6610-29-3; **3** ($R^1 = CH_3$, $R^2 = C_2H_5$), 21198-37-8; **3** ($R_1 = R_2 = H$), 79-19-6; **3** ($R^1 = CH_3$, $R_2 = n\text{-}C_3H_7$), 21198-40-3; **5** (Ar = $p\text{-}ClC_6H_4$), 16794-67-5; **6** ($R^1 = CH_3$), 60-34-4; **7** (Ar = $p\text{-}ClC_6H_4$, $R^1 = C_2H_5$, $R_2 = CH_3$), 114058-88-7; **8**, 38942-51-7; **9**, 114058-89-8; **10**, 114058-93-4; **11**, 114058-90-1; **12**, 110623-32-0; **13**, 110623-33-1; **14**, 114058-91-2; **15**, 110623-34-2; **16**, 110623-35-3; **17**, 110623-36-4; **18**, 110623-25-1; **19**, 26028-65-9; **20**, 29527-27-3; **21**, 27349-24-2; **22**, 110623-24-0; **23**, 114058-92-3; **24**, 110623-26-2; **25**, 110623-27-3; **26**, 110623-29-5; **27**, 110623-31-9; **28**, 110623-30-8; **29**, 110623-38-6; **30**, 110623-37-5; **31**, 110623-39-7; **32**, 110623-40-0.

Facile N-Oxygenation of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine by the Flavin-Containing Monooxygenase. A Convenient Synthesis of Tritiated [*methyl*-^{3}H]-4-Phenyl-2,3-dihydropyridinium Species

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A rapid, efficient procedure useful for the radiosynthesis of [*Me*-^{3}H]-MPDP$^+$ ([*methyl*-^{3}H]-4-phenyl-2,3-dihydropyridinium species) is described. Hog liver microsomes or the highly purified flavin-containing monooxygenase from hog liver quantitatively biotransforms [*Me*-^{3}H]-MPTP to its corresponding radiolabeled *N*-oxide. For the small-scale synthesis required for radiolabeling procedures, this enzymatic process is superior to H_2O_2-mediated N-oxygenation of MPTP. In the presence of 0.5 mM NADPH, 4.5 mM *n*-octylamine, and 2 μCi [*Me*-^{3}H]-MPTP, the only product detected in extracts from incubations performed with hog liver microsomes or purified hog liver flavin-containing monooxygenase is [*Me*-^{3}H]-MPTP *N*-oxide. [*Me*-^{3}H]-MPTP *N*-oxide is almost completely converted to [*Me*-^{3}H]-MPDP$^+$ by the action of trifluoroacetic anhydride. This procedure has the advantage of using a commercially available tritiated starting material, efficient transformations, and easily accomplished purification to afford a rapid synthesis of [*Me*-^{3}H]-MPDP$^+$.

There is considerable current interest in radiolabeled metabolites of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a thermal decomposition product of a "street narcotic" that is responsible for a parkinsonism-like syndrome in humans.[1-3] A convenient procedure for the synthesis of tritiated 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP$^+$) may be useful in delineating the involvement of MPDP$^+$ in the neurotoxicity of MPTP.

The mechanism of neurotoxicity of MPTP is not clear, but it is apparent that any explanation must include the facts that (1) MPTP requires metabolic bioactivation by monoamine oxidase (MAO) to exert its toxic effect,[4-7] (2) the primary initial metabolite of MAO biotransformation of MPTP is MPDP$^+$,[8,9] (3) MPDP$^+$ is reactive to nucleophiles such as cyanide ion and is readily oxidized,[7,10-12] and (4) MPDP$^+$ is further transformed to 1-methyl-4-phenylpyridinium species (MPP$^+$).[7,13]

