive control; untreated animals provided baseline MAO-A and MAO-B activities. The animals were sacrificed on day 4 of the experiment, that is 24 hours after receiving the third and final dose of the (S)-carbamate and the single dose of (R)-deprenyl. The results are summarized in Table 1.

TABLE 1. MAO activity* of brain mitochondrial homogenates obtained from mice following the indicated treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total MAO Activity</th>
<th>MAO-A Activity</th>
<th>MAO-B Activity</th>
<th>Residual Activity</th>
<th>MAO-B/A Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N=3) Control</td>
<td>3.76±1.19</td>
<td>0.58±0.55</td>
<td>3.45±0.89</td>
<td>0.21±0.08</td>
<td>5.95</td>
</tr>
<tr>
<td>(R)-deprenyl</td>
<td>0.84±0.61</td>
<td>0.64±0.57</td>
<td>0.46±0.06</td>
<td>0.24±0.06</td>
<td>0.72</td>
</tr>
<tr>
<td>Carbamate</td>
<td>3.75±0.25</td>
<td>0.76±0.39</td>
<td>3.35±0.16</td>
<td>0.20±0.10</td>
<td>4.40</td>
</tr>
<tr>
<td>(S)-(16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Units: nmol 1-methyl-4-(1-methylpyrrol-2-y)-2,3-dihydropyridinium metabolite (23-Scheme 5) formed/min-mg protein.

b Untreated.

c 1 µmol/kg day 3 only.

d 25 µmol/kg/day x 3 days.

The values for the control mice show that the majority of brain MAO activity is due to MAO-B and only about 15% to MAO-A. These values compare favorably with those reported in the literature (see, Youngster et al., *J. Neurochem.* 53:1837-1842 (1989)). The corresponding measurements with the (R)-deprenyl treated positive control animals show a reduction of total MAO activity to almost 20% of the control level. Furthermore, essentially all of the lost activity is due to inhibition of MAO-B. The enzyme activities in the (S)-carbamate treated animals, however, are essentially identical to those observed in the untreated control animals. Consequently, at the dose employed in this experiment, the (S)-carbamate does not inhibit brain MAO.
The results of the in vivo experiments with the (R)-carbamate (R)-(16) are summarized in Table 2.

TABLE 2. MAO activity\(^a\) of brain mitochondrial membranes obtained from mice following the indicated treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total MAO Activity (N=6)</th>
<th>MAO-A Activity</th>
<th>MAO-B Activity</th>
<th>Residual Activity</th>
<th>MAO-B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline(^b)</td>
<td>3.55±0.54</td>
<td>1.13±0.14</td>
<td>3.46±0.87</td>
<td>0.46±0.09</td>
<td>3.06</td>
</tr>
<tr>
<td>(R)-deprenyl(^c)</td>
<td>1.36±0.11</td>
<td>1.06±0.11</td>
<td>0.70±0.23</td>
<td>0.44±0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>(R)-nor-deprenyl(^d)</td>
<td>1.31±0.15</td>
<td>1.07±0.09</td>
<td>0.68±0.24</td>
<td>0.46±0.10</td>
<td>0.71</td>
</tr>
<tr>
<td>Carbamate(^e)</td>
<td>1.36±0.08</td>
<td>1.10±0.11</td>
<td>0.78±0.15</td>
<td>0.45±0.11</td>
<td>0.63</td>
</tr>
</tbody>
</table>

\(^a\) Units: nmol 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium metabolite (23-Scheme 5) formed/min-mg protein.

\(^b\) 0.1 mL saline once daily x 3 days.

\(^c\) 0.1 mL saline days 1 and 2 followed by 11 μmol drug/kg day 3.

\(^d\) 120 μmol/kg/day x 3 days.

\(^e\) 124 μmol/kg/day x 3 days.

