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Interactions of 1-methyl-3-phenylpyrrolidine and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane with monoamine oxidase B

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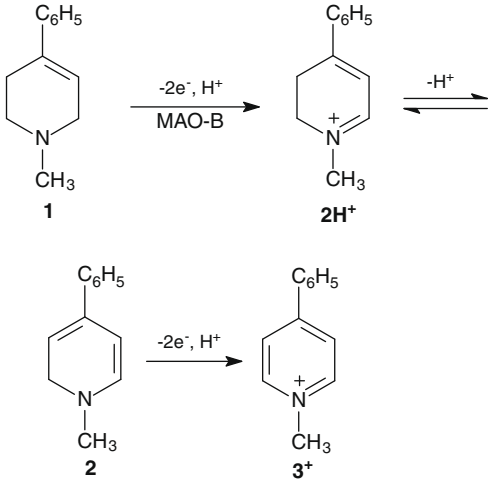
ABSTRACT

The parkinsonian inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its corresponding five-membered ring analogue 1-methyl-3-phenyl-3-pyrroline are cyclic tertiary allylamines and good substrates of monoamine oxidase B (MAO-B). The MAO-B catalyzed 2-electron α-carbon oxidation of this class of substrates appears to be dependent on the presence of the allylic π-bond since the corresponding saturated piperidinyl analogue of MPTP is reported not to be an MAO-B substrate. The only saturated cyclic tertiary amine known to act as an MAO-B substrate is the 3,4-cyclopropyl analogue of MPTP, 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane. As part of our ongoing studies we have examined the MAO-B substrate properties of the corresponding pyrrolidinyl analogue, 1-methyl-3-phenylpyrrolidine, and the 3,4-cyclopropyl analogue, 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane. The results document that both the pyrrolidinyl analogue [K_m = 234 μM; V_{max} = 8.37 nmol/(min-mg mitochondrial protein)] and the 3,4-cyclopropyl analogue [K_m = 148 μM; V_{max} = 16.9 nmol/(min-mg mitochondrial protein)] are substrates of baboon liver mitochondrial MAO-B. We also have compared the neurotoxic potential of these compounds in the C57BL/6 mouse. The results led us to conclude that these compounds are not MPTP-type neurotoxins.

1. Introduction

The flavoenzyme monoamine oxidase B (MAO-B) catalyzes the 2-electron α-carbon oxidation of a variety of endogenous and exogenous arylalkylamines such as dopamine, epinephrine and benzylamine.[1] Also among its substrates is the parkinsonian inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] (Scheme 1).[2] MPTP is a member of the cyclic tertiary allylaminyl class of MAO-B substrates and is oxidized at C-6 of the tetrahydropyridinyl ring to yield the conjugated eniminiumyl metabolite, the 1-methyl-4-phenyl-2,3-dihydropyridiniumyl species [MPDP$^+$ (**2H**$^+$)].[3] Presumably the corresponding conjugate base **2** undergoes a second 2-electron oxidation to generate the 1-methyl-4-phenylpyridiniumyl metabolite MPP$^+$ (**3**$^+$) that is thought to act as the ultimate neurotoxin.[2,4,5] Inhibitors of MAO-B that block the conversion of MPTP to MPP$^+$ protect experimental animals against the neurotoxic action of MPTP.[6] The MAO-B substrate properties of this cyclic tertiary aminyl class of substrates are thought to be dependent on the presence of the allylic π-bond since the corresponding saturated piperidinyl analogue **4** of MPTP is reported



Scheme 1. The MAO-B-catalyzed oxidation of MPTP (**1**) at C-6 of the tetrahydropyridinyl ring to yield the corresponding conjugated eniminiumyl metabolite MPDP$^+$ (**2H**$^+$). MPDP$^+$, via the conjugate base **2**, is further oxidized to yield the pyridiniumyl product MPP$^+$ (**3**$^+$).

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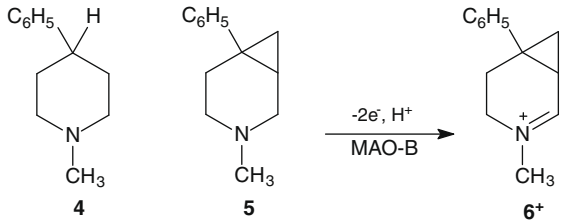
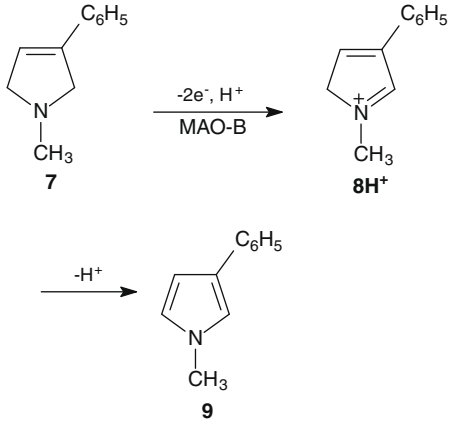
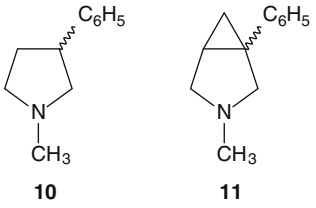


Scheme 2. The structures of 1-methyl-4-phenylpiperidine (**4**) and 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**5**). The MAO-B catalyzed oxidation of **5** yields the iminiumyl metabolite $\mathbf{6}^{+}$.



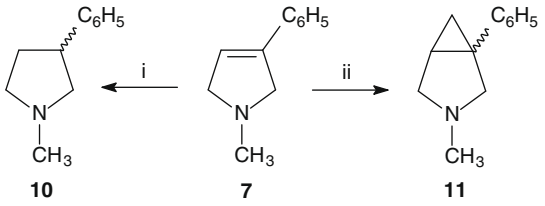
Scheme 3. The MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline (**7**) to yield the iminiumyl $\mathbf{8H}^{+}$ that, following deprotonation, gives the stable product 1-methyl-3-phenylpyrrole (**9**).



