Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1685–1689

BIOORGANIC & MEDICINAL CHEMISTRY

Rat Liver Microsomal Enzyme Catalyzed Oxidation of 4-Phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine

Simon Kuttab,[a] Jackie Shang[b] and Neal Castagnoli, Jr.[b,*]

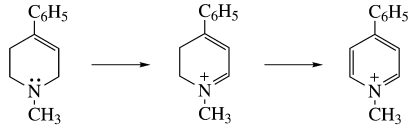
[a]*Birzeit University, Department of Chemistry, Birzeit, West Bank, Via Israel*
[b]*Department of Chemistry, Virginia Tech, 107 Davidson Hall, Blacksburg, VA 24061, USA*

Received 27 December 2000; accepted 17 February 2001

Abstract—As part of our ongoing studies to characterize the catalytic pathway(s) for the monoamine oxidase and cytochrome P450 catalyzed oxidations of 1,4-disubstituted 1,2,3,6-tetrahydropyridinyl derivatives, we have examined the metabolic fate of 4-phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine in NADPH supplemented rat liver microsomes. Three metabolic pathways have been identified: (1) allylic ring α-carbon oxidation to yield the dihydropyridinium species, (2) nitrogen oxidation to yield the *N*-oxide and (3) *N*-dealkylation to yield 4-phenyl-1,2,3,6-tetrahydropyridine and cinnamaldehyde. A possible mechanism to account for the formation of cinnamaldehye involves an initial single electron transfer from the nitrogen lone pair to the iron oxo system $Fe^{+3}(O)$ to form the corresponding cyclopropylaminyl radical cation that will be processed further to the final products. The reaction pathway leading to the dihydropyridinium metabolite may also proceed via the same radical cation intermediate but direct experimental evidence to this effect remains to be obtained.

Introduction

The monoamine oxidase B (MAO-B) catalyzed oxidation of the parkinsonian inducing proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] generates, via the dihydropyridinium species **2**, the ultimate toxic pyridinium metabolite **3** which accumulates in dopaminergic nigrostriatal nerve terminals where it inhibits the mitochondrial electron transport system (Scheme 1).[1] In an effort to characterize the reaction pathway responsible for the initial 2-electron α-carbon oxidation of MPTP, we have examined the interactions of the corresponding 1-cyclopropyl analogue **4** with a solubilized form of beef liver MAO-B (Scheme 2).[2] According to Silverman, the catalytic pathway of this flavoenzyme proceeds by an initial single electron transfer (SET) step[3] which, in the case of **4**, would generate a cyclopropylaminyl radical cation **5**. Ring opening of **5**, which should proceed at near diffusion rate limits,[4] results in the formation of the highly reactive distonic primary radical cation **6** that bioalkylates an enzyme active site functionality and inactivates the enzyme. Enzyme kinetic studies confirmed the expected mechanism based inactivation properties of **4** (k_{inact} 0.7 min^{-1}).[2] Also consistent with an SET-based mechanism, cyclopropylaminyl radical cations generated from various tetrahydropyridinyl derivatives under model SET reaction conditions were converted to ring opened products without any evidence of dihydropyridinium formation.[5]



Scheme 1. Metabolic bioactivation pathway of MPTP (**1**) to the neurotoxic pyridinium metabolite **3** via the MAO-B generated dihydropyridinium intermediate **2**.

Unlike the behavior observed under the model SET reaction conditions, the MAO-catalyzed reaction of **4** also leads to the dihydropyridinium metabolite **8**; the estimated partition ratio was 17.[6] Furthermore, not all *N*-cyclopropyltetrahydropyridines are inactivators of MAO. For example, 1-cyclopropyl-4-phenoxy-1,2,3,6-tetrahydropyridine (**9**) is converted efficiently to the corresponding dihydropyridinium metabolite **11** ($V_{max}/K_m = 1650$ min^{-1} mM^{-1}) without any evidence of loss of enzyme activity.[7] Based on this behavior, we have considered the possibility that the MAO catalytic pathway may also proceed via an initial hydrogen atom