Preliminary toxicity studies showed that a dose of 124 μmol/kg/day x 3 days was well tolerated. In this series of experiments, a second positive control [120 μmol (R)-nordeprenyl/kg/day x 3 days] was included to provide a direct comparison with the active drug that should be released following bioactivation of the (R)-carbamate. The control mice received sterile saline only. The (R)-deprenyl treated mice were administered only sterile saline on days 1 and 2 followed by 11 μmol drug/kg on day three. All mice were sacrificed on day 4, the brains were excised and the mitochondrial membranes were prepared and assayed for MAO activity.

The values for the total MAO activity as well as the MAO-A, MAO-B and residual activity for the control animals were essentially the same as the
corresponding values for the control animals observed in the first experiment
(Table 1) as were the values for the (R)-deprenyl treated animals. Under these
dosing conditions, (R)-nordeprenyl was as effective as (R)-deprenyl in inhibiting
brain MAO-B activity as measured by the reduction of the ratio of
MAO-B/MAO-A from 3.06 to 0.66 [(R)-deprenyl treatment] and 0.71
[(R)-nordeprenyl treatment]. Again, no effect was observed on MAO-A
activity. Treatment with (R)-(16) also led to the selective loss of brain MAO-B
activity (reduction to 23% of control level) with complete retention of brain
MAO-A activity (1.10 vs 1.13 nmoles product/min-mg protein). Thus, with
these doses, the (R)-carbamate is as active and as selective an inhibitor of brain
MAO-B as is (R)-deprenyl. However, the prodrug configuration of the present
invention, which utilizes host systems to release (R)-nordeprenyl, may have
enhanced utility in the treatment of Parkinson’s disease or other disorders in the
central nervous system and avoid mortality problems encountered with (R)-
deprenyl. In addition, there is a possibility that the prodrug properties observed
with the R-carbamate will lead to the selective inhibition of brain MAO-B
activity with a sparing of peripheral MAO-B activity.

It is expected that the dose provided to a patient will vary widely
depending on the patient, the condition being treated, and the delivery mode.
The (R)-(16) carbamate can be administered by a variety of routes including i.v.,
i.p., subcutaneous, i.m., oral, sublingual, suppository, etc., and can be combined
with a variety of carrier fluids such as saline, oils, elixirs, emulsions, etc., or
solids such as lactose, lozenges, etc. The Parkinson disease patient, or other
patient that would benefit from selective inhibition of MAO-B, would be
administered a quantity of (R)-(16) sufficient to selectively inhibit brain MAO-B
activity for a desired period of time.
EXAMPLE 2

Neuroprotection Experiments

Introduction-The prion toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes dopamine depletion in the striata of the C57BL/6 mouse, which is a generally accepted model for neurotoxicity. This toxicity can be prevented by pretreating the animals with (R)-deprenyl, a monoamine oxidase B inhibitor which prevents the biotransformation of MPTP to the ultimate neurotoxin, the 1-methyl-4-phenylpyridinium species. The results reported above discuss the effects of two 1-methyl-1,2,3,6-tetrahydropyrid-4-yl carbamate derivatives on the brain mitochondrial activity of monoamine oxidase A and B. As noted above, it was determined that following injection of the (S)-enantiomer, MAO-A and B activity remained unchanged. Injection of the (R)-enantiomer led to decreased B but not A activity. This indicated that the prodrug was bioactivated by MAO-A which led to the liberation of either (S-) or (R)-nordeprenyl in the mouse brain. Here, studies are described which evaluate the in vivo neuroprotective properties of 1-methyl-(1,2,3,6-tetrahydropyridyl)-(R)-N-(1-methyl-2-phenylethyl)-N-propargylcarbamate [(R)-carbamate] which, upon bioactivation, should liberate the active (R)-enantiomer in this animal model and lead to neuroprotection.

Experimental Section

Dopamine Determinations. Dopamine (DA) determinations were performed using a BAS 4C HPLC-EC system and detector, a BAS C8 column, a Rheodyne injector and a Kipp and Zonen BD 41 strip chart recorder. A standard curve was obtained using calibration standards containing three concentrations of DA and the internal standard, dihydroxybenzylamine (DHBA, IS) and a base line standard containing IS only (n = 3). The mobile phase consisted of: 24.0 g sodium phosphate, monobasic; 291.91 mg 1-octanesulfonic acid sodium salt,
7.41 mg ethylenediamine-tetraacetic acid disodium salt dihydrate; 55 ml methanol; acetonitrile 15 ml added to a 1 L volumetric flask and diluted to the mark with Milli-Q water. Striatal samples (20 μl) were injected into the HPLC and quantitative estimations of dopamine were made by comparing the peak-height ratios of samples (DA/IS) to peak-height ratios of the standard curve.