Scheme 4. The structures of (±)-1-methyl-3-phenylpyrrolidine (**10**) and (±)-3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**11**).

not to be an MAO-B substrate (Scheme 2).[7,8] The only known saturated cyclic tertiary aminyl MAO-B substrate is the 3,4-cyclopropyl analogue of MPTP, racemic 3-methyl-6-phenyl-3-azabicyclo[4.1.0]-heptane (**5**),[9] that is converted to the corresponding iminiumyl metabolite $\mathbf{6}^{+}$. The π-bond characteristics of the cyclopropyl group may be responsible for the good substrate properties of **5**.[9]

Another member of the cyclic tertiary allylaminyl class of MAO-B substrates is the five-membered ring MPTP analogue 1-methyl-3-phenyl-3-pyrroline (**7**) (Scheme 3).[10–12] MAO-B catalyzes the ring α-carbon oxidation of **7** to yield 1-methyl-3-phenylpyrrole (**9**) as the final product. This 2-electron oxidation process most likely proceeds via $\mathbf{8H}^{+}$, the short lived conjugate acid of the pyrrolyl product **9**.[13] As part of our ongoing investigations of five-membered azacyclic systems, we have examined the interactions of the corresponding saturated pyrrolidinyl [(±)-1-methyl-3-phenylpyrrolidine (**10**)] and 3,4-cyclopropyl [(±)-3-methyl-1-phenyl-3-azabicyclo[3.1.0]-hexane (**11**)] analogues of 1-methyl-3-phenyl-3-pyrroline with MAO-B (Scheme 4). In view of the π-bond characteristics of the cyclopropyl group, we expected that **11** would be a good MAO-B substrate. On the other hand, the pyrrolidinyl analogue **10**, being a homolog of **4**, should be devoid of MAO-B substrate properties.



Scheme 5. Synthetic pathway to compounds racemic **10** and racemic **11**. Key: (i) H_2 (atm), PtO_2, rt; (ii) $(C_2H_5)_2Zn$, CH_2I_2.

We also have examined the possibility that the saturated analogues **10** and **11** may act as neurotoxins in the C57BL/6 mouse.[14] The neurotoxic action of MPTP is dependent upon its metabolic conversion by MAO-B to yield the charged pyridinium species MPP^{+} ($\mathbf{3}^{+}$).[2] After accumulating in the inner mitochondrial membrane of the nigrostriatal nerve terminals, MPP^{+} is thought to act by inhibiting complex I of the mitochondrial respiratory chain.[6,15] The mitochondrial accumulation is driven by the transmembrane electrochemical gradient which appears to rely upon the presence of the permanent charge of MPP^{+}.[16,17] Since the MAO-B catalyzed 2-electron oxidations of substrates **10** and **11** may yield charged intermediates and end products, the possibility exists that these substrates could display MPTP-type neurotoxicity. In this study we have tested this hypothesis by measuring ex vivo striatal dopamine concentrations in C57BL/6 mice 7 days after administration of **10** and **11**.[13] In addition, the possibility that **10** and **11** may be converted to charged intermediates by MAO-B was further investigated using mass spectrometry.

2. Results

2.1. Chemistry

Both **10** and **11** were synthesized from 1-methyl-3-phenyl-3-pyrroline (**7**)[13] as shown in Scheme 5. Atmospheric pressure hydrogenation of **7** in the presence of PtO_2 gave **10** in high yield (71%).[18] In a reaction similar to that previously described for the cyclopropylation of MPTP,[9] treatment of **7** with an excess of CH_2I_2 and diethylzinc yielded **11** (24%). Both substrates were converted to their respective oxalate salts. Compounds **10**[19] and **11**[20] have been prepared previously by alternative synthetic pathways. N-Methylation of 4-phenylpiperidine with formic acid/formaldehyde (Eschweiler–Clarke reaction) gave compound **4**.[13,21]

2.2. General enzymology

In the present study we have examined the MAO-B substrate properties of the saturated cyclic tertiary amines **10** and **11**. As enzyme source we have employed the mitochondrial fraction obtained from baboon liver tissue since it exhibits a high degree of MAO-B catalytic activity.[22] Also, based on the similarity of the steady-state kinetic parameters (K_m and V_{max}) between baboon and human MAO-B, the interactions of substrates with MAO-B obtained from baboon liver tissue appears to be similar to those of the human form of the enzyme.[22] The rates of oxidation of **10** and **11** by baboon liver mitochondrial MAO-B were measured by monitoring the amount of H_2O_2 generated in the presence of the enzyme. In the catalytic cycle of MAO-B, one mole of O_2 is reduced to H_2O_2 for each mole of substrate oxidized. The concentration of H_2O_2 in the enzyme reactions can be conveniently measured by the peroxidase-coupled spectrophotometric assay system described previously.[23] For the purpose of this study we have chosen a discontinuous assay protocol with an incubation time of 20 min (for substrate **10**) or 15 min (for substrate **11**). As illustrated in

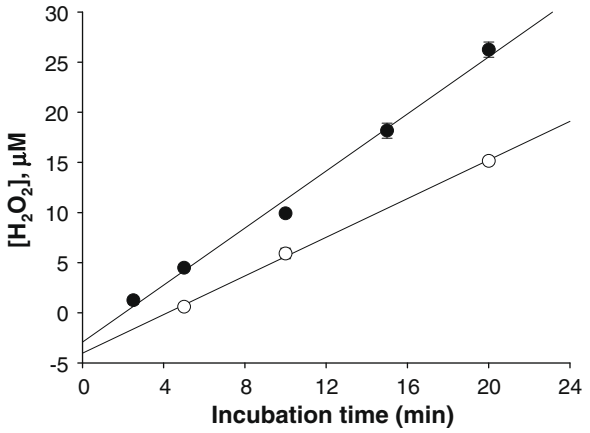
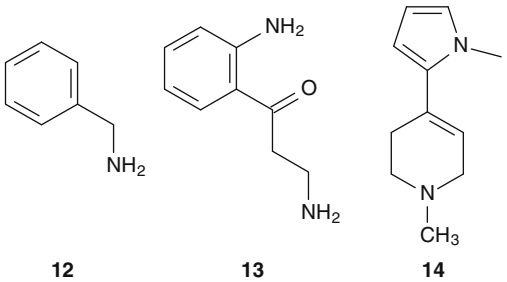


