Bioorganic & Medicinal Chemistry 17 (2009) 3104–3110

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Inhibition of monoamine oxidase B by *N*-methyl-2-phenylmaleimides

Clarina I. Manley-King [a], Gisella Terre'Blanche [a], Neal Castagnoli Jr. [b], Jacobus J. Bergh [a], Jacobus P. Petzer [a,*]

[a] Pharmaceutical Chemistry, School of Pharmacy, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa
[b] Department of Chemistry, Virginia Tech and Edward Via College of Osteopathic Medicine, Blacksburg, VA 24061, USA

ARTICLE INFO

Article history:
Received 15 January 2009
Revised 2 March 2009
Accepted 3 March 2009
Available online 9 March 2009

Keywords:
Monoamine oxidase B
Reversible inhibitors
Competitive inhibition
Maleimide
Hydrolysis

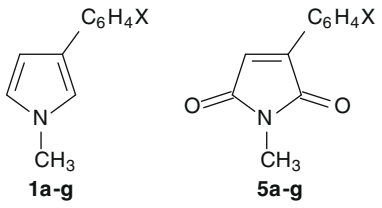
ABSTRACT

Based on a recent report that 1-methyl-3-phenylpyrrolyl analogues are moderately potent reversible inhibitors of the enzyme monoamine oxidase B (MAO-B), a series of structurally related *N*-methyl-2-phenylmaleimidyl analogues has been prepared and evaluated as inhibitors of MAO-B. In general, the maleimides were more potent competitive inhibitors than the corresponding pyrrolyl analogues. *N*-Methyl-2-phenylmaleimide was found to be the most potent inhibitor with an enzyme–inhibitor dissociation constant (K_i value) of 3.49 μM, approximately 30-fold more potent than 1-methyl-3-phenylpyrrole (K_i = 118 μM). This difference in activities may be dependent upon the ability of the maleimidyl heterocyclic system to act as a hydrogen bond acceptor. This is in correspondence with literature reports which suggest that hydrogen bond formation is involved in stabilizing inhibitor–MAO-B complexes. Also reported here is a brief kinetic study of the hydrolysis of the *N*-methyl-2-phenylmaleimidyl analogues in aqueous solution. The findings of the inhibition studies are discussed with reference to the rate and extent of hydrolysis.

1. Introduction

It was recently demonstrated that a series of 1-methyl-3-phenylpyrrolyl analogues (**1a**–**g**) act as moderately potent competitive inhibitors of the enzyme, monoamine oxidase B (MAO-B) (Fig. 1).[1] The most potent analogue was 1-methyl-(3-trifluoromethylphenyl)pyrrole (**1e**) with an enzyme–inhibitor dissociation constant (K_i value) of 6.55 μM. The least potent inhibitor was 1-methyl-3-phenylpyrrole (**1a**) with a K_i value of 118 μM. Since 1-methyl-3-phenylpyrroles probably bind to the active site of MAO-B principally via hydrophobic interactions, we speculate that modification of the structure to include hydrogen bond acceptors may enhance binding affinity. Literature supports the idea that reversible MAO-B inhibitors may be stabilized via hydrogen bonding in the active site of the enzyme.[2] For example, the three-dimensional structure of recombinant human MAO-B co-crystallized with safinamide (**2**) (Fig. 2) has shown that the amidyl functional group acts as both hydrogen bond donor and acceptor with Gln206 and an ordered water molecule in the substrate cavity of the enzyme.[3] Likewise, 7-(3-chlorobenzyloxy)-4-formylcoumarin (**3**) binds with its coumarin moiety in the substrate cavity space, where the aldehyde oxygen acts as a hydrogen bond acceptor for the hydroxyl group of Tyr435 and a conserved water molecule.[3] Structural analysis of the small molecule inhibitor, isatin (**4**), in complex with human MAO-B, shows that the C-2 carbonyl oxygen and pyrrole NH are hydrogen bonded to ordered water molecules present in the active site.[4]

Therefore, the inclusion of hydrophilic substituents that could displace active site water molecules and establish hydrogen bond interactions with active site residues and residual water molecules should result in increased affinity of a reversible inhibitor for MAO-B.[4] *N*-Methyl-2-phenylmaleimide (**5a**) (Fig. 1) is an example of a modified 1-methyl-3-phenylpyrrolyl system that could lead to hydrogen bonding in the active site of MAO-B. In contrast to the pyrrolyl analogues *N*-methyl-2-phenylmaleimides may interact with MAO-B via both hydrogen bonding and hydrophobic burial and hence may be more potent inhibitors of the enzyme. In the



a: X = H
b: X = 3-Cl
c: X = 3-Br
d: X = 3-F
e: X = 3-CF_3
f: X = 3-CH_3
g: X = 3-OCH_3

Figure 1. The structures of compounds discussed in the text: 1-methyl-3-phenylpyrrolyl (**1a**–**g**) and *N*-methyl-2-phenylmaleimidyl (**5a**–**g**) analogues.

* Corresponding author. Tel.: +27 18 2992206; fax: +27 18 2994243.
E-mail address: jacques.petzer@nwu.ac.za (J.P. Petzer).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.03.005

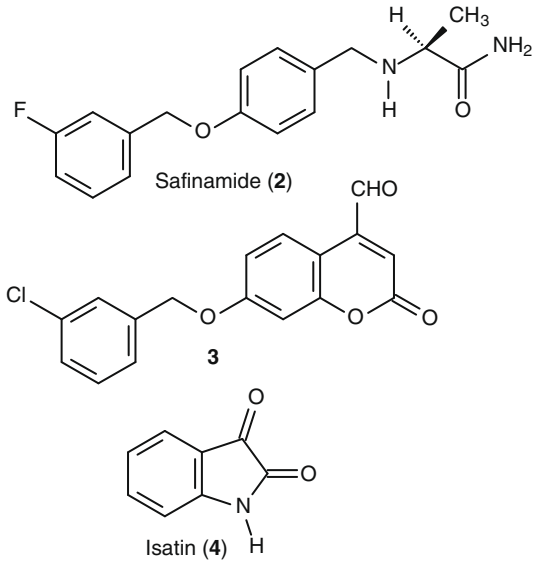


Figure 2. The structures of compounds discussed in the text: safinamide (**2**), 7-(3-chlorobenzyloxy)-4-formylcoumarin (**3**) and isatin (**4**).