**Animal Studies.** C57BL/6 male retired breeder mice (25 - 35 g; Harlan/Sprague Dawley) were housed 1/cage in the Laboratory Animal Resource Facility at 21-23°C with free access to standard laboratory chow and tap water on a 12 h day/night cycle. All compounds were dissolved in sterile saline, and injections were administered intraperitoneally in a volume of 0.15, 0.2 or 0.3 ml.

**Animal Protocols.** Following are the protocols from three different experiments; Prodrug groups (P): Animals received one injection of the (R)-carbamate as the oxalate salt (5mg/Kg, 15 mg/Kg, 30 mg/Kg, or 50 mg/Kg) on days 1, 2 and 3, followed on day 3 at 1 h post treatment with MPTP-HCl (40 mg/Kg); MPTP only group (M): Animals received sterile saline days 1, 2 and 3, followed on day 3 at 1 h post treatment with MPTP-HCl (40 mg/Kg); Control Animals (C): Control animals received sterile saline only. Seven days post MPTP treatment, animals were sacrificed by cervical dislocation and the striata were dissected on ice. Ten μL IS in 5% trichloroacetic acid was added per mg striata.

Results Expressed as Percent DA; Controls = 100%

**Experiment #1**

- Controls (n=4) 100.0±7.1
- MPTP (n=8) 41.6±4.8
- Prodrug 50 (n=8) 102.8±7.1

**Experiment #2**

- Controls (n=4) 100.0±5.9
- MPTP (n=7) 45.0±18.6
- Prodrug 5 (n=7) 35.5±14.0
Experiment #3

Controls (n=4)  100.0±6.6
MPTP (n=7)  17.6±6.9
Prodrug 15 (n=8)  69.5±22.6
Prodrug 30 (n=8)  100.5±25.3

Each experiment has its own control group of animals, both positive and negative as the degree of MPTP depletion varies considerably from batch to batch of mice. Thus, the results must be evaluated with respect to the MPTP depletion within each experiment. From experiment #1, it can be seen that a dose of 50 mg/Kg of the (R)-carbamate protects the mice completely from MPTP neurotoxicity. In experiment #2 the dose of 5 mg/Kg did not afford protection. Experiment #3 more clearly defined the doses necessary for protection in the mouse with 15 mg/Kg affording considerable protection and 30 mg/Kg affording complete protection. These experiments show results consistent with the putative biotransformation of the (R)-carbamate by MAO-A to the active neuroprotectant, which upon release should protect against the MAO-B mediated MPTP neurotoxicity.

EXAMPLE 3

Tetrahydropyridyl phosphorodiamidates are synthesized according to the general synthesis scheme 6:

Reaction Scheme 6
R₁ and R₂ are substituted amine moieties and can be either primary or secondary amines. One compound according this aspect of the invention has R₁ as N(CH₂CH₂Cl)₂ and R₂ as NHCH₂C₆H₅. The synthesis of this compound is set forth below:

**Oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl-N-benzyl-N', N'-bis(2-chloroethyl)phosphorodiamidate**

A 1.4M solution of methylithium in diethyl ether (45.4 ml, 63.5 mmol) was added dropwise to a solution of silyl enol ether (10.70g, 55.7 mmol) in 140 ml THF at room temperature with stirring under nitrogen. After four hours, the reaction mixture was transferred under pressure via a cannula to a dropping funnel from which was added to a solution of the phosphoramic dichloride (19.42 g, 75.1 mmol) in 140 ml THF dropwise at 0°C. Following two hours at 0°C, benzylamine (15.46 g, 0.144 mol) was added dropwise to the solution and the resulting suspension was stirred overnight at room temperature. The solid benzylamine hydrochloride that formed was removed by filtration and the filtrate was evaporated to dryness. Chromatography of the residue [150 g silica gel, CHCl₃/CH₃OH (6:1, v/v)] gave 13.10 g (37.7%) of the oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl-N-benzyl-N’N’-bis(2-chloroethyl)phosphorodiamidate as an oil. ¹H NMR (CDCl₃) δ 7.27-7.37 (m, 5H, PhH), 5.56 (unresolved m, 1H, C5), 4.11-4.21 (m, 2H, PhCH₂), 3.31-3.62 (m, 14H, CH₂CH₂Cl, C2, C3, and C6), 2.84 (s, 3H, N-CH₃). The solid oxalate was prepared in CH₃CN and was recrystallized from EtOAc/CH₃CN: mp 133-134°C; ¹H NMR (CD₃OD) δ 7.17-7.28 (m, 5H, PhH), 5.39 (unresolved m, 1H, C5), 3.99-4.05 (dd, 2H, PhCH₂) 3.28-3.58 (m, 12H, C6, C2, and CH₂CH₂Cl), 2.86 (s, 4.5H, N-CH₃ + 0.5 CH₃CN), 2.52 (unresolved m, 2H, C3). Anal. Calcd. for C₁₉H₁₈Cl₂N₂O₄P·0.25 CH₃CN: C, 46.29; H, 5.73; N, 9.45%. Found C 46.13; H, 6.11; N, 9.41%.
The tetrahydropyridyl phosphorodiamidate discussed above were tested in a series of enzyme substrate studies. In these studies, the isolation and purification of MAO-A from human placenta and MAO-B from beef liver were carried out using the procedures reported in Salach et al., *Methods in Enzymology*, 142:627-637 (1987), with the following modifications. The phospholipase A used in the preparation was obtained commercially (Sigma, St. Louis, MO), rather than from the crude venom. The MAO-A preparation was not subjected to the sephadex purification, and the MAO-B preparation was not subjected to the glucose gradient purification step. In both cases, however, highly active preparations were obtained. The specific activity of MAO-A (17 nmol/ml) was established with kynuramine as the substrate at 30°C ($k_{cat} = 146$ min$^{-1}$). The specific activity of MAO-B (10 nmol/ml) was established with MPTP as the substrate at 30°C ($k_{cat} = 204$ min$^{-1}$). Solutions of the phosphorodiaminates in phosphate buffer (pH 7.4, 0.5 mM, final volume 500μl) in a 1 ml quartz cuvette were treated with 20μl of the MAO-A preparation (final concentration 0.16μm) or 5μl of the MAO-B preparation (final concentration 0.10μm) and the cuvette was placed in a Beckman model DU-50 spectrophotometer maintained at 37°C. The substrate properties were evaluated qualitatively by obtaining a series of UV scans vs. time over a 1 hour period.

Kinetic studies were carried out using a Beckman DU-50 spectrophotometer. Solutions of the test compounds (final volume 500μl, final substrate concentrations 500-8000 μm) in 100μM sodium phosphate (pH=7.4) were incubated in the presence of 0.16μm MAO-A. The $k_{cat}$ and $K_m$ values were calculated from double reciprocal plots.

Repeated UV scans (400-200 nm) of incubation mixtures shows that the phosphorodiamidates are stable in the presence of MAO-B. This outcome was not expected since poor MAO-B substrate properties have been observed with THP derivatives bearing bulky substituents on C-4. The phosphorodiamidates
proved to be MAO-A substrates as evidenced by the time dependent increase in a chromophore corresponding to the expected aminoenone. The MAO-A dependent formation of the aminoenone species should coincide with the release of the phosphoramidate mustard from the hydrolysis of the dihydropyridinium metabolite. Kinetic studies on the MAO-A catalyzed oxidation of the phosphorodiamidates demonstrated a linear initial rate plot at concentrations that bracketed the $K_m$ values. The oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl-N-benzyl-N'N'-bis(2-chloroethyl)phosphorodiamidate was a good substrate of MAO-A with $k_{cat}/K_m = 38 \text{min}^{-1}\text{mM}^{-1}$. This value is somewhat better than that reported for the cytochrome P450 catalyzed oxidation of cyclophosphamide (about 10 min$^{-1}$mM$^{-1}$). The studies indicate that the phosphorodiamates may find utility in targeting MAO-rich cells with a cytotoxic agent. For instance, the compounds may be used for the selective destruction of MAO-A rich neurons in the central nervous system.