Figure 1. The linear plots of the baboon liver mitochondrial MAO-B (0.15 mg protein/mL) catalyzed oxidations of substrates **10** (500 μM; open circles) and **11** (250 μM; filled circles) as measured by H_2O_2 production.[23]

Figure 1, the H_2O_2 generated as a result of the oxidation of **10** and **11** in the presence of baboon liver mitochondrial MAO-B remained linear for at least 20 min at substrate concentrations of 500 and 250 μM, respectively. Control incubations carried out in the absence of the test substrates documented that the background H_2O_2 generation by the mitochondrial fractions was less than 2.3 μM. This is approximately 6–11% of the H_2O_2 measured in 20 min incubations of substrate **10** (21–24 μM) and in 15 min incubations of substrate **11** (33–39 μM) at V_{max} concentrations. This result verifies that the H_2O_2 generated (89–94%) by the mitochondrial fractions are dependent upon the presence of the test substrates.

To estimate the extent of the H_2O_2 produced by non-MAO-B mediated oxidation of substrates **10** and **11**, the baboon liver mitochondrial MAO-B were pre-inactivated with the MAO-B selective inactivator (*R*)-deprenyl.[22] Following incubation of substrate **11** (250 μM) with the (*R*)-deprenyl pre-inactivated mitochondria, only low concentrations of H_2O_2 were observed. For example, the H_2O_2 concentration (1.79 ± 0.11 μM) with the inactivated mitochondrial preparation was only 6.6% of that formed with the fully active preparation (27.3 ± 0.53 μM). Similarly, when substrate **10** (500 μM) was incubated with the (*R*)-deprenyl pre-inactivated mitochondria only 1.92 ± 0.06 μM of H_2O_2 was observed which is 9.3% of the amount formed (20.6 ± 0.08 μM) with the fully active preparation. These results confirm that the oxidations of **10** and **11** in the presence of the baboon liver mitochondrial fraction used here as enzyme source are MAO-B dependent. The oxidation activity not suppressed by (*R*)-deprenyl has been observed previously where tetrahydropyridinyl analogues served as the MAO-B substrates.[22] At least for baboon liver mitochondria the residual activity is not related to the action of the MAO-A isoform since the baboon liver mitochondria are known to be devoid of MAO-A activity.[22] Using the same experimental protocol, analogous results were obtained with the known MAO-B substrates benzylamine (**12**), kynuramine (**13**), MPTP (**1**) and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine [MMTP (**14**)] (Scheme 6). For example, following incubations of **12** (500 μM), **13** (500 μM), MPTP (500 μM) and MMTP (250 μM) with the (*R*)-deprenyl pre-inactivated mitochondria, the concentrations of H_2O_2 measured were 5–13% of those measured with fully active preparations.



12 13 14

Scheme 6. The structures of benzylamine (**12**), kynuramine (**13**) and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine [MMTP (**14**)].

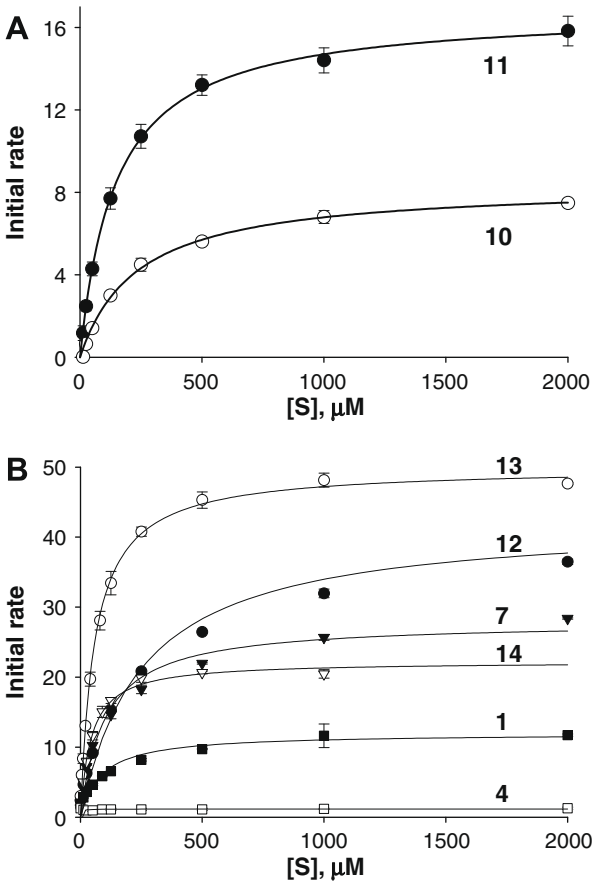


Figure 2. Plots of initial rate versus substrate concentration for the steady-state baboon liver mitochondrial MAO-B (0.15 mg protein/mL) catalyzed oxidation of test substrates **10** (open circles, 20 min incubation period) and **11** (filled circles, 15 min incubation period) at 37 °C (Panel A). The corresponding plots of initial rate vs. substrate concentration for kynuramine (**13**, open circles), benzylamine (**12**, filled circles), pyrroline **7** (filled triangles), MMTP (**14**, open triangles), MPTP (**1**, filled squares) and **4** (open squares) (Panel B). Incubation periods varied from 6 to 15 min (see Section 4) and the initial rates are expressed as nmoles H_2O_2 formed/min-mg protein.