*Corresponding author. Fax: +1-540-231-8890; e-mail: ncastagn@vt.edu

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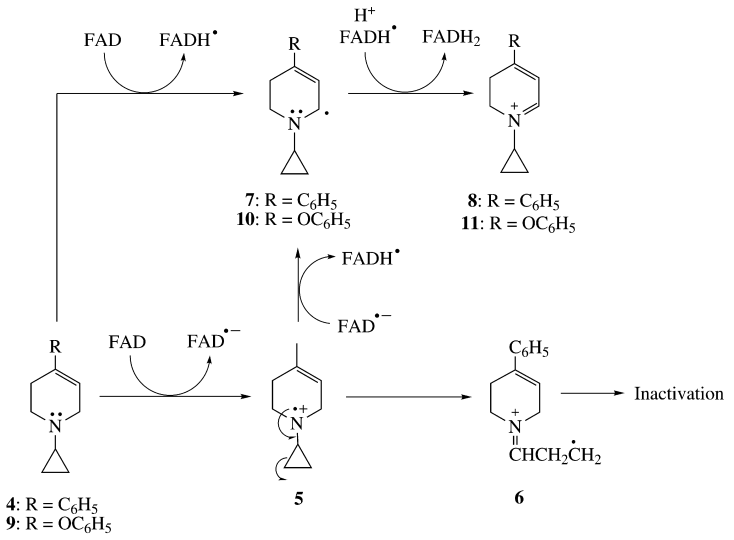
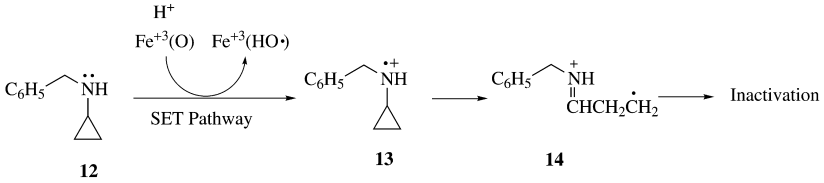
PII: S0968-0896(01)00091-8



Scheme 2. Proposed MAO catalyzed oxidation pathways of 1-cyclopropyl-4-substituted tetrahydropyridinyl derivatives.



Scheme 3. The proposed SET pathway for the cytochrome P450 mediated bioactivation of *N*-benzylcyclopropylamine (**12**).

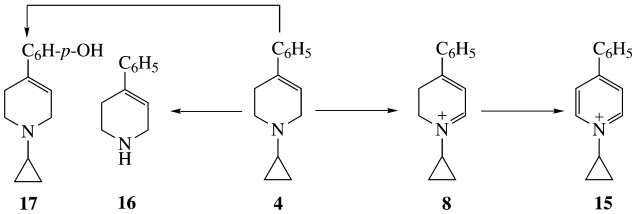
transfer (HAT) step that would convert the substrate molecule to the carbon radical intermediate (in this case **10**) without passing through the aminyl radical cation.

The SET pathway also has been proposed to account for the cytochrome P450 catalyzed oxidative *N*-dealkylation of amines.[8] Evidence supporting this hemoprotein mediated pathway is illustrated in Scheme 3 with *N*-benzylcyclopropylamine (**12**). One electron initially is transferred from the nitrogen lone pair to the P450 iron oxo group $Fe^{+3}(O)$. The resulting cyclopropylaminyl radical cation **13** ring opens to form a primary carbon centered radical (**14**) that inactivates the enzyme by alkylating an active site functionality.[9] The SET proposal, however, has been challenged.[10] For example, in support of a HAT mechanism, Dinnocenzo et al. have demonstrated a perfectly linear relationship between the isotope effects observed with a series of substrates in the P450 catalyzed reaction and the corresponding values observed in an HAT chemical model reaction.[11]

The results of our studies on the oxidative metabolism of the tetrahydropyridinyl cyclopropylamine **4** by NADPH supplemented rat liver microsomes also may be viewed as evidence supporting a HAT mechanism.[12] Three metabolic pathways were observed: (1) α-carbon oxidation leading to the dihydropyridinium (**8**) and pyridinium (**15**) metabolites, (2) loss of the cyclopropyl group to give the *N*-dealkylated metabolite **16**, and (3) aromatic hydroxylation to give the phenolic metabolite **17** (Scheme 4).