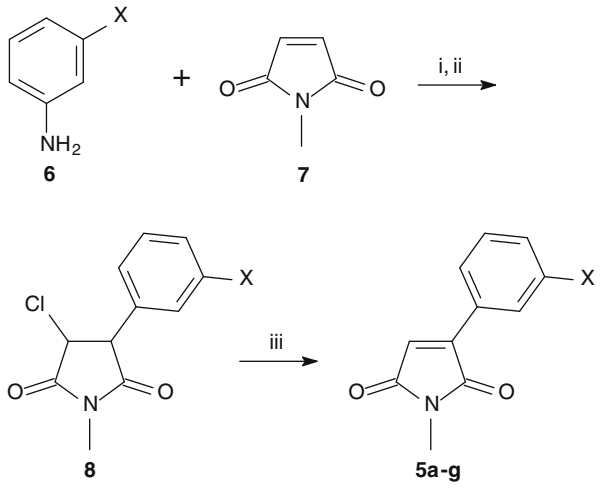
present study we have prepared and evaluated a series of *N*-methyl-2-phenylmaleimides (**5a**–**g**) as potential MAO-B inhibitors and compared the potencies of inhibition to that of the corresponding pyrrolyl analogues (**1a**–**g**). As with the series of pyrrolyl analogues different substituents at C-3 of the phenyl ring were examined.

The double bond of the maleimide ring has been shown to undergo conjugate addition reactions with thiol groups at physiological pH to form a stable thio ether bond. This chemistry has been exploited in the modification, quantitation and analysis of cysteine containing proteins.[5] Since MAO-B contains an active site cysteinyl residue (Cys172),[6] the possibility exists that the maleimides investigated here may interact irreversibly with the enzyme and hence the time-dependency of the interaction between *N*-methyl-2-phenylmaleimide (**5a**) and MAO-B has been studied. In accordance with this idea both recombinant human MAO-A and MAO-B are inactivated by *N*-ethylmaleimide.[7] Also, since *N*-alkylmaleimides are reported to undergo hydroxide ion-mediated hydrolysis,[8] the rate of hydrolysis of *N*-methyl-2-phenylmaleimide in aqueous solution also was examined. The findings of the inhibition studies are discussed with reference to the reversibility of inhibition and rate and extent of hydrolysis.

2. Results

2.1. Chemistry

The *N*-methyl-2-phenylmaleimidyl analogues (**5a**–**g**) examined in this study were conveniently prepared according to a previously reported procedure (Scheme 1).[9] In the first step the appropriately substituted aniline (**6**) is diazotized and the resulting diazo intermediate treated at −10 °C with *N*-methylmaleimide (**7**), a modified Meerwein reaction.[10,11] The target *N*-methyl-2-phenylmaleimides (**5a**–**g**) were obtained following thermal dehydrohalogenation of the intermediate chlorosuccinimides (**8**) in the presence of 2,6-lutidine. The formation of Diels–Alder adducts has been reported as side products in the preparation of maleimides according to this synthetic route.[12] This side reaction could be partially suppressed by dilution of the lutidine with isopropanol and by reduction of the heating time.[9] Following purification by column chromatography[13] to remove substantial amounts of 'diazo resins', the final



Scheme 1. Synthetic pathway to *N*-methyl-2-phenylmaleimides (**5a**–**g**). Key: (i) $NaNO_2$, HCl, 0 °C; (ii) sodium acetate, $CuCl_2$, 0 °C; (iii) 2,6-lutidine, isopropanol.

N-methyl-2-phenylmaleimides were recrystallized twice from ethanol. Even though this resulted in relatively low yields, it was found to be effective in removing residual amounts of the Diels–Alder adduct as judged by ^{1}H NMR. The presence of these contaminants could also be conveniently detected via neutral aluminum oxide TLC (see Section 4). The structures and purity of the target compounds were verified by mass spectrometry, ^{1}H NMR and ^{13}C NMR. For those compounds previously reported, the physical data and melting points obtained were compared to the corresponding literature values as cited in Section 4.

2.2. Enzymology

In the present study we have examined the MAO-B inhibition potential of *N*-methyl-2-phenylmaleimides (**5a**–**g**) using the mitochondrial fraction obtained from baboon liver tissue as enzyme source. Baboon liver tissue exhibits a high degree of MAO-B catalytic activity while being devoid of MAO-A activity.[14] Also, the interaction of reversible inhibitors with MAO-B obtained from baboon liver tissue appears to be similar to the interaction with the human form of the enzyme since inhibitors are approximately equipotent with both enzyme sources.[15] As substrate for the MAO-B activity measurements, 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP) was used. MMTP undergoes MAO-B catalyzed ring α-carbon oxidation to yield the corresponding dihydropyridinium species $MMDP^+$.[14] Since $MMDP^+$ absorbs light maximally at a wavelength of 420 nm, the concentration of this species can be conveniently estimated via spectrophotometry. None of the *N*-methyl-2-phenylmaleimides (**5a**–**g**) evaluated here absorbs light at this wavelength (see Section 4).