2.3. Steady-state MAO-B substrate properties of 10 and 11

The values of the steady-state kinetic parameters (K_m and V_{max}) for the baboon liver mitochondrial MAO-B catalyzed oxidation of the test substrates **10** and **11** were determined by measuring the initial rates of H_2O_2 production at eight substrate concentrations spanning at least two orders of a magnitude.[23] Plots of initial rates versus substrate concentration for **10** and **11** (Fig. 2A) and for **1**, **7**, **12**, **13** and **14** (Fig. 2B) gave the K_m and V_{max} values reported in Table 1. While the steady-state baboon liver mitochondrial MAO-B substrate properties of MPTP (**1**) and **7** have been previously reported,[13] the kinetic parameters for these substrates were reevaluated in the present study since different mitochondrial preparations may exhibit differing activities. The kinetic values

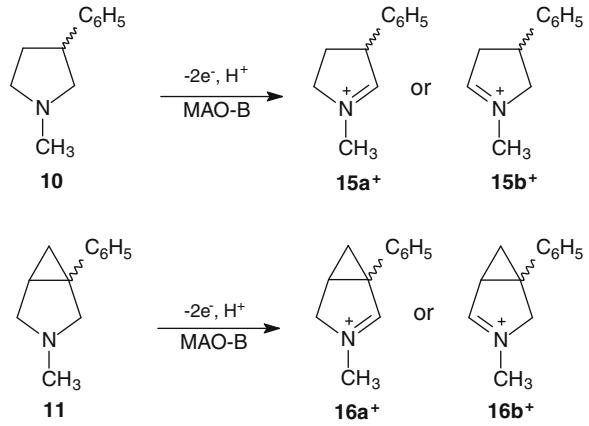
Table 1
Values of the steady-state kinetic parameters for the MAO-B catalyzed oxidation of selected aminyl substrates

	V_{max}[a]	K_m (μM)	V_{max}/K_m[b]
10	8.37 ± 0.25	234 ± 23.5	0.04
11	16.9 ± 0.60	148 ± 7.36	0.11
MPTP (**1**)	11.7 ± 0.15; 6.7[c]	81.8 ± 7.01; 173[c]	0.14
7	27.9 ± 0.13; 9.2[c]	100 ± 8.40; 54.6[c]	0.28
MMTP (**14**)	22.2 ± 0.37	43.4 ± 3.20	0.51
Benzylamine	42.4 ± 0.41	249 ± 0.30	0.17
Kynuramine	50.0 ± 0.41	58.9 ± 4.89	0.85
4	not a substrate		

[a] Values are expressed in nmol/(min-mg mitochondrial protein).
[b] Values are expressed in (min-mg mitochondrial protein)$^{-1}$.
[c] Values obtained from Ref.13.

that were recorded for substrate **11** (K_m = 148 μM; V_{max} = 16.9 nmol/min-mg protein) documents that **11** acts as a good substrate of baboon liver MAO-B. Based on the V_{max}/K_m values, it is apparent that the cyclopropyl analogue **11** [V_{max}/K_m = 0.11 (min-mg protein)$^{-1}$] and MPTP (**1**) [V_{max}/K_m = 0.14 (min-mg protein)$^{-1}$] have comparable substrate properties and that both **1** and **11** are poorer substrates than the pyrrolinyl analogue **7** [V_{max}/K_m = 0.28 (min-mg protein)$^{-1}$].

Although poorer than the corresponding pyrrolinyl (**7**) and fused cyclopropyl (**11**) analogues, the saturated pyrrolidinyl analogue **10** also proved to be an MAO-B substrate [V_{max}/K_m = 0.04 (min-mg protein)$^{-1}$]. This was an unexpected outcome in view of the report that the piperidinyl homolog **4**, the saturated analogue of MPTP (**1**), is stable in the presence of mouse MAO-B.[7] Compound **4** also proved to be stable in the presence of baboon liver mitochondrial MAO-B (Fig. 2B and Table 1). These results should prove useful for the further characterization of cyclic tertiary aminyl structural features required for MAO-B substrates.



Scheme 7. The proposed MAO-B-catalyzed oxidation of **10** and **11** to yield the iminiumyl metabolites **15a**$^+$/**15b**$^+$ and **16a**$^+$/**16b**$^+$, respectively.

2.4. Mass spectrometry

To investigate the structures of the metabolites that form upon the MAO-B catalyzed ring α-carbon oxidation of **10** and **11**, reactions containing 100 μM of the substrates, respectively, and baboon liver mitochondrial MAO-B were incubated for 14 h at 37 °C. LC-ESI-MS analysis of the incubation mixtures showed two major peaks in the total ion tracings of both incubations. For substrate **10**, the parent compound eluted at 6.77 min with the expected MH$^+$ ion at *m*/*z* 162. The metabolite derived from **10** eluted at 6.48 min and displayed M$^+$ at *m*/*z* 160 (Fig. 3A). This mass of this metabolite is consistent with the isomeric 2-electron oxidation products, **15a**$^+$ and **15b**$^+$ (Scheme 7), that may form as a result of the action of MAO-B on **10**. Similarly, for the incubations containing **11**, the parent substrate eluted at 6.76 min and displayed