Processing of MPTP by MAO appears to be critical for the expression of MPTP neurotoxicity. Results from a number of laboratories examining a variety of tissues suggest that high specific binding of MPTP parallels the presence of MAO and especially MAO B in the tissues examined.[4] Specific binding of MPTP to MAO B could result from at least two mechanisms, including (1) covalent binding to MAO B itself, or (2) high affinity and slow enzymatic turnover of MPTP to MPDP$^+$. The evidence is that MAO B is irreversibly inactivated by MPTP[8] and that this process is relatively inefficient: 1 mol of enzyme is inactivated per 17 000 mol of product formed.[9] In contrast, MAO A is only competitively inhibited by MPTP.[8] MAO B catalyzes the oxidation of MPTP 10 times faster than MAO A[8,9] and the rate of MAO B oxidation of MPTP compares favorably with benzylamine, an excellent MAO type B substrate. That the neurotoxicity observed for MPTP requires MAO-catalyzed oxidation is supported by the finding that MAO B inhibitors protect against the neurotoxicity of MPTP.[4,15,16] A number of studies have demonstrated that MPTP or derivatives of MPTP that are neurotoxic are MAO substrates, but, of course, not all MAO substrates are toxic.[5,6] It is clear that MPDP$^+$ is the primary initial metabolite arising from MAO oxidation of MPTP and it is this metabolism that appears to parallel the neurotoxicity of MPTP.[7] MPDP$^+$, the major initial metabolite of MPTP in the brain, was found to serve as an electrophile for cyanide.[11,13] An analogue of MPDP$^+$ stable to oxidation was observed not to react with various

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amine and thiol nucleophiles, and it is possible that $MPDP^+$ can serve as an electrophile only if the unstable adducts are stabilized by the biological environment.[10,11,17] MPTP-induced neurotoxicity may be a consequence of the depletion of glutathione in the brain and the subsequent accumulation of oxidized byproducts of biological macromolecules.[18] $MPDP^+$ may be neurotoxic, perhaps via further oxidation to MPP^+, a cytotoxic agent in its own right.[19] Another possibility is that MPP^+ may participate in "redox cycling", leading to toxic O_2-derived products.[20] That the oxidation of MPTP to $MPDP^+$ and further oxidation products that lead to specific binding interactions occurs is supported by numerous observations in vivo and in vitro.[8,12,21-24] The covalent interaction of $MPDP^+$ with macromolecules in tissues relevant to Parkinson's disease has received less attention. MPTP binds with high affinity to rat tissue[12] and rat and human brain slices in vitro,[25] and the distribution of MPTP binding sites in rat brain corresponds to the localization of MAO.[26] Recently, it has been demonstrated that, unlike MPP^+, MPTP and $MPDP^+$ potently inhibit mouse brain neostriatal synaptic transmission.[27] The fact that the MAO inhibitor pargyline blocks the irreversible inhibition of synaptic transmission of MPTP[27] and that pargyline blocks the irreversible effects of $MPDP^+$[28] suggests that both $MPDP^+$ and a further metabolite of $MPDP^+$, possibly MPP^+, may be involved in the ultimate expression of MPTP neurotoxicity. A radiolabeled form of $MPDP^+$ should be quite useful for a variety of investigations.

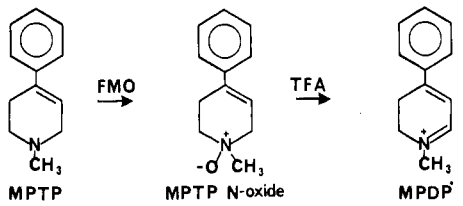
Herein is described a useful two-step procedure for the biosynthetic preparation of [*Me*-^{3}H]-$MPDP.^+$ The method allows for extremely efficient transformations and has the advantage that simple purifications are accomplished. In addition, the simple two-step synthetic method, in principle, may be applied to the radiosynthesis of a wide variety of other potentially neurotoxic dihydropyridinium compounds.