The MAO-B inhibitory properties of **5a**–**g** first were investigated in order to determine whether the test inhibitors act as reversible inhibitors or time-dependent inactivators of the enzyme. As stated in the introduction, enzyme inactivation could be mediated if the maleimide double bond were to undergo a conjugate addition reaction with the active site cysteinyl residue (Cys172) of MAO-B. For this study **5a** was selected as a representative test compound. Baboon liver mitochondria were preincubated with **5a** (14 μM) for periods of 0, 15, 30, and 60 min and the rates of MAO-B catalyzed oxidation of MMTP (90 μM) to $MMDP^+$ were measured.[16] As shown in Figure 3 the MAO-B oxidation rate slightly increases with increased preincubation time of **5a** with the enzyme. The increase of catalytic activity may be due to the hydrolysis of the maleimidyl inhibitor in aqueous solution to yield

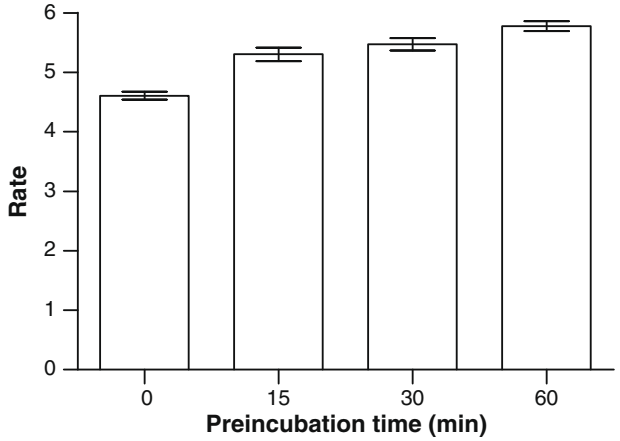
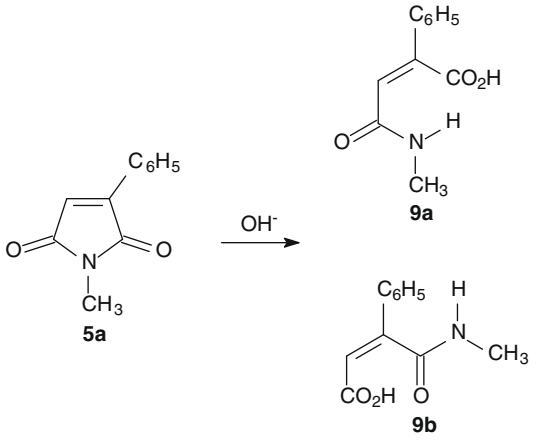


Figure 3. The rates of the baboon liver mitochondrial MAO-B catalyzed oxidation of MMTP (90 μM). The enzyme preparation was preincubated for various periods of time (0–60 min) with **5a** (14 μM). The rates are expressed as nmol $MMDP^+$ formed/mg protein/min.



Scheme 2. The proposed hydrolysis of *N*-methyl-2-phenylmaleimides (**5a**) in aqueous solution to yield the isomeric maleamic acids **9a** and/or **9b**.

one or a mixture of the corresponding carboxy acids (Scheme 2). Should such hydrolysis render the maleimide inhibitor inactive, the result would be an increase of MAO-B catalytic activity with increased preincubation time. This analysis is consistent with time dependency of the putative hydrolysis (see below). Since there is no decrease of MAO-B activity with increased incubation time, it can also be concluded that **5a** interacts reversibly with the active site of MAO-B. The reversibility of enzyme inhibition by **5a**–**g** was well supported by the linear Lineweaver–Burk plots constructed from the kinetic data. As shown by example with *N*-methyl-2-(3-chlorophenyl)maleimide (**5b**), the Lineweaver–Burk plots were indicative of competitive inhibition for the inhibitors tested (Fig. 4). Although this result suggests that *N*-methyl-2-phenylmaleimides are not alkylated by the thiol group of the active site cysteinyl residue (Cys172), the possibility that MAO-B is inactivated at a very slow rate can not be excluded. For example, *N*-ethylmaleimide at a concentration of 0.8 mM is reported to inactivate recombinant human MAO-B with a half-time of 8 h.[7]

To evaluate the notion that the products generated upon hydrolysis of the maleimidyl analogues are not MAO-B inhibitors, **5b** at a concentration of 13 μM (approximately $2 \times K_i$) was preincubated in the aqueous incubation buffer (100 mM sodium phosphate buffer, pH 7.4) for various time periods (0–240 min). Baboon liver

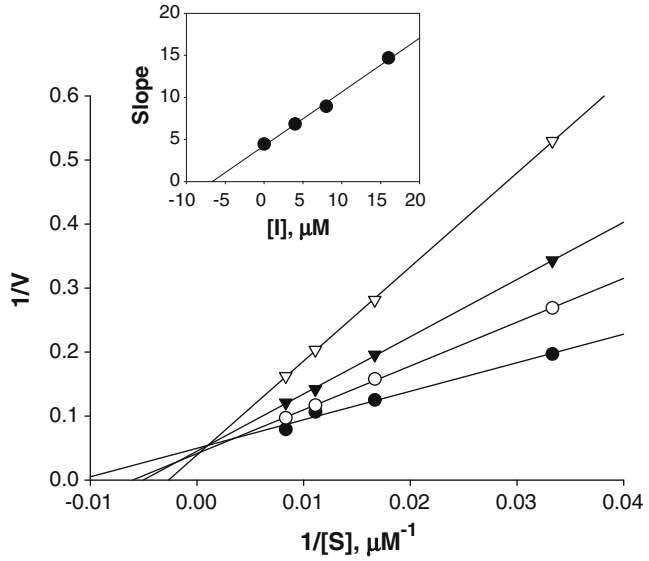
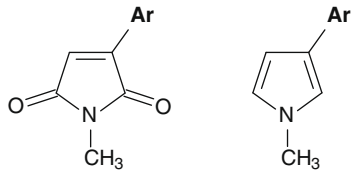


Figure 4. Lineweaver–Burk plots of the oxidation of MMTP by baboon liver MAO-B in the absence (filled circles) and presence of various concentrations of **5b** (open circles, 4 μM; filled triangles, 8 μM; open triangles, 16 μM). The rates are expressed as nmol $MMDP^+$ formed/mg protein/min and the inset is the replot of the slopes versus the inhibitor concentration.

mitochondria (0.15 mg protein/mL) and MMTP at a final concentration of 90 μM were added and the rates of the MAO-B catalyzed oxidation of MMTP to $MMDP^+$ were measured (see Section 4). When **5b** was preincubated for a period of 0 min, the rate of $MMDP^+$ formation was 47% (±0.3%) of the rate measured in the absence of **5b**. When **5b** was preincubated for 30, 60, 120, 180 and 240 min the rates of $MMDP^+$ formation were 52% (±1.4%), 66% (±0.4%), 68% (±0.4%), 80% (±0.4%) and 84% (±0.9%), respectively, of the rate measured in the absence of **5b**. These data show that an increase in preincubation time results in a reduction of the inhibition potency of **5b**. Since an increase in preincubation time is associated with a larger extent of hydrolysis, these results are in accordance with the notion that the hydrolysis products are not MAO-B inhibitors or at least weak inhibitors compared to the parent maleimide.