Linear rates of formation for all three metabolites were observed during the first 10 min of the incubations. The absence of effective enzyme inactivation properties of **4** in this system argues that, if formed, the distonic radical cation **6** does not react rapidly with a sensitive group at the active site of the enzyme. This behavior might be rationalized by assuming that rapid radical recombination occurs between **6** and the partially reduced P450 iron oxo group $[Fe^{+3}(HO\cdot)]$ to form the β-hydroxyiminium species **18** (Scheme 5), a pathway not available in the MAO catalyzed reaction. Subsequent hydrolysis of **18** would yield **16** and 3-hydroxypropanal (**19**) that could dehydrate to acrolein (**20**). Efforts to detect **19** and/or **20** to add support to the proposed pathway, however, were not successful.

In order to gain further insight in the metabolic pathway leading to loss of the cyclopropyl group in this system, we have examined the metabolic fate of 4-phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine (**21**) in NADPH supplemented rat liver microsomal enzyme

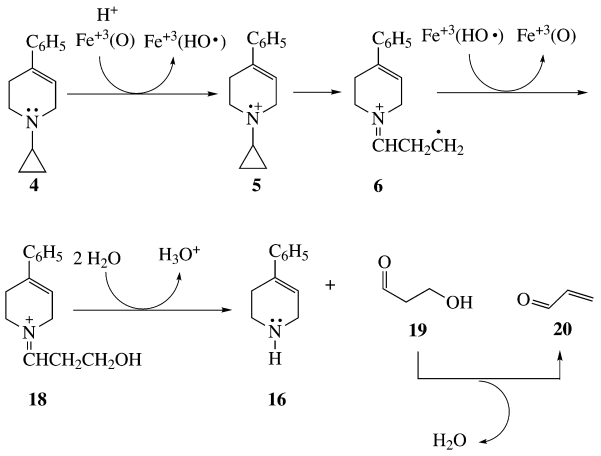


Scheme 4. The cytochrome P450 catalyzed metabolic profile of 1-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine (**4**).

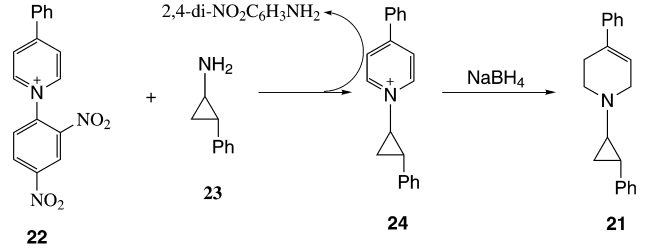
preparations. We anticipated that it would be possible to determine the metabolic fate of the phenylcyclopropyl group present in **21** more easily than was the case with the unsubstituted cyclopropyl group present in **4**.

Results and Discussion

The synthesis of the substrate **21** was readily achieved as shown in Scheme 6. The known 4-phenyl-1-(2,4-dinitrophenyl)pyridinium intermediate **22** was treated with *trans*-2-phenyl-cyclopropylamine (**23**) to yield the corresponding



Scheme 5. Proposed pathway for the cytochrome P450 catalyzed oxidative *N*-decylopropylation of 1-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine (**4**).



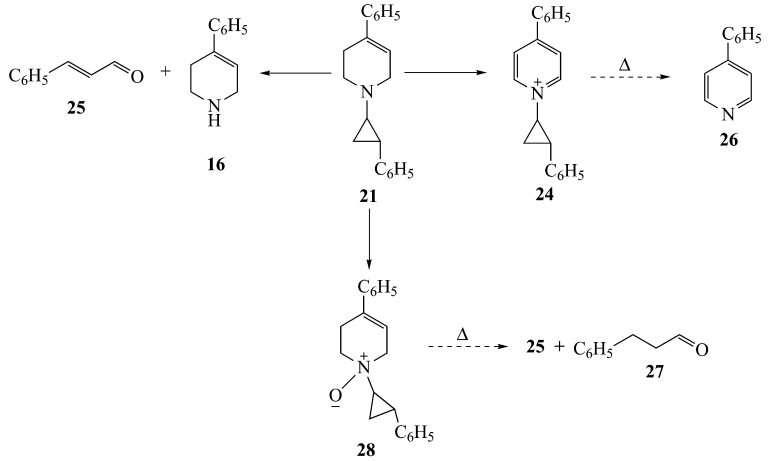
Scheme 6. Synthetic route to 4-phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine (**21**).

trans-2-phenylcyclopropylpyridinium species **24**. This compound proved to be very hygroscopic although spectroscopic data were consistent with the assigned structure (see Experimental). Crude **24** was reduced directly with $NaBH_4$ to gave the desired tetrahydropyridinyl product **21** that was stored as its stable oxalate salt.