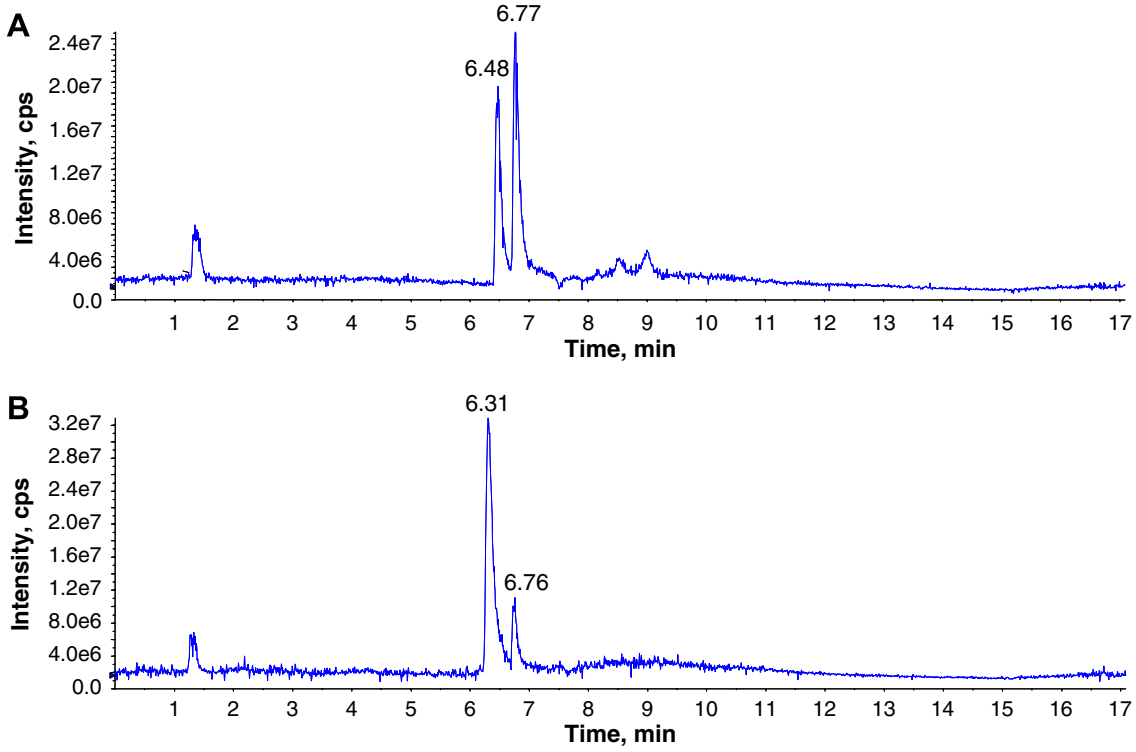


Figure 3. LC-ESI-MS total ion chromatograms of incubations containing 100 μM of **10** (Panel A) and **11** (Panel B), respectively, and baboon liver mitochondrial MAO-B (0.6 mg protein/mL). The incubations were carried out for 14 h at 37 °C.

the expected MH^+ ion at m/z 174 while the metabolite eluted at 6.31 min and had M^+ at m/z 172 (Fig. 3B). Again the mass of the metabolite is consistent with the isomeric iminiumyl species, **16a**$^+$ and **16b**$^+$ (Scheme 7), that are expected to form upon the action of MAO-B on **11**. The detection of only one metabolite peak for each of **10** and **11**, however, suggests that ring α-carbon oxidation of these substrates is highly regioselective. These metabolite peaks were absent from the total ion chromatograms of incubations of **10** and **11** in the absence of enzyme (data not shown).

2.5. Neurotoxicity studies

It has been documented that the administration of MPTP to C57BL/6 mice causes degeneration of nigrostriatal neurons.[14] The extent of neuronal damage can be estimated by measuring the degree of dopamine depletion in the striatum 7–10 days following treatment. In a frequently used protocol, aged mice are treated with single intraperitoneal injections of MPTP (95–238 μmol/kg).[24,25] In this study we investigated whether similar treatment of C57BL/6 mice with **10** or **11** also results in nigrostriatal injury as indicated by a loss of dopamine in the striatum. For each of the test compounds a group of control mice (n = 10) was treated with saline (0.20 mL/30 g mouse). Again for each test compound, a positive control group (n = 10 mice) was treated with MPTP hydrochloride (167 μmol/kg). A third group of mice (n = 10 for each test compound) was treated with the oxalate salts of **10** or **11** (238 μmol/kg). The animals were sacrificed 7 days after treatment and the dopamine concentrations in the dissected striata were determined by HPLC-ECD analysis as described in Section 4.[26]

As expected MPTP treatment significantly (p <0.01) depleted the dopamine levels to 36% (Fig. 4A) and 27% (Fig. 4B) of the control values. In contrast, treatment with neither **10** nor **11** depleted striatal dopamine levels with the measured dopamine concentrations at 99% (Fig. 4A) and 111% (Fig. 4B) of the control values, respectively.

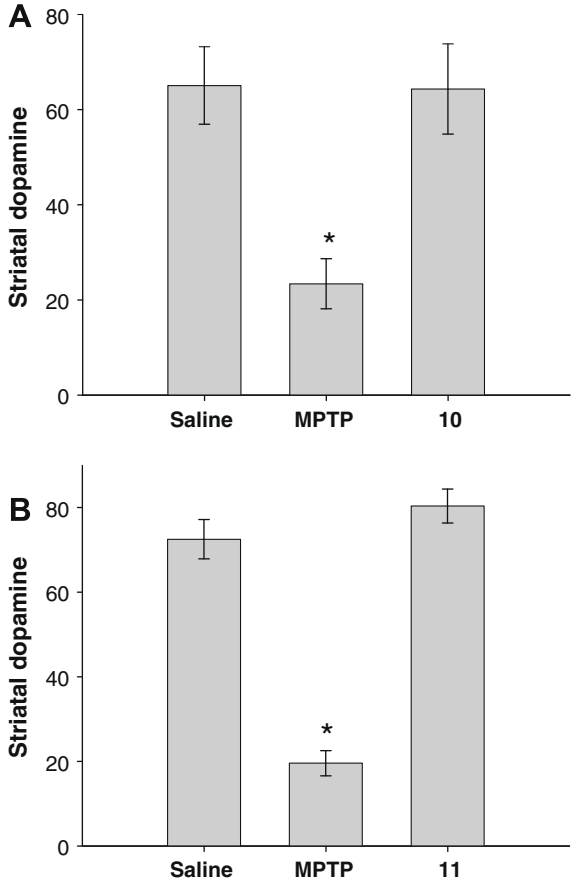


Figure 4. Striatal dopamine levels of C57BL/6 male mice (n = 10 mice/group) measured seven days after treatment (ip) with saline, MPTP·HCl (167 μmol/kg), **10**·oxalate (238 μmol/kg) (Panel A) or **11**·oxalate (238 μmol/kg) (Panel B). Dopamine levels are expressed as pmol/mg tissue. *Significantly different (p <0.01) from the saline treated group.