The enzyme–inhibitor dissociation constants (K_i values) for the inhibition of MAO-B by the *N*-methyl-2-phenylmaleimides (**5a**–**g**) evaluated here as well as those previously reported[1] for the corresponding 1-methyl-3-phenylpyrrolyl analogues (**1a**–**g**) are listed in Table 1. The same assay conditions, enzyme source and substrate that were used for the evaluation of the pyrrolyl inhibitors[1] were also employed here for the evaluation of the maleimidyl inhibitors. In general, the maleimides were more potent competitive inhibitors of MAO-B than the corresponding pyrrolyl analogues with K_i values ranging from 3.49 to 11.0 μM for the maleimides and 6.55–118 μM for the pyrrolyl analogues. *N*-Methyl-2-phenylmaleimide (**5a**) was found to be the most potent inhibitor with a K_i value of 3.49 μM, approximately 30-fold more potent than 1-methyl-3-phenylpyrrole (**1a**) (K_i = 118 μM). The differences in inhibition potencies between the C-3 phenyl substituted maleimidyl (**5b**–**g**) and the pyrrolyl (**1b**–**g**) inhibitors were more modest. For example, *N*-methyl-2-(3-bromophenyl)maleimide (**5c**) (K_i = 6.99 μM) was only approximately twofold more potent than 1-methyl-3-(3-bromophenyl)pyrrole (**1c**) (K_i = 14.3 μM). The only exception was observed for the 3-CF_3 phenyl substituted analogues with pyrrolyl **1e** (K_i = 6.55 μM) inhibiting MAO-B more potently than the corresponding maleimidyl **5e** (K_i = 11.0 μM). Interestingly, in contrast to the pyrrolyl analogues considered here,[1] substitution on the phenyl ring of the *N*-methyl-2-phenylmaleimidyl analogues leads

Table 1
The K_i values for the inhibition of MAO-B by the *N*-methyl-2-phenylmaleimides (**5a–g**) and the corresponding 1-methyl-3-phenylpyrrolyl analogues (**1a–g**)[a]



5 **1**

	Ar	K_i value for **5**[b]	K_i value for **1**[c]
a	C_6H_5	3.49	118
b	3-ClC_6H_4	6.66	20.9
c	3-BrC_6H_4	6.99	14.3
d	3-FC_6H_4	5.73	38.9
e	3-$CF_3C_6H_4$	11.0	6.55
f	3-$CH_3C_6H_4$	8.60	56.0
g	3-$OCH_3C_6H_4$	8.56	41.7

[a] The K_i values are expressed in μM.
[b] The K_i values of **5b–g** may be an underestimation of the potencies due to high rates of aqueous hydrolysis (see text for details).
[c] Values obtained from Ref. 1.

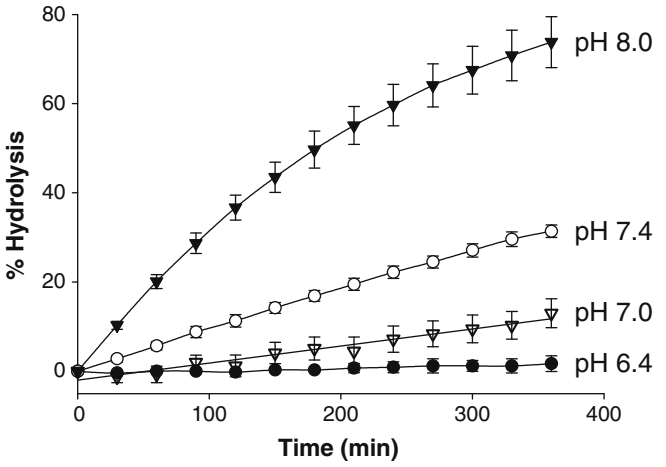


Figure 5. The rates of hydrolysis of **5a** (100 μM) in 100 mM sodium phosphate buffer at pH 6.4, 7.0, 7.4 and 8.0. The temperature of the incubations was 37 °C and the extent of hydrolytic cleavage was estimated spectrophotometrically from the decrease in absorbance at 349 nm of the aqueous solutions of **5a**. Because of the limited solubility of **5a** in water, all incubations contained 4% DMSO as cosolvent.

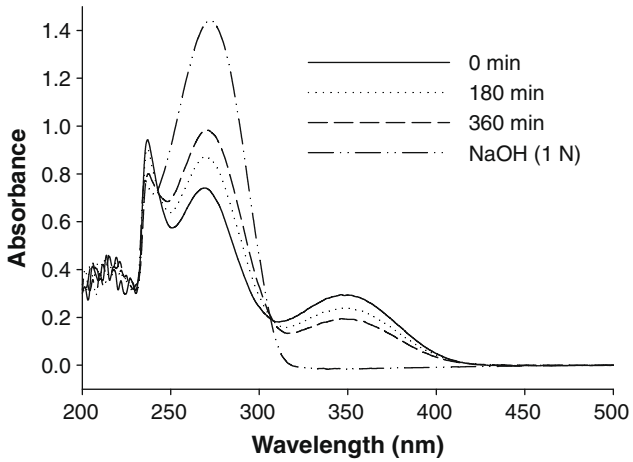


Figure 6. The absorption spectra of a solution of **5a** (100 μM, starting concentration) following incubations for different time periods (0, 180 and 360 min) in 100 mM sodium phosphate buffer at pH 7.4. The absorption spectrum of a solution of **5a** (100 μM, starting concentration) incubated for 45 min in 1 N NaOH is also shown. The temperature of the incubations was 37 °C and DMSO (4%) was added as cosolvent.

to inhibitors with reduced inhibition potencies since unsubstituted **5a** was found to be the best inhibitor. As discussed in the next section, the relatively modest inhibition potencies of **5b–g** may be a result of a higher rate of hydrolysis in the aqueous incubation medium used for the inhibition studies compared to the hydrolysis rate of **5a**.