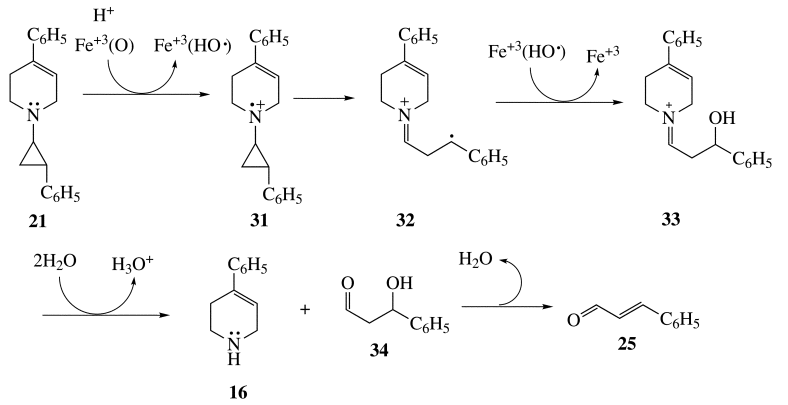
Compound **21** was stable in rat liver microsomal incubation mixtures in the absence of NADPH. Analyses by gas chromatography–electron ionization mass spectrometry (GC–EIMS) of acetonitrile isolates of incubation mixtures that had been supplemented with NADPH showed the presence of several new compounds. In addition to the starting amine, three major peaks and one minor peak were present in the total ion chromatogram (TIC) tracing. Based on comparison of the mass spectral properties observed with those of standards, the three major peaks corresponded to the structures (Scheme 7) of cinnamaldehyde (**25**), 4-phenyl-1,2,3,6-tetrahydropyridine (**16**) and 4-phenylpyridine (**26**); the minor peak was identified as 3-phenylpropanal (**27**).

The same acetonitrile isolate also was subjected to HPLC-diode array (HPLC-DA) analysis. Comparison of retention times and UV chromophores confirmed the presence of cinnamaldehyde (**25**) and the *N*-dealkylated product **16**. Neither 4-phenylpyridine (**26**) nor 3-phenylpropanal (**27**), however, were observed. Instead, the pyridinium species **24** (identified by comparison with the synthetic standard) and a second compound, with UV spectral properties similar to those of the parent substrate molecule **21** but with more polar chromatographic characteristics, were observed. Based on the metabolic fate of the corresponding 1-methyl analogue **1**,[13] we suspected this metabolite to be the *N*-oxide **28**. A synthetic standard of **28** (as a 1:1 mixture of diastereomers) was prepared by treatment of **21** with *m*-chloroperoxybenzoic acid. The UV-DA characteristics of this synthetic material confirmed the structure of this metabolite as the *N*-oxide.

The differences between the GC–EIMS and HPLC-DA results were resolved by examining the GC–EIMS



Scheme 7. Metabolic fate of 4-phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine **21** and pyrolytic degradation products of two metabolites.



Scheme 8. Proposed metabolic pathway for the conversion of the phenylcyclopropylamine **21** to cinnamaldehyde (**25**).

behavior of the synthetic pyridinium species **24** and *N*-oxide **28**. As expected, **24** underwent thermolytic *N*-dealkylation to produce 4-phenylpyridine (**26**) which accounts for the presence of this compound in the GC–EIMS tracing of the metabolic incubation isolate. The tracing obtained with the *N*-oxide showed several thermolytic products including 3-phenylpropanal and cinnamaldehyde. Consequently, the 3-phenylpropanal observed in the metabolite mixture extract by GC–EIMS may be an artifact obtained from thermolysis of **28**.