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims.
CLAIMS

We claim:

1. A prodrug compound having the chemical formula:

\[
\begin{array}{c}
\text{X} \\
\text{N} \\
\text{C H}_3
\end{array}
\]

where X is a moiety having at least one chiral center.

2. The prodrug of claim 1 wherein X has the general structural formula:

\[
\begin{array}{c}
\text{R}_1 \\
\text{N} \\
\text{R}_2
\end{array}
\]

where at least one of R_1 and R_2 has chiral center, and wherein R_1 and R_2 are each selected from the group consisting of hydrogen, halogen, substituted alkyl moieties, unsubstituted alkyl moieties, substituted aryl moieties, unsubstituted aryl moieties, and moieties connecting R_1 and R_2.
3. The prodrug of claim 2 wherein the chemical structure is:

![Chemical Structure Image]

4. The prodrug of claim 2 wherein the chemical structure is:

![Chemical Structure Image]

where $R_3$ is selected from the group consisting of $\text{CH}_3$ and $H$. 


5. The prodrug of claim 2 wherein the chemical structure is:

\[ R_3 \]

where \( R_3 \) is selected from the group consisting of \( \text{CH}_3 \) and \( \text{H} \).

6. The prodrug of claim 2 wherein the chemical structure is:

\[ \text{H} \]

\[ \text{N} \]

\[ \text{CO} \]

\[ \text{O} \]

\[ \text{CH}_3 \]
1. The prodrug of claim 2 wherein the chemical structure is:

2.

1. The prodrug of claim 2 wherein the chemical structure is:

2.

1. The prodrug of claim 2 wherein the chemical structure is:
10. The prodrug of claim 2 wherein the chemical structure is:

![Chemical Structure 1]

11. The prodrug of claim 2 wherein the chemical structure is:

![Chemical Structure 2]

12. The prodrug of claim 1 wherein X has the structure:

![Chemical Structure 3]

where $R_3$ and $R_4$ are selected from the group consisting of substituted and substituted primary and secondary amines.

13. The prodrug of claim 12 wherein $R_3$ is $N(CH_2CH_2Cl)_2$ and $R_4$ is $NHCH_2C_6H_5$. 
patient comprising the step of providing a patient in need thereof with a quantity
sufficient to selectively inhibit monoamine oxidase-B activity of a prodrug
compound having following the chemical formula:

\[
\begin{array}{c}
\text{X} \\
\text{C}_2\text{H}_3
\end{array}
\]

where X is a moiety having at least one chiral center.

15. A method of treating a patient suffering from Parkinson’s disease,
comprising the step of providing a patient suffering from Parkinson’s disease
with a sufficient quantity of a prodrug compound having the following chemical
formula:

\[
\begin{array}{c}
\text{X} \\
\text{C}_2\text{H}_3
\end{array}
\]

where X is a moiety having at least one chiral center.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
  IPC(6) : A61K 31/44; C07D 401/00, 413/00, 211/70; C07C 261/00, 229/00  
  US CL. : 514/332, 333, 340, 343; 546/256, 283, 276,329; 558/166, 169

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
  U.S. : 514/332, 333, 340, 343; 546/256, 283, 276,329; 558/166, 169

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US 4,251,538 A (HAUSBERG ET AL.) 17 February 1981.</td>
<td>1-15</td>
</tr>
<tr>
<td>A</td>
<td>US 4,301,168 A (STIRLING et al.) 17 November 1981.</td>
<td>1-15</td>
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</tbody>
</table>

See patent family annex.

Date of the actual completion of the international search: 07 JANUARY 1998
Date of mailing of the international search report: 02 FEB 1998

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