2.3. The rate of hydrolysis of *N*-methyl-2-phenylmaleimide

As discussed in the previous section, the rates of the MAO-B catalyzed oxidation of MMTP in the presence of **5a** increase slightly with increased preincubation time of **5a** with the enzyme (Fig. 3). Since *N*-alkylmaleimides are reported to undergo hydroxide ion dependent hydrolysis,[8] the possibility exists that the hydrolysis of **5a** in the aqueous incubation medium to yield products that are not MAO-B inhibitors could affect its apparent inhibition potency. This process would reduce the concentration of inhibitor **5a** and therefore result in an increase of the rates of MAO-B catalyzed oxidation of MMTP. The observation that MAO-B catalytic activity increases with increased preincubation time is in agreement with the time dependency of the hydrolysis process. To determine if hydrolysis of **5a**, the most potent inhibitor of the series, may have significantly affected the measured K_i value (Table 1), the extent and rate of hydrolysis of **5a** were examined in the aqueous incubation medium used in the enzymology. Solutions of **5a** (100 μM) in 100 mM sodium phosphate buffer (pH 6.4, 7.0, 7.4 and 8.0) were incubated at 37 °C and the remaining concentrations of **5a** in the incubations were measured spectrophotometrically (Fig. 5). Compound **5a** absorbs maximally at 349 nm. After incubations in 1 N NaOH this chromophore is replaced by a new chromophore with λ_{max} at 269 nm (Fig. 6). The reduction of absorbance at 349 nm is associated with the hydrolysis of the maleimide ring since hydrolysis of *N*-methylmaleimide results in a similar reduction in absorbance at its longest wavelength of maximal absorbance (300 nm).[8] Therefore the residual concentration of **5a** in the incubations could be calculated from the molar absorptivity (2820 M^{-1}) of **5a** at 349 nm (Fig. 6).

The results (Fig. 5) show that while **5a** is stable at pH 6.4, at pH 7–8 a time– and pH–dependent reduction of **5a** concentration is observed. As discussed above, the reduction of **5a** concentration may be attributed to the hydrolysis of the maleimidyl ring. Since the rate of hydrolysis increases with increasing pH, the process is hydroxide ion dependent. This is similar to what has been reported for the hydrolysis of *N*-alkylmaleimides.[8] At 360 min after initiation of the experiment, the extent of hydrolysis at pH 7.0, 7.4 and 8.0 were 13%, 31.4% and 73.8%, respectively. After 30 min at a pH of 7.4 the extent of hydrolysis was only 2.8% for a 100 μM solution of **5a**. Similarly, slow hydrolysis was also observed at lower concentrations of **5a**. After 30 min at a pH of 7.4 the extent of hydrolysis was approximately 1% for a 10 μM solution (results not shown). Since the time period used for the measurement of the K_i values was only 10 min, it may be concluded that no significant reduction of **5a** concentration due to hydrolysis in the aqueous incubation medium occurred during this time and that hydrolytic cleavage of **5a** therefore did not significantly affect the measured K_i value. However, a similar analysis of the aqueous stability of **5b–g** revealed that, in contrast to **5a**, these congeners underwent rapid hydrolysis in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C (Fig. 7). After a 30 min incubation period, the ex-

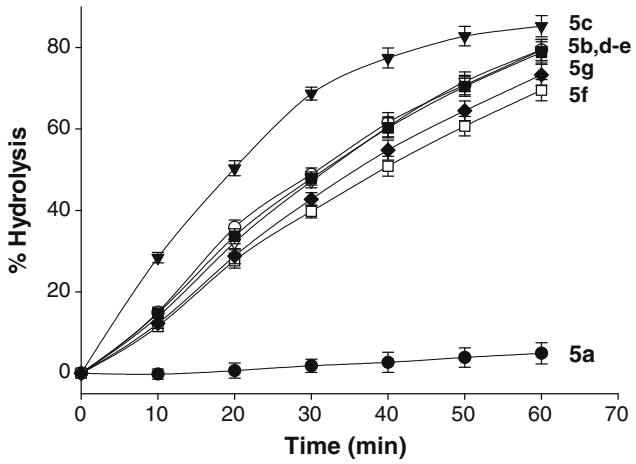


Figure 7. The rates of hydrolysis of **5a**–**g** (100 μM) in 100 mM sodium phosphate buffer at pH 7.4. The temperature of the incubations was 37 °C and the extent of hydrolytic cleavage was estimated spectrophotometrically from the decrease in absorbance at 337–354 nm (see Section 4) of the aqueous solutions of **5a**–**g**. Because of the limited solubility of **5a**–**g** in aqueous solvent, all incubations contained 4% DMSO as cosolvent.

tents of hydrolysis were 40–69% for 100 μM solutions of **5b**–**g**. It can therefore be concluded that significant reductions of the concentrations of **5b**–**g** in the aqueous incubation medium occurred during the MAO-B inhibition studies and that, assuming that the respective hydrolysis products are not MAO-B inhibitors, the measured K_i values (Table 1) are an underestimation of the inhibition potencies of these inhibitors. An interesting observation is that the set of absorption spectra generated for the hydrolysis of **5a** exhibits a stable isosbestic point at 308 nm (Fig. 6). This suggests that the hydrolysis reaction of **5a** proceeds without forming intermediates or multiple products.[17] The absorption spectra generated from the aqueous incubations of **5b**–**g**, however, do not exhibit isosbestic points which suggest that mechanisms of degradation of **5b**–**g** are different from that of **5a**. This may underlie the difference in aqueous stability between **5a** and **5b**–**g**.