The biotransformation of the phenylcyclopropyl substrate **21** to cinnamaldehyde is of particular interest. This conversion may be rationalized according to the SET pathway (Scheme 8). Thus, the intermediate cyclopropylaminyl radical cation **29** would ring open to form the distonic primary radical cation **30**. Since there is little evidence of enzyme inactivation, we assume that **30** reacts preferentially by radical recombination with the hydroxyl radical associated with the heme moiety of the enzyme to give the β-hydroxyiminium species **31**. Subsequent hydrolysis of **31** would yield the β-hydroxyaldehyde **32** that would dehydrate spontaneously to cinnamaldehyde. The pyrolytic conversion of the *N*-oxide **28** to cinnamaldehyde, while of chemical interest, is unlikely to be related to the enzymatic formation of cinnamaldehyde. Consequently, we view these results as evidence in support of the SET pathway for the cytochrome P450 catalyzed oxidations of cyclopropylamines.

Experimental

General methods

All reagents (with the exception of solvents) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). UV–vis absorption spectra were recorded in a Beckman DU-7000 spectrophotometer. Proton and ^{13}C NMR spectra were recorded on a Bruker WP 270-MHz spectrophotometer. Chemical shifts (δ) are reported relative to the tetramethylsilane as an internal standard. Spin multiplicities are given as s (singlet) brs (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). GC–EIMS was performed on a Hewlett-Packard 5890 GC fitted with an HP-1 methylsilicon capillary column (20 m×200 μm×0.33 μm film thickness) which was coupled to a Hewlett-Packard 5870 mass selective detector and on a Fisons GC 8000 fitted with an identical HP-1 column which was coupled to a Fisons Quattro mass selective detector. The temperature program employed was as follows: 60 °C for 1 min, then 25 °C/min to 275 °C. High resolution chemical ionization mass spectrometry (HR-CIMS) was performed on a VG 7070 HF instrument using methane as the reagent gas. The HPLC system consisted of a Beckman 110 A pump set at a flow rate of 1 mL/min, a Zorbox C18 HPLC column (4.6 mm id×25 cm 5 μm particle size) and an LC-235C diode array (LC-DA) detector. The mobile phase consisted of 0.1 M acetate buffer adjusted to pH 4.5/acetonitrile (80:20, v/v). Melting points were performed on a Thomas-Hoover melting point apparatus and are uncorrected. Microanalysis were performed by Atlantic Microlabs, Inc., Norcross, GA, USA.

Microsomal preparations. Microsomal fractions were prepared from saline rinsed whole livers, freshly obtained and pooled from untreated male CRL:Cd BR Sprague–Dawley rats (250–300 g) following standard procedures.[14] A suspension of the final microsomal pellet in 20% glycerol solution (20 mM potassium phosphate buffer, pH 7.4 in 20% w/v glycerol containing 0.1 mM EDTA) was stored at −65 °C until use. Microsomal protein concentrations were determined by the bicinclonic acid method[15] and standardized relative to bovine serum albumin.

Microsomal incubations. The incubation system consisted of 1 μM total cytochrome P450 in a final volume of 1 mL 50 mM Tris–HCl buffer containing 0.1 mM EDTA, pH 7.4 at 37 °C. Substrate was added in 10 μL methanol for a final concentration of 250 μM. After preincubation at 37 °C for 2 min, the reaction was initiated by addition of 100 μL of an NADPH generating system [5 units isocitric dehydrogenase (ICDH), 10 mM $NADP^+$] to maintain a steady state concentration of 1 mM NADPH in the incubation mixture. After gentle swirling in a constant temperature water bath for 30 min the reaction was quenched by the addition of an equal volume of acetonitrile and the resulting mixture was centrifuged for 5 min (15,000×*g*). Control incubations using a denatured enzyme and a zero time sample also were conducted. The supernatants were analyzed by GC–EIMS and/or HPLC-DA.