Results

In the course of studies aimed at investigating the N-oxygenation of allylic tertiary amines, MPTP was evaluated in vitro for N-oxygenation substrate ability in microsomal preparations and with the purified flavin-containing monooxygenase.[29,30]

MPTP is a cyclic allylic tertiary amine similar in structure to other *N*-methylpiperidine substrates for this enzyme.[31] Kinetic constants for the N-oxygenation of MPTP catalyzed by untreated rat liver microsomes were calculated from the rate of *N*-oxide formation by a radiometric procedure.[29] The K_m and V_{max} values obtained from double-reciprocal plots of velocity versus substrate are 45 μM and 4.8 nmol min^{-1} (mg of protein)$^{-1}$, respectively. MPTP is also an excellent substrate for the purified hog liver flavin-containing monooxygenase. The K_m and V_{max} values are 32 μM and 730 nmol min^{-1} (mg of protein)$^{-1}$ and compare favorably with those of the better amine substrates for this enzyme.[29,31] It was envisaged that efficient formation of radiolabeled MPTP *N*-oxide followed by treatment with trifluoroacetic anhydride (the modified Polonovski reaction) would readily afford radiolabeled $MPDP^+$ (Scheme I). This reaction sequence was utilized as a practical means to synthesize [*Me*-^{3}H]-$MPDP.^+$ The data of Table I show that [*Me*-^{3}H]-MPTP is converted almost quantitatively to its *N*-oxide by both purified hog liver flavin-containing monooxygenase and hog liver microsomes. These results are similar to the N-oxygenation of MPTP observed for rat or mouse liver microsomes described previously.[29,30] It is clear from this paper and other studies[29-31,33-36] that employing uninduced liver microsomes (or purified flavin-containing monooxygenase), in the presence of *n*-octylamine and NADPH, efficiently converts

Scheme I. Modified Polonovski Reaction for the Conversion of MPTP to $MPDP^+$[a]



[a] FMO is the flavin-containing monooxygenase and TFA is trifluoroacetic anhydride.

Table I. N-Oxygenation of MPTP by the Flavin-Containing Monooxygenase or H_2O_2

condition	% product[a] MPTP	MPTP *N*-oxide	$MPDP^+$	MPP^+
purified FMO[b]	1.0 ± 1	99.0 ± 2		
hog liver microsomes[c]	2.0 ± 1	98.0 ± 1		
H_2O_2 (2 equiv)[d]	8.3 ± 2	66.1 ± 8	14.5 ± 2	1.8 ± 1
H_2O_2 (10 equiv)[e]	14.0 ± 3	58.7 ± 7	10.8 ± 3	2.8 ± 1
H_2O_2 (100 equiv)[f]	6.3 ± 1	65.4 ± 4	12.3 ± 1	5.5 ± 1

[a] Reactions were quantitated by HPLC and scintillation counting of the isolated products and yields are the mean of at least three experiments ± standard deviation. [b] 150 μg of purified flavin-containing monooxygenase (FMO) was incubated with 2 μCi [*Me*-^{3}H]-MPTP as described in the Experimental Section. [c] 3.75 mg of hog liver microsomes was incubated with 2 μCi [*Me*-^{3}H]-MPTP as described in the Experimental Section. [d] Chemical oxidations of 2 μCi [*Me*-^{3}H]-MPTP were performed as described in the Experimental Section and stopped after or 24 h or [e] 10 h or [f] 1 h.

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tertiary amines like MPTP to their corresponding *N*-oxides. In the presence of NADPH and *n*-octylamine, experiments indicated that (1) *n*-octylamine stimulated microsomes and purified enzyme to catalyze the formation of MPTP *N*-oxide more than 2-fold, (2) in the presence of this primary alkylamine, MPTP *N*-oxide is the only metabolite detected in extracts of metabolic reactions, and (3) MPTP *N*-oxide was recovered quantitatively and did not undergo oxidation, reduction, or other subsequent chemical reactions during metabolic incubations or isolation. The only product detected in dichloromethane extracts of metabolic reactions eluted with retention volume identical with that of authentic MPTP *N*-oxide upon separation by HPLC. No nor-MPTP or other metabolites could be detected in reactions employing *n*-octylamine, a good inhibitor of cytochrome P-450[37] and a known positive effector for the flavin-containing monooxygenase.[31]