3. Discussion

In the present study a series of *N*-methyl-2-phenylmaleimides (**5a**–**g**) was evaluated as inhibitors of MAO-B and the inhibition potencies were compared to those of the corresponding 1-methyl-3-phenylpyrrolyl analogues (**1a**–**g**). *N*-Methyl-2-phenylmaleimide (**5a**) was found to be the most potent inhibitor, approximately 30-fold more potent than 1-methyl-3-phenylpyrrole (**1a**). The enhanced inhibition potency of **5a** may be dependent upon the ability of the carbonyl oxygen atoms of the maleimidyl heterocyclic system to act as hydrogen bond acceptors. The idea that the maleimides may be stabilized via hydrogen bonding in the active site is in agreement with the literature. The three-dimensional structures of human recombinant MAO-B co-crystallized with several reversible inhibitors have documented that enzyme–inhibitor complexes are frequently stabilized by hydrogen bonding.[3,4] Several ordered water molecules and amino acid residues (for example Gln206 and Tyr435), which have been shown to act as hydrogen bond donors and acceptors, are present in the active site of MAO-B. The suggestion that, in contrast to pyrrolyl inhibitor **1a**, maleimidyl inhibitor **5a** is stabilized by hydrogen bonding within the active site of MAO-B is further supported by the observation that the difference between the K_i values of **5a** (K_i = 3.49 μM) and **1a** (K_i = 118 μM) corresponds to a $\Delta\Delta G^\circ$ of approximately 2.1 kcal/mole. This difference in energy is within the range expected for hydrogen bond formation. We conclude that the formation of hydrogen bonds is an important factor in stabilizing MAO-B–ligand complexes and should be taken into consideration when designing MAO-B inhibitors.

The relatively modest differences between the inhibition potencies of **5b**–**g** and their corresponding pyrrolyl analogues (**1b**–**g**) is most likely the result of a high rate of hydrolysis in the aqueous medium used for the inhibition studies. The results document that **5b**–**g** undergo rapid hydrolysis in aqueous media and, assuming that the respective degradation products are not MAO-B inhibitors, the measured K_i values are likely to be an underestimation of the true inhibition potencies of these inhibitors. In contrast, **5a** exhibited a relatively slow rate of hydrolysis and the measured K_i value for the inhibition if MAO-B may be a more accurate estimation of its inhibition potency.

4. Experimental

Caution: MMTP is a structural analogue of the nigrostriatal neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously.[18]

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma-Aldrich and were used without purification. The oxalate salt of MMTP was prepared as described previously.[19] 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. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to deuterated chloroform ($CDCl_3$). Spin multiplicities are given as s (singlet), d (doublet), q (quartet) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Direct insertion electron impact ionization (EIMS) and high resolution (HRMS) mass spectra were obtained with an AutoSpec ETOF (Micromass) mass spectrometer. 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 and a Shimadzu MultiSpec-1501 photodiode array spectrophotometer. Thin layer chromatography (TLC) was carried out with neutral aluminum oxide 60 (Merck) containing UV_{254} fluorescent indicator and benzene as mobile phase. Column chromatography was carried out with Fluka aluminum oxide (Brockmann Activity I).

4.2. Synthesis of *N*-methyl-2-phenylmaleimidyl analogues (5a–g)

The *N*-methyl-2-phenylmaleimidyl analogues (**5a**–**g**) were synthesized according to a previously reported procedure.[9] The appropriately C-3 substituted aniline derivative (102 mmol) was dissolved in 12 N hydrochloric acid (30 mL) and water (10 mL) and then cooled in an ice–salt bath (−10 °C). Upon cooling, the aniline hydrochloric acid salt precipitates from solution. To this, a solution of sodium nitrite (153 mmol) in 16 mL water was added dropwise over a period of 10 min with vigorous stirring. The aniline derivative is completely diazotized when the reaction returns to a complete solution of the diazonium salt. *N*-Methylmaleimide (102 mmol) was dissolved in 80 mL of acetone, cooled on an ice–salt bath (−10 °C) and the diazotized aniline was added over a period of 2 min. The pH of the reaction was adjusted to 3.0 with solid

sodium acetate followed by the addition of solid $CuCl_2$ (2.5 g) as a single portion.[10,11] The reaction was stirred for a period of 3 h in the ice–salt bath while the pH was periodically adjusted to 2.9. The reaction was allowed to return to room temperature and was stirred for an additional 18 h. If a precipitate forms (**5b**–**e**, **g**), it was collected by filtration while for an oil (**5a** and **5f**), 100 mL water was added and the crude product was extracted to benzene (100 mL). The benzene phase was dried over anhydrous $MgSO_4$ and removed under reduced pressure, while the precipitate was air dried. To the crude product was added 2,6-lutidine (2.5 g) and isopropanol (12.5 mL) and the resulting solution was heated for 15 min at 100 °C. For the synthesis of **5a** and **5f** a solution of 100 mL of 1 N hydrochloric acid solution was added to the reaction. The resulting mixture was extracted to benzene (100 mL) and washed with an aqueous solution of 1 N hydrochloric acid (2×50 mL). Following drying of the benzene over anhydrous $MgSO_4$, the solvent was removed under reduced pressure. For the synthesis of **5b**–**e**, **g**. the reaction mixture was cooled and filtered to obtain a dark brown solid. The crude products were dissolved in a minimum amount of benzene, applied to a short aluminum oxide column (35×50 mm) and were eluted with benzene as mobile phase. The collected fractions were recrystallized twice from boiling ethanol to afford the yellow or brown crystalline products. For previously described **5a** and **5g** we found the melting points to be 146–148 °C and 145–148 °C while the reported melting points are 147–148 °C and 146–148 °C, respectively.[9] The characterizations of compounds that are previously unreported are summarized below.