4 - Phenyl - *trans* - 1 - (2 - phenylcyclopropyl) - 1,2,3,6 - tetrahydrozpyridine (21). A solution of the dinitropyridinium salt **22**[16] (0.9 g, 2.6 mmol) and tranylcypromine (obtained from 0.9 g, 5.2 mmol of the corresponding hydrochloride salt) in anhydrous butanol (30 mL) was heated under reflux for 12 h. The dark red color that formed initially slowly turned dark brown. The butanol was removed in vacuo and an aqueous solution of the residue was washed with dichloromethane (4×30 mL) The water was removed under reduced pressure to afford the crude hygroscopic pyridinium chloride **24**: ^{1}H NMR (DMSO-d_6) δ 9.25 (d, 2H, 2,6-PyH), 8.53 (d, 2H, 3,5-PyH), 8.15 (m, 2H, PhH), 7.65 (m, 4H, PhH), 7.35 (m, 4H, PhH), 4.72 (m, 1H, C_3H_4), 3.12 (m, 1H, C_3H_4), 2.50 (s, H_2O), 2.25 (m, 1H, C_3H_4) and 1.86 (m, 1H, C_3H_4); ^{13}C NMR (DMSO-d_6) δ 155.0, 145.0, 137.5, 132.0, 131.8, 130.0, 126.5, 126.0, 127.8, and 123.8; UV (MeOH) λ_{max} 305 nm. A portion of this product was treated directly with excess $NaBH_4$ in 25 mL methanol at 0 °C. After stirring for 30 min, the solvent was removed in vacuo and the residue in 25 mL H_2O was extracted with dichloromethane (2×20 mL). The combined organic layer was dried over Na_2SO_4, filtered, evaporated and the residue in 25 mL of ether was treated with ethereal oxalic acid. The precipitated oxalate salt was recrystallized from methanol/ether (0.36 g, 45%): mp 181–182 °C; ^{1}H NMR (DMSO-d_6) δ 7.46–7.27 (m, 10H, PhH), 6.17 (m, 1H, C5H), 3.55 (bs, 2H, C6H), 3.10 (bs, 2H, C2H) 2.59 (bs, 2H, C3H), 2.37 (bs, 1H, NCH), 2.23 (bs, 1H, NCHC*H*), 1.28 (m, 1H, NCHCH_2), 1.16 (m, 1H, NCHCH_2); ^{13}C NMR (DMSO-d_6) δ 139.5, 134.0, 128.2, 127.2, 125.7, 124.5, 119.9, 51.5, 47.4, 44.2, 26.0, 22.8; UV (MeOH) λ_{max} 209, 245 nm; GC (t_R = 8.7 min)-EIMS *m*/*z* (%) 275 (30, $M^{\cdot+}$), 184 (100), 155 (20), 115 (40), 91(45), 54 (18). Anal. calcd for $C_{22}H_{23}NO_4 \cdot 0.16H_2O$: C, 71.73; H, 6.25; N, 3.80. Found: C, 71.78; H, 6.34; N, 3.78.

4-Phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyiridine-*N*-oxide (28). A solution of *m*-chloroperoxybenzoic acid (0.63 g, 55%, 2 mmol) in dichloromethane (10 mL) was added dropwise to a solution of **21** (0.58 g, 2.11 mmol) in dichloromethane at 0 °C. The resulting mixture was stirred at 0 °C for 1 h then at room temperature for 3 h. The reaction mixture was chromatographed on a basic alumina column (30 g, eluent 5% methanol in dichloromethane) to afford **28** (0.4 g, 64%) as a powder: ^{1}H NMR ($CDCl_3$) δ 7.3–7.0 (m, 10 H, PhH), 5.9 (m, 0.5 H, C4H of one diastereomer), 5.7 (m, 0.5 H, C4H of second diastereomer), 4.03–3.94, (m, 2H, C6H), 3.6–3.5 (m, 2H, C2H), 3.07 (m, 1H, NCH), 2.88 (m, 2H, C3H), 2.15 (m, 0.5H, NHCHC*H* of one diastereomer), 1.95 (m, 1H, NHC*H* of second diastereomer), 1.05 (m, 2H, NCH_2CHCH). HR-CIMS. calcd for $C_{20}H_{21}N0$: 292.1704. Found: 292.1701.

Acknowledgements

This work was supported by the National Institute of Neurological and Communicative Disorders and Strokes (NS 28792) and by the Harvey W. Peters Center for the Study of Parkinson's Disease.

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