3. Discussion

The results document that the fused 3,4-cyclopropylpyrrolidinyl analogue **11** has substrate properties comparable to those of the known MAO-B pyrrolinyl substrate **7** when examined with baboon liver mitochondrial MAO-B. This result is analogous to that observed with the corresponding six-membered series, that is, MPTP (**1**) and its fused 3,4-cyclopropylpiperidinyl analogue **5**.[9] As mentioned in the introduction, the π-bond characteristics of the cyclopropyl group may be responsible for the observation that the saturated substrates **5** and **11** are approximately as good MAO-B substrates as their corresponding allylaminyl analogues MPTP (**1**) and **7**, respectively.[9] The saturated pyrrolidinyl analogue **10** also was shown to be a relatively good substrate for baboon liver mitochondrial MAO-B, with a turnover comparable to that of MPTP. This result is in contrast to the behavior of the saturated piperidinyl analogue **4** of MPTP that is not an MAO-B substrate,[7,8] behavior that was confirmed in the present study. This result, therefore, suggests that the presence of an allylic π-bond or 3,4-cyclopropyl-fused system is not a prerequisite for cyclic tertiary amines to display MAO-B substrate properties. This finding should provide additional opportunities to map the nature of the interactions of MAO-B with tertiary aminyl substrates and should alert investigators to the possibility of MAO-B substrate potential of compounds under development as future therapeutic agents.

Also noteworthy is the observation that the α-carbon oxidation of MPTP and its 3,4-cyclopropyl analogue **5** occurs regioselectively at C-6.[9] This suggests that the electronic properties of the allylic π-bond and cyclopropyl group may contribute to the stabilization of the corresponding α-carbon radicals that may be intermediary in the catalytic mechanism of MAO-B.[9] This analysis could explain why the piperidinyl derivative **4** is stable in the presence of MAO-B. The finding of the present study that the pyrrolidinyl analogue **10** acts as a relatively good MAO-B substrate, however, does not support this hypothesis. In addition to electronic properties, the three dimensional structures and binding orientations within the active site are likely to contribute to the substrate properties and the regioselectivity of oxidation of this class of compounds. The factors that determine the substrate properties of cyclic tertiary amines remain to be identified. In the present study, mass spectral analysis suggests that the MAO-B catalyzed two-electron oxidations of **10** and **11** also occur regioselectively with the formation of only a single product for each substrate. Evidence suggests that the products are the corresponding iminiumyl metabolites **15a**$^+$/**15b**$^+$ and **16a**$^+$/**16b**$^+$ for the metabolism of **10** and **11**, respectively.

The neurotoxicity of MPTP is thought to be the consequence of its MAO-B-catalyzed metabolism in the brain that ultimately yields the pyridiniumyl mitochondrial toxin MPP^+.[2,6] Evidence suggests that the mitochondrial toxicity and subsequent neurotoxic action of MPP^+ rely on it being permanently charged.[16,17] Consistent with this view, 1-methyl-3-phenyl-3-pyrroline (**7**), which is metabolized to a neutral pyrrolyl species, is not neurotoxic.[13] In this investigation we also have evaluated **10** and **11** as potential MPTP-type nigrostriatal neurotoxins. The results document that, unlike MPTP, these cyclic tertiary amines are not neurotoxic. While the reasons for this lack of neurotoxicity are not known, an explanation may be

that the pharmacokinetic properties and in vivo molecular interactions of **10** and **11** and their respective MAO-B-generated products are different from those of MPTP and MPP^+. Among the pharmacokinetic factors important for the neurotoxic action of MPTP are a high degree of blood–brain barrier permeability of MPTP and slow clearance of MPP^+ from the brain.[27] Among the molecular interactions that lead to neuronal death are the accumulation of MPP^+ in the nigrostriatal nerve terminals via the plasma membrane dopamine transporter (DAT),[28,29] the energy-dependent accumulation of MPP^+ within the inner mitochondrial membrane[16] and inhibition of complex I of the mitochondrial respiratory chain.[15] The possibility also exists that the proposed iminiumyl metabolites that arise as a consequence of the MAO-B catalyzed oxidation of **10** and **11** may be converted to neutral species in vivo. Such neutral species are not expected to be mitochondrial toxins. For example, deprotonation of **15a**/**b**$^+$ would yield the corresponding 2-pyrrolinyl analogue which may undergo a second α-carbon oxidation and deprotonation to generate the pyrrolyl species. Iminiumyl **16a**/**b**$^+$ in turn may rearrange to form the neutral six-membered dihydropyridine species. These proposed transformations require further investigation.

In conclusion, the present study shows that the structural requirements for cyclic tertiary amines to act as MAO-B substrates are less stringent than previously thought. Additionally, the data presented in this paper demonstrate that the MAO-B-catalyzed oxidations of cyclic tertiary amines leading to charged metabolites do not necessarily lead to neurotoxic outcomes. The complex sequence of events required for the parkinsonian inducing properties observed with MPTP does extend to the five-membered azacyclic systems reported here and in earlier publications. The findings reported here may alert investigators to the possibility that drugs under development which incorporate pyrrolidinyl and azabicyclo[3.1.0]hexane moieties are potential substrates for the MAO enzymes. It is however unlikely that the α-carbon oxidations of such drugs would lead to MPTP-type neurotoxic events.