The chemical oxidation of MPTP with aqueous H_2O_2 indicated that (1) inefficient formation of MPTP *N*-oxide occurs, (2) a variety of MPTP *N*-oxide oxidation or disproportionation products are produced before MPTP is completely consumed, and (3) mixtures of products are observed for a number of different reaction conditions employed (Table I). The best reaction conditions[38] afforded only a 66% yield of the desired MPTP *N*-oxide. The chemical oxidation yields reported here are not isolated yields, but rather they are product distributions arising from direct HPLC analysis. Presumably, isolation and purification of [*Me*-^{3}H]-MPTP *N*-oxide from these reactions would decrease this yield further. As a result of this study, investigations with [*Me*-^{3}H]-MPTP *N*-oxide derived from enzymatic procedures only were used in the further elaboration of [*Me*-^{3}H]-MPDP$^+$. Purified [*Me*-^{3}H]-MPTP *N*-oxide isolated from incubations of either hog liver microsomes or purified flavin-containing monooxygenase was combined with trifluoroacetic anhydride according to the procedure described in the Experimental Section to afford [*Me*-^{3}H]-MPDP$^+$ in overall yields of 60 ± 8% and 82 ± 6%, respectively. The only product detected in extracts from reactions of [*Me*-^{3}H]-MPTP *N*-oxide with trifluoroacetic anhydride eluted with a retention volume identical with that of authentic synthetic MPDP$^+$. The conversion of [*Me*-^{3}H]-MPTP *N*-oxide to [*Me*-^{3}H]-MPDP$^+$ was achieved in average yields of 90 ± 8%. The lower apparent overall yield of the microsomal reactions probably reflects the inefficient extraction of MPTP *N*-oxide from microsome suspensions.

In an attempt to investigate the stability of the [*Me*-^{3}H]-MPDP$^+$ synthesized, dilute acetonitrile solutions of radiolabeled material were periodically analyzed by HPLC for decomposition or disproportionation. After 10 h at room temperature, less than 6 ± 2% of the [*Me*-^{3}H]-MPDP$^+$ disproportionates to [*Me*-^{3}H]-MPP$^+$, as determined by HPLC analysis.[29] Storage in frozen benzene under an atmosphere of argon effectively inhibits decomposition of [*Me*-^{3}H]-MPDP.$^+$

Discussion

Herein is described a rapid, convenient procedure for the synthesis of [*Me*-^{3}H]-MPDP$^+$ from commercially available [*Me*-^{3}H]-MPTP. The method is based on facile bioconversion of [*Me*-^{3}H]-MPTP to [*Me*-^{3}H]-MPTP *N*-oxide and chemical rearrangement to [*Me*-^{3}H]-MPDP$^+$ (the modified Polonovski reaction). Several advantageous features of this overall transformation should be pointed out. First, as has been demonstrated in a number of laboratories, MPTP is efficiently converted to MPTP *N*-oxide by hepatic flavin-containing monooxygenase activity from a variety of species including mouse,[30] rat,[29] rabbit,[38] hog,[29] and human[29]. As outlined in this paper and as described previously, use of uninduced liver preparations in the presence of *n*-octylamine, which inhibits cytochrome P-450[37] and stimulates flavin-containing monooxygenase activity,[31] provides a convenient method for quantitatively converting MPTP to MPTP N-oxide. This is especially apparent when performing the synthesis with small quantities of radiolabeled MPTP. In contrast, chemical oxidation of small amounts of radiolabeled MPTP with excess H_2O_2 results in mixtures of products, presumably resulting from the strong oxidizing agents present. MPTP *N*-oxide is formed from H_2O_2-catalyzed N-oxygenation but excess H_2O_2 (or other species present) convert MPTP *N*-oxide to MPDP$^+$, which disproportionates to MPP$^+$ and MPTP.[13,32] Significant byproducts are apparent under reaction conditions that do not consume all of the starting MPTP. Under large-scale multigram reaction conditions, the contribution of these side reactions presumably cannot compete with H_2O_2-mediated MPTP *N*-oxide formation. [*Me*-^{3}H]-MPTP *N*-oxide is readily isolated and purified from metabolic incubation reactions. Addition of trifluoroacetic anhydride quantitatively converts the radiolabeled *N*-oxide to [*Me*-^{3}H]-MPDP.$^+$ Trifluoroacetic acid provides a stabilizing counterion for MPDP$^+$ and allows for ease of handling and isolation of the radiolabeled material. As described by other investigators, MPDP$^+$ is significantly more stable under acidic conditions. Under basic conditions, MPDP$^+$ disproportionates to MPP$^+$ and MPTP.[10,11,13,39] The studies described here support this concept and suggest that [*Me*-^{3}H]-MPDP$^+$$CF_3COO^-$ at very low concentrations does not readily disproportionate and may be useful in mechanistic and in vitro metabolism studies.