N-Methyl-2-(3-chlorophenyl)maleimide (**5b**) was prepared from 3-chloroaniline and *N*-methylmaleimide (**7**) in a yield of 6%: mp 113–115 °C; ^{1}H NMR ($CDCl_3$) δ 3.07 (s, 3H), 6.74 (s, 1H), 7.35–7.44 (m, 2H), 7.78–7.81 (m, 1H), 7.89–7.91 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 23.94, 125.02, 126.64, 128.48, 130.19, 130.35, 131.05, 135.04, 142.60, 169.92, 170.26; EIMS m/z; 221 ($M^{\cdot+}$); HRMS calcd 221.024356, found 221.023664.

N-Methyl-2-(3-bromophenyl)maleimide (**5c**) was prepared from 3-bromoaniline and *N*-methylmaleimide (**7**) in a yield of 16%: mp 116–119 °C; ^{1}H NMR ($CDCl_3$) δ 3.06 (s, 3H), 6.73 (s, 1H), 7.28–7.33 (m, 1H), 7.55–7.58 (m, 1H), 7.82–7.85 (m, 1H), 8.03–8.04 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 23.91, 123.00, 125.01, 127.07, 130.39, 130.58, 131.30, 133.92, 142.42, 169.85, 170.19; EIMS m/z; 265, 267 ($M^{\cdot+}$); HRMS calcd 264.973840, found 264.974289.

N-Methyl-2-(3-fluorophenyl)maleimide (**5d**) was prepared from 3-fluoroaniline and *N*-methylmaleimide (**7**) in a yield of 31%: mp 155–158 °C; ^{1}H NMR ($CDCl_3$) δ 3.05 (s, 3H), 6.73 (s, 1H), 7.11–7.17 (m, 1H), 7.36–7.44 (m, 1H), 7.63–7.69 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 23.87, 115.47 (d), 117.99 (d), 124.21, 124.93, 130.50 (d), 142.61 (d), 161.12, 164.40, 169.93, 170.26; EIMS m/z; 205 ($M^{\cdot+}$); HRMS calcd 205.053907, found 205.053180.

N-Methyl-2-(3-trifluoromethylphenyl)maleimide (**5e**) was prepared from 3-trifluoromethylaniline and *N*-methylmaleimide (**7**) in a yield of 9%: mp 103–105 °C; ^{1}H NMR ($CDCl_3$) δ 3.07 (s, 3H), 6.81 (s, 1H), 7.55–7.60 (m, 1H), 7.68–7.71 (m, 1H), 8.07–8.10 (m, 1H), 8.15–8.18 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 23.93, 121.82, 125.25 (d), 125.38 (d), 127.45 (q), 129.48 (d), 131.56 (q), 131.65, 142.45, 169.75, 170.19; EIMS m/z; 255 ($M^{\cdot+}$); HRMS calcd 255.050713, found 255.053127.

N-Methyl-2-(3-methylphenyl)maleimide (**5f**) was prepared from 3-methylaniline and *N*-methylmaleimide (**7**) in a yield of 4%: mp 91–93 °C; ^{1}H NMR ($CDCl_3$) δ 2.38 (s, 3H), 3.05 (s, 3H), 6.68 (s, 1H), 7.24-7.34 (m, 2H), 7.68–7.70 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 21.37, 23.78, 123.72, 125.71, 128.70, 128.79, 129.04, 131.88, 138.64, 144.14, 170.45, 170.76; EIMS m/z; 201 ($M^{\cdot+}$); HRMS calcd 201.078979, found 201.078442.

4.3. MAO-B inhibition studies

MAO-B activity measurements were carried out in sodium phosphate buffer (100 mM, pH 7.4) with mitochondria isolated from baboon liver tissue.[1,20,21] The MAO-A and -B mixed substrate, MMTP[14] served as substrate for the inhibition studies. The final volume of the incubations were 500 μL and contained the mitochondrial isolate (0.15 mg protein/mL), MMTP (30–120 μM), and various concentrations of the test inhibitors. The limited solubility of the test compounds in aqueous solution required the use of 4% DMSO as cosolvent. Following incubation at 37 °C for 10 min, the enzyme reactions were terminated by the addition of 10 μL perchloric acid (70%) and the samples were cleared via centrifugation at 16,000*g* for 10 min. The concentrations of the MAO-B generated product, $MMDP^+$, were measured spectrophotometrically at a wavelength of 420 nm ($\varepsilon = 25{,}000\ M^{-1}$).[14] K_i values were determined by reploting the slopes of the Lineweaver–Burk plots versus the inhibitor concentration and the K_i value was determined from the *x*-axis intercept (intercept = $-K_i$). Each K_i value reported here is representative of a single determination where the correlation coefficient (R^2 value) of the replot of the slopes versus the inhibitor concentrations was at least 0.99.

4.4. Time-dependent inhibition studies

To determine whether maleimide **5a** acts as a reversible inhibitor or as a time-dependent inactivator of MAO-B, baboon liver mitochondria (0.3 mg of protein/mL) were preincubated with **5a** for periods of 0, 15, 30 and 60 min at 37 °C.[14] The preincubation solvent was 100 mM sodium phosphate buffer (pH 7.4) and the concentration of **5a** was 14 μM. MMTP, at final concentration of 90 μM, was then incubated at 37 °C for 15 min with 0.15 mg protein/mL of the preincubated mitochondria. The final volumes of these incubations were 500 μL and the final concentration of the **5a** was 7 μM, approximately double the K_i value (3.49 μM) of **5a** for the inhibition of MAO-B. Following termination of the reactions by the addition of 10 μL of perchloric acid (70%), the concentrations of $MMDP^+$ were measured as outlined above. These experiments were carried out in triplicate and the values are expressed as mean ± standard error of the mean (SEM).[1]

To determine whether the products generated upon hydrolysis of maleimide **5b** act as inhibitors of MAO-B, **5b** was preincubated in the aqueous incubation buffer (100 mM sodium phosphate buffer, pH 7.4) for various time periods (0, 30, 60, 120, 180, 240 min) at 37 °C. Baboon liver mitochondria (0.15 mg protein/mL) and MMTP at a final concentration of 90 μM were added and incubation was continued at 37 °C for 15 min. The final volumes of these incubations were 500 μL and the final concentration of **5b** was 13 μM, approximately double the K_i value (6.66 μM). Incubations carried out in the absence of **5b** were included as controls. All incubations contained 4% DMSO as cosolvent. The reactions were terminated by the addition of 10 μL of perchloric acid (70%) and the concentrations of $MMDP^+$ were measured as outlined above. These experiments were carried out in triplicate and the values are expressed as mean ± standard error of the mean (SEM).