4. Experimental

Caution. MPTP is a nigrostriatal neurotoxin and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously.[30]

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma–Aldrich and were used without purification. MPTP·HCl (**1**) and kynuramine·2HBr (**13**) were purchased from Sigma–Aldrich while the oxalate salts of 1-methyl-3-phenyl-3-pyrroline (**7**)[13] and MMTP (**14**)[31] were prepared as described previously. Benzylamine (**12**) (Merck) was converted to the HCl salt in ethanol. Petroleum ether used in this study had a distillation range of 40–60 °C. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Varian Gemini 300 spectrometer at frequencies of 300 MHz and 75 MHz, respectively, and on a Bruker Avance III 600 spectrometer at frequencies of 600 and 150 MHz, respectively. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the deuterated methanol (CD_3OD) or DMSO-d_6. Spin multiplicities are given as s (singlet), d (doublet) or m (multiplet). Fast atom bombardment mass spectra (FAB-MS) were recorded with a VG 7070E mass spectrometer while direct insertion electron impact ionization mass spectra (EI-HRMS) were obtained with an AutoSpec ETOF (Micromass). Melting points (mp) were determined on a Stuart SMP10 melting point apparatus and are uncorrected. UV–vis spectra were recorded on a Shimadzu UV-2100 double-beam spectrophotometer. Column chromatography was carried out with Fluka aluminum oxide (Brockmann Activity I). To determine the purity of the oxalic acid salts of **10**, **11** and **4**, HPLC analyses were carried out with an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector (see Supplementary data). HPLC grade acetonitrile (Merck) and Milli-Q water (Millipore) was used for the chromatography.

4.2. Synthesis of the oxalic acid salt of 1-methyl-3-phenylpyrrolidine (10)

1-Methyl-3-phenyl-3-pyrroline (**7**) (12.04 mmol, 1.92 g) and PtO_2 (1.32 mmol, 0.3 g) in 60 mL methanol was stirred at room temperature in an atmosphere of hydrogen.[18] After 14 h of stirring the catalyst was removed via filtration and the solvent was evaporated to yield the free base **10** as a light yellow oil (71%). Compound **10** was converted into its oxalic acid salt in diethyl ether and recrystallized from boiling methanol to give white powdery crystals: yield 36%; mp 148–149 °C; ^{1}H NMR (Varian Gemini 300, DMSO-d_6) δ 2.05 (m, 1H), 2.37 (m, 1H), 2.85 (s, 3H), 3.23 (m, 1H), 3.41 (m, 2H), 3.63 (m, 2H), 7.23–7.34 (m, 5H); ^{13}C NMR (Varian Gemini 300, DMSO-d_6) δ 32.0, 40.6, 42.2, 54.8, 60.0, 127.0, 127.3, 128.63, 140.2, 165.0; EI-HRMS *m*/*z*: calcd 161.1205, found 161.1201 (M^+).

4.3. Synthesis of the oxalic acid salt of 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (11)

Compound **11** was synthesized from 1-methyl-3-phenyl-3-pyrroline (**7**)[13] according to the procedure previously reported for the cyclopropylation of MPTP.[9] The crude product (24%) was purified by aluminum oxide chromatography (petroleum ether/ethyl acetate 50:50) and converted into its oxalic acid salt in diethyl ether. Compound **11** has been previously reported as the hydrochloric acid salt:[20] yield 8.4%; mp 141–143 °C; ^{1}H NMR (Varian Gemini 300, CD_3OD) δ 1.22 (m, 1H), 1.37 (m, 1H), 2.16 (m, 1H), 2.96 (s, 3H), 3.59 (m, 2H), 3.82 (m, 1H), 4.01 (m, 1H), 7.23–7.37 (m, 5H); ^{13}C NMR (Varian Gemini 300, CD_3OD) δ 24.4, 32.4, 41.1, 49.5, 58.6, 61.5, 128.1, 128.3, 129.8, 139.8, 166.5; FAB-MS *m*/*z*: 174 (MH^+); EI-HRMS calcd 173.1205, found 173.1200 (M^+).

4.4. Synthesis of the oxalic acid salt of 1-methyl-4-phenylpiperidine (4)

4-Phenylpiperidine (1.55 mmol; 0.25 g) was cooled to 0 °C and 98% formic acid (9.30 mmol; 358 μL) followed by 36.5% aqueous formaldehyde (9.30 mmol; 702 μL) were carefully added. The reaction mixture was stirred at 80 °C for 3 h and water (14 mL) followed by an aqueous solution (14 mL) of sodium carbonate (33 mmol) was added to the reaction. The reaction was extracted to diethylether (3 × 20 mL) and dried over anhydrous magnesium sulfate (4 g). Upon removal of the solvent a light yellow oil remained which was converted into the oxalic acid salt in diethyl ether. The oxalate salt was recrystallized from boiling methanol to give colorless crystals: yield 62%; mp 159–160 °C; ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.93 (m, 4H), 2.74 (m, 4H), 3.00 (m, 2H), 3.43 (d, 2H, J = 10.8 Hz), 7.22 (m, 3H), 7.30 (m, 2H); ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 29.7, 38.5, 42.5, 53.5, 126.6, 126.6, 128.6, 144.3, 165.2; EI-HRMS *m*/*z*: calcd 175.1361, found 175.1360 (M^+).

4.5. Steady-state MAO-B activity measurements

Baboon liver mitochondria were isolated according to the previously reported procedure and stored at −70 °C.[32] To the mitochondrial isolates were added one volume sodium phosphate buffer