Experimental Section

Chemicals. Synthetic metabolites of MPTP, MPTP *N*-oxide, and MPDP$^+$ were synthesized by the method of Weissman et al.[38] MPTP, MPP$^+$, *n*-octylamine, and H_2O_2 were purchased from Aldrich Chemical Co. The synthetic compounds were characterized by normal spectroscopic techniques and the spectra were identical with published values. EDTA, NADP$^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. All other chemicals and reagents used were of the highest quality commercially available. *N*-Methyl-tritiated MPTP ([*Me*-^{3}H]-MPTP) was purchased from New England Nuclear (Boston, MA) at a specific activity of 82.3 Ci/mmol.

Metabolic Incubations. Hog liver microsomes were a generous gift of Prof. D. M. Ziegler (University of Texas at Austin). Purified hog liver flavin-containing monooxygenase was prepared by published procedures.[40,41] Purified hog liver flavin-containing monooxygenase and hog liver microsomes used in this study were shown to N-oxygenate dimethylaniline at a rate of 262 and 9.6 nmol min^{-1} (mg of protein)$^{-1}$, respectively.[42] The enzyme or microsome preparations were kept cold until the reactions were initiated because the flavin-containing monooxygenase from this tissue is unusually sensitive to thermal inactivation.[31,43] Incu-

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bation conditions were described previously:[43] 4.5 mM *n*-octylamine, 1 mM EDTA, 0.5 mM $NADP^+$, 2 mM glucose 6-phosphate, and 1 IU of glucose-6-phosphate dehydrogenase was dissolved in 50 mM potassium buffer (pH 7.8) in a total of 0.5 mL, the mixture was cooled to 4 °C, and flavin-containing monooxygenase (100 μg of purified enzyme or 2.5 mg of hog liver microsomal protein) was added. Reactions were initiated by the addition of 2 μCi [*Me*-^{3}H]-MPTP and were carried out in triplicate at 33 °C under air with constant shaking. After 20 min of incubation, another 50 μg of purified flavin-containing monooxygenase or 1.25 mg of hog liver microsomal protein was added to insure that maximal conversion to [*Me*-^{3}H]-MPTP *N*-oxide was achieved. After an additional incubation of 15 min, two 1-mL portions of ice-cold dichloromethane was added and mixed, and the layers were separated by centrifugation. The dichloromethane layer was reduced in volume and applied to alumina preparative TLC plates and developed in chloroform/methanol (9:1) as described previously.[29] The bands corresponding to the *N*-oxide and MPTP were scraped and filtered through a small column of Celite (100 mg, 0.4 × 1.0 cm), eluted with ethyl acetate/dichloromethane/methanol (60:30:10), and evaporated in vacuo to afford purified [*Me*-^{3}H]-MPTP *N*-oxide and recovered [*Me*-^{3}H]-MPTP. While the results described herein concerning microsomal N-oxygenation were obtained with hog liver microsomes and purified hog liver flavin-containing monooxygenase, very similar results were obtained with rat and mouse liver microsomes.[30] In principle, hog, rat, or mouse liver microsomes can be utilized to very efficiently biotransform MPTP to MPTP *N*-oxide, by using the procedure described above.