4.5. Hydrolysis studies

Measurements of the extent of hydrolysis of **5a** (5–100 μM) were carried out in 100 mM sodium phosphate buffer at pH 6.4, 7.0, 7.4 and 8.0. All incubations were conducted in quartz absorption cuvettes (Hellma) to a volume of 2 mL and contained a final concentration of 4% DMSO as cosolvent. The incubation temperature was maintained at 37 °C with a Shimadzu CPS controller. UV–vis scans were recorded at 30 min intervals over a range of 230–500 nm with a Shimadzu MultiSpec-1501 photodiode array

spectrophotometer. Since hydrolysis of the maleimide ring results in bleaching of absorbance at 349 nm, the residual concentration of **5a** in the incubations were calculated from the molar absorptivity (2820 M^{-1}) of **5a** at this wavelength. The same procedure was followed for the aqueous stability measurements of **5b–g** and the reduction of absorbance at the following wavelengths were recorded at 10 min intervals: **5b** 342 nm (3100 M^{-1}), **5c** 343 nm (3240 M^{-1}), **5d** 343 nm (3210 M^{-1}), **5e** 337 nm (2860 M^{-1}), **5f** 352 nm (3860 M^{-1}), **5 g** 354 nm (3780 M^{-1}). All values are expressed as mean ± SEM of duplicate determinations.

Acknowledgments

The NMR spectra were recorded by André Joubert while the MS spectra were recorded by Johan Jordaan and Louis Fourie of the SASOL Centre for Chemistry, North-West University. This work was supported by grants from the National Research Foundation and the Medical Research Council, South Africa.

References and notes

1. Ogunrombi, M. O.; Malan, S. F.; Terre'Blanche, G.; Castagnoli, N., Jr.; Bergh, J. J.; Petzer, J. P. *Bioorg. Med. Chem.* **2008**, *16*, 2463.
2. Novaroli, L.; Daina, A.; Favre, E.; Bravo, J.; Carotti, A.; Leonetti, F.; Catto, M.; Carrupt, P. A.; Reist, M. *J. Med. Chem.* **2006**, *49*, 6264.
3. Binda, C.; Wang, J.; Pisani, L.; Caccia, C.; Carotti, A.; Salvati, P.; Edmondson, D. E.; Mattevi, A. *J. Med. Chem.* **2007**, *50*, 5848.
4. Binda, C.; Li, M.; Hubálek, F.; Restelli, N.; Edmondson, D. E.; Mattevi, A. *Proc. Natl. Acad. Sci. U.S.A* **2003**, *100*, 9750.
5. Knight, P. *Biochem. J.* **1979**, *179*, 191.
6. Binda, C.; Newton-Vinson, P.; Hubálek, F.; Edmondson, D. E.; Mattevi, A. *Nat. Struct. Biol.* **2002**, *9*, 22.
7. Hubálek, F.; Pohl, J.; Edmondson, D. E. *J. Biol. Chem.* **2003**, *278*, 28612.
8. Matsui, S.; Aida, H. *J. Chem. Soc., Perkin Trans. 2* **1978**, 1277.
9. McKenzie, T. C.; Epstein, J. W.; Fanshawe, W. J.; Dixon, J. S.; Osterberg, A. C.; Wennogle, L. P.; Regan, B. A.; Abel, M. S.; Meyerson, L. R. *J. Med. Chem.* **1984**, *27*, 628.
10. Rondestvedt, C. S. *Org. React* **1976**, *24*, 225.
11. Izzo, P. T. *J. Org. Chem.* **1963**, *28*, 1713.
12. Epstein, J. W.; McKenzie, T. C.; Lovell, F. M.; Perkinson, N. A. *J. Chem. Soc., Chem. Commun.* **1980**, 314.
13. Rondestvedt, C. S.; Kalm, M. J.; Vogl, O. *J. Am. Chem. Soc.* **1956**, *78*, 6115.
14. Inoue, H.; Castagnoli, K.; Van der Schyf, C. J.; Mabic, S.; Igarashi, K.; Castagnoli, N., Jr. *J. Pharmacol. Exp. Ther.* **1999**, *291*, 856.
15. Petzer, J. P.; Steyn, S.; Castagnoli, K. P.; Chen, J. F.; Schwarzschild, M. A.; Van der Schyf, C. J.; Castagnoli, N., Jr. *Bioorg. Med. Chem.* **2003**, *11*, 1299.
16. Khalil, A. A.; Steyn, S.; Castagnoli, N., Jr. *Chem. Res. Toxicol.* **2000**, *13*, 31.
17. Berlett, B. S.; Levine, R. L.; Stadtman, E. R. *Anal. Biochem.* **2000**, *287*, 329.
18. Pitts, S. M.; Markey, S. P.; Murphy, D. L.; Weisz, A. In *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome*; Markey, S. P., Castagnoli, N., Jr., Trevor, A. J., Kopin, I. J., Eds.; Academic: New York, 1986; pp 703–716.
19. Bissel, P.; Bigley, M. C.; Castagnoli, K.; Castagnoli, N., Jr. *Bioorg. Med. Chem.* **2002**, *10*, 3031.
20. Van den Berg, D.; Zoellner, K. R.; Ogunrombi, M. O.; Malan, S. F.; Terre'Blanche, G., ; Castagnoli, N., Jr.; Bergh, J. J.; Petzer, J. P. *Bioorg. Med. Chem.* **2007**, *15*, 3692.
21. Vlok, N.; Malan, S. F.; Castagnoli, N., Jr.; Bergh, J. J.; Petzer, J. P. *Bioorg. Med. Chem.* **2006**, *14*, 3512.