(100 mM, pH 7.4) containing 50% glycerol (w/v) and the protein concentrations were determined by the method of Bradford.[33] The K_m and V_{max} values for the MAO-B catalyzed oxidation of the oxalate salts of the test substrates **10** and **11** were determined by measuring the initial rates of H_2O_2 generation at eight substrate concentrations spanning at least two orders of magnitude (12.5–2000 μM). The reactions contained 85 μL of the chromogenic solution[23] and were carried out in a final volume of 500 μL (in 100 mM sodium phosphate buffer, pH 7.4). The chromogenic solution was prepared in 100 mM sodium phosphate buffer (pH 7.4). The enzyme concentration used was 0.15 mg mitochondrial protein/mL. Following incubation at 37 °C for a period of 20 min (substrate **10**) or 15 min (substrate **11**), the reactions were terminated by the addition of 10 μL (*R*)-deprenyl (5 mM) and placing the reactions on ice. The samples were centrifuged at 16,000*g* for 10 min and the H_2O_2 concentrations were measured by the peroxidase-coupled spectrophotometric assay system described previously.[23] Quantitative estimations were made by means of a linear calibration curve that ranged from 11 to 88 μM of H_2O_2. The initial rates as a function of substrate concentration were fitted to the Michaelis–Menten equation using the one site binding model incorporated into the GraphPad Prism software package (GraphPad Software Inc.). All measurements were carried out in triplicate and the K_m and V_{max} values were expressed as means ± standard error of the mean (SEM). The steady-state MAO-B catalyzed oxidation properties of benzylamine (**12**), kynuramine (**13**), MPTP (**1**), MMTP (**14**), **7** and **4** were determined following the same protocol with the exception that the experiments were carried out in duplicate and the incubation times (the time intervals for which oxidation remains linear) were chosen as follows: benzylamine (8 min), kynuramine (6 min), MPTP (12.5 min), MMTP (10 min) and **7** (10 min). For **4** the incubation time was 15 min.

4.6. Linearity of oxidation of the aminyl substrates

To determine the time interval for which MAO-B catalyzed production of H_2O_2 remains linear, the oxalate salts of test substrates **10** (500 μM) and **11** (250 μM) and 85 μL of the chromogenic solution[23] were incubated at 37 °C with 0.15 mg protein/mL of the baboon liver mitochondrial fraction. The reactions were carried out in 100 mM sodium phosphate buffer (pH 7.4) to a final volume of 500 μL. At the required time points (2.5–20 min), the reactions were terminated with the addition of 10 μL (*R*)-deprenyl (5 mM) and the MAO-B-generated H_2O_2 were measured as described previously.[23] All measurements were conducted in triplicate and the concentrations of H_2O_2 were expressed as means ± SEM.

4.7. (*R*)-Deprenyl studies

To estimate the extent of the H_2O_2 produced by non-MAO-B mediated oxidation of substrates **10** and **11**, baboon liver mitochondria (0.3 mg protein/mL) were preincubated with the hydrochloric acid salt of the MAO-B selective inactivator (*R*)-deprenyl (3×10^{-6} M). These preincubations were carried out at 37 °C for 30 min in sodium phosphate buffer (100 mM, pH 7.4).[22] The preinactivated mitochondrial fractions were then added to the test substrates **10** (500 μM) and **11** (250 μM) to yield final concentrations of 0.15 mg mitochondrial protein/mL. The volume of these incubation mixtures were 500 μL and contained 85 μL of the chromogenic solution.[23] The reactions were incubated for 15 min at 37 °C and terminated with the addition of 10 μL (*R*)-deprenyl (5 mM). Control incubation reactions with the fully active enzyme preparation were carried out following the same procedure with the exception that for the preincubations the addition of (*R*)-deprenyl was omitted. The concentrations of the MAO-B-generated H_2O_2 were measured as described previously.[23] All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM. The extent of the H_2O_2 produced by non-MAO-B mediated oxidation of benzylamine (500 μM), kynuramine (500 μM), MPTP (500 μM), MMTP (250 μM) and **7** (500 μM) were determined following the same protocol.

4.8. Mass spectrometry

Reactions containing baboon liver mitochondrial MAO-B (0.6 mg protein/mL) and 100 μM of the substrates, **10** and **11**, respectively, were incubated for 14 h at 37 °C in sodium phosphate buffer (100 mM, pH 7.4). The samples were subsequently centrifuged at 16,000*g* for 10 min and the supernatant fractions were subjected to LC-ESI-MS analysis. Control reactions containing only mitochondrial MAO-B (0.6 mg protein/mL) or substrate (**10** or **11**) were included to facilitate the identification of the metabolite peaks.

The LC-ESI-MS system consisted of an Agilent 1100 series binary gradient pump, an autosampler and a vacuum solvent degasser coupled to an API 2000 triple quadrupole mass spectrometer (Applied Biosystems). Analyst 1.4 data acquisition and analysis software was employed and mass spectra were acquired over the scan range *m*/*z* 158–177 with the instrument operating in positive ion mode. Nitrogen (purity 99.995%) was used as both nebulizer (20 psi) and the drying gas (15 L/min, 350 °C). The capillary voltage and the vaporizer temperature were set at 5500 V and 300 °C, respectively. The declustering, focusing and entrance potentials were set to 50, 230 and 8.0 V, respectively. Separation was achieved with a Gemini C18 column (2 × 150 mm, 5 μm) (Phenomenex, Torrance, CA) and a mobile phase which consisted initially of 95% Milli-Q water (containing 0.1% ammonium formate, pH 5.0) and 5% acetonitrile. The solvent was delivered at a flow rate of 250 μL/min and the injection volume was 10 μL. One minute after injection of the sample, a time-gradient program was initiated and the composition of acetonitrile in the mobile phase was increased linearly to 90% over a period 7 min and maintained at this percentage for 4 min. A period of 5 min was allowed for the instrument to re-equilibrate at the initial conditions before injection of the next sample.

4.9. Animal studies and striatal dopamine measurements

Male C57BL/6 mice (30–35 g, 9–11 months of age) were provided by the Laboratory animal center of the Potchefstroom campus. The protocols for all animal trials were reviewed and approved by the Research Ethics Committee of the North-West University. Five animals were housed per cage in a temperature (21 ± 0.5 °C) and humidity (50 ± 5% relative humidity) controlled room on a 12 h light–12 h dark cycle with free access to food and water. All injections were intraperitoneal (ip) in a volume of 0.2 ml per 30 g mouse. Sterile saline was used as vehicle for all of the test compounds. Mice were sacrificed by rapid cervical dislocation. The dopamine concentrations in the dissected mouse striata were determined as described previously[26] and were expressed as means ± SEM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.079.

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