Chemical Oxidation of MPTP with H_2O_2. Two microcuries of [*Me*-^{3}H]-MPTP (6.5 ng, 0.024 nmol) was combined with 475 μL of ethanol. A 25-μL aliquot of diluted 30% aqueous H_2O_2 was added to the reaction in order to achieve the concentrations of H_2O_2 listed in Table I. The reaction proceeded with shaking under an atmosphere of air at room temperature. At the appropriate time, as described in Table I, a 50-μL aliquot was withdrawn from the reaction and combined with 450 μL of dimethyl sulfoxide/acetonitrile (20:80). A 50-μL aliquot of the quenched reaction mixture was analyzed directly by high-performance liquid chromatography (HPLC) by monitoring the amount of radioactivity with scintillation counting of material coeluting with authentic synthetic MPTP and MPTP oxidation products, as described previously.[29]

Transformation of [*Me*-^{3}H]-MPTP *N*-Oxide to [*Me*-^{3}H]-MPDP$^+$. Typically, two μCi (7 ng, 0.024 nmol) of purified [*Me*-^{3}H]-MPTP *N*-oxide from the enzymatic incubates was dried by azeotroping with toluene. [*Me*-^{3}H]-MPTP *N*-oxide was combined with 2 mL of dichloromethane and cooled to −20 °C and 50 μL of trifluoroacetic anhydride (74 μg, 0.35 μmol) was added. The reaction mixture was allowed to warm to room temperature and after 10 min was evaporated to dryness and was taken up in 500 μL of acetonitrile. The acetonitrile fraction was analyzed directly by HPLC.

Analytical Procedures. The composition of chemical and enzymatic N-oxygenation reaction products were determined by TLC and HPLC analysis. HPLC analysis was performed as previously described,[29] except that 50 mM *N,N*-dimethyloctylamine was added to the elution buffer to improve the chromatographic separations. Analysis was performed on an IBM Model 9300 Ternary Gradient HPLC system with a 5-μm Altex ultrasphere-ODS reverse-phase analytical HPLC column with UV detection at 242 nm for MPTP *N*-oxide and 342 nm for MPDP$^+$. The structures of radiolabeled reaction products were confirmed by pooling the materials from preparative HPLC runs and determining their UV–vis spectra with a Perkin-Elmer Model 595 UV–vis spectrophotometer. Radioactivity was quantitated by scintillation counting with a Searle Mark III scintillation counter. During the handling and use of MPTP, special precautions as outlined previously[44] were employed.

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C-Nor-9,11-secoestranes as Modified Estrogens and Fertility Regulation†

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The synthesis of *C*-nor-9,11-secoestradiol (4) has been achieved from 17β-acetoxy-11-chloro-3-methoxy-*C*-nor-9,11-secoestra-1,3,5(10)-trien-9-one (1) through a sequence of reactions without affecting the stereochemistry of estradiol-17β. Removal of the 9-keto function of 1 by hydrogenolysis and its subsequent treatment with Na/NH_3 gives *C*-nor-9,11-secoestradiol 3-(methyl ether) (3), which has been demethylated under alkaline conditions to furnish *C*-nor-9,11-secoestradiol (4). Pyridinium chlorochromate oxidation of 3 gives the corresponding 17-ketone 6. Jones' oxidation of 4 to the ketone 5 and reaction of 5 and 6 with lithium acetylide gives corresponding 17α-ethynyl derivatives 7 and 8. Relative binding affinity to estradiol-17β receptors and uterotropic, antiuterotrophic, and antiimplantation activities of compounds 3–8 have been studied. The effect of conformational flexibility on ligand–receptor interaction of these compounds is discussed.

The action of hormones is mediated[1] through their interaction with specific receptors and presumably is a measure of a complimentarity existing between the receptor and the interacting molecule. Therefore, a study of structure–activity relationships (SAR) of secoestradiols was undertaken as an approach to estrogen receptor mapping and toward the development of modified estrogens for fertility regulation.

In the estradiol (E_2-17β) molecule, oxygen functions at C-3 and C-17 and its specific stereochemistry are consid-

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