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Inhibition of monoamine oxidase by selected phenylalkylcaffeine analogues

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Keywords
caffeine; competitive; monoamine oxidase; reversible inhibition; structure–activity relationship

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Abstract

Objectives Caffeine represents a useful scaffold for the design of monoamine oxidase (MAO) type B inhibitors. Specifically, substitution on the C8 position yields structures which are high-potency MAO-B inhibitors. To explore the structure–activity relationships of MAO-B inhibition by caffeine-derived compounds, this study examines the MAO inhibitory properties of a series of phenylalkylcaffeine analogues.

Methods Employing the recombinant human enzymes, the potencies (IC50 values) by which the caffeine analogues inhibit MAO-A and MAO-B were measured. The reversibility of inhibition of a selected inhibitor was determined by measuring the recovery of enzyme activity after dilution and dialysis of enzyme-inhibitor mixtures.

Key findings The results document that the phenylalkylcaffeine analogues are reversible and selective MAO-B inhibitors with a competitive mode of inhibition. The most potent analogue, 8-(7-phenylheptyl)caffeine, exhibits IC50 values for the inhibition of MAO-A and MAO-B of 3.01 μM and 0.086 μM, respectively. Increasing the length of the alkyl side chain leads to enhanced MAO-A and MAO-B inhibitory potency while introduction of a carbonyl group reduces MAO-B inhibitory potency.

Conclusions Phenylalkylcaffeines represent a new class of high-potency MAO-B inhibitors with the longer alkyl side chains yielding enhanced inhibitory activity. Such compounds may represent useful leads for the development of anti-parkinsonian therapies.

Introduction

The monoamine oxidases (MAOs) are mitochondrial bound enzymes, which are present in most mammalian tissues.[1] The two known MAO isoforms, MAO-A and MAO-B, are products of different genes and are expressed in a tissue-selective manner.[2] While MAO-A and MAO-B are both found in human liver tissue,[3] MAO-A is the main isoform in human intestinal[4] and placental tissues.[5] Both isoforms are present in the human brain, with MAO-B present in higher amounts.[6,7] The primary function of MAO is the catabolism of monoamine neurotransmitters in central and peripheral tissues. The oxidative deamination by MAO-B is considered to be one of the major catabolic pathways of dopamine in the human brain, and this process is therefore a target in the treatment of Parkinson's disease.[8] Inhibitors of MAO-B may safeguard the depleted supply of dopamine in the parkinsonian brain and prolong the activity of dopamine derived from its metabolic precursor, levodopa.[9,10] MAO-B inhibitors are thus recommended as adjunctive therapy in patients treated with levodopa. It is noteworthy that MAO-B activity, as well as density, increase with age in most brain regions while MAO-A activity remains unchanged.[7,11,12] The inhibition of the MAO-B-catalysed oxidation of dopamine is therefore of enhanced relevance in the aged parkinsonian brain. It should be noted that both MAO-A and MAO-B oxidize dopamine in the brain.[1] MAO-A inhibition, however, is in general not a desired property of drugs used in Parkinson's disease therapy since inhibitors of MAO-A potentiates the sympathomimetic effects of dietary amines such as tyramine, which may lead to serious adverse effects.[13,14]

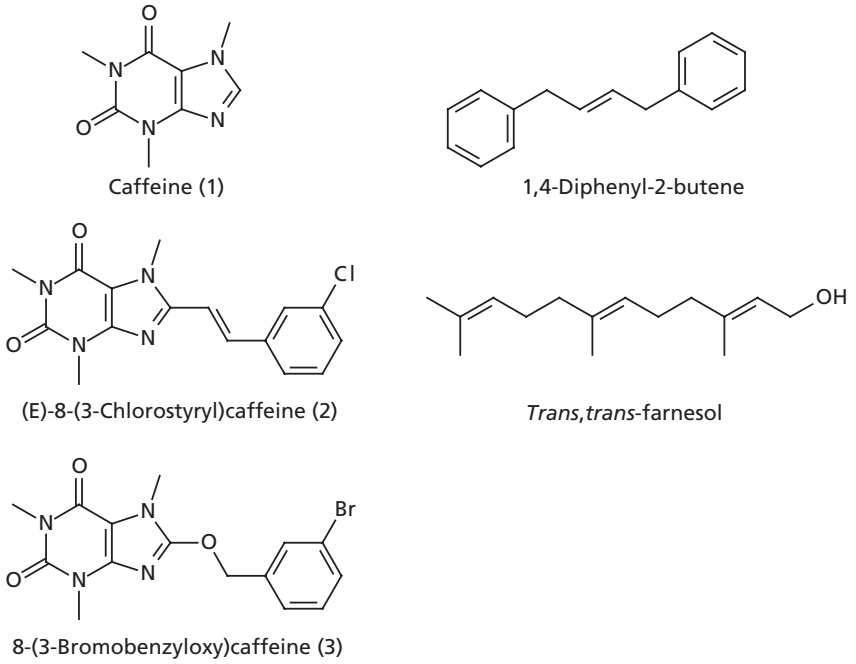


Figure 1 The structures of caffeine, (E)-8-(3-chlorostyryl)caffeine, 8-(3-bromobenzyloxy)caffeine, 1,4-diphenyl-2-butene and *trans*,*trans*-farnesol.

MAO inhibitors used for Parkinson's disease therapy should therefore be selective for the B isoform.

In Parkinson's disease, MAO-B inhibitors may also preserve dopaminergic nigrostriatal neurons by protecting against underlying neurodegenerative processes. MAO-B inhibitors may reduce the formation of potentially neurotoxic metabolic by-products of the MAO catalytic cycle.[1] The catalytic cycle of MAO-B stoichiometrically yields one mole each of an iminium intermediate that is hydrolysed to the aldehyde product (which may react with exocyclic amino groups of nucleosides and N-terminal and lysine ε-amino groups of proteins), and H_2O_2 (which may lead to oxidative damage) for each mole of monoamine substrate oxidized.[1]

Based on the above considerations, several research groups pursue the design of MAO-B selective inhibitors for Parkinson's disease therapy. While irreversible inhibitors of MAO-B have been used clinically, reversible inhibitors may have a number of potential advantages. For example, in contrast to reversible inhibition, at high drug concentrations or following repeated drug administration, irreversible inhibitors may lead to the loss of isoform selectivity.[15] Also, following withdrawal of an irreversible inhibitor, the rate by which the activity of the MAO enzyme is recovered may be variable and full recovery may require several weeks.[16,17] Following the administration of reversible inhibitors, enzyme activity is recovered when the inhibitor is cleared from the tissues. A variety of oxygen and nitrogen containing heterocycles has been employed as MAO-B inhibitors. Among these, caffeine (1) has emerged as a useful scaffold for the design of potent and reversible MAO-B inhibitors (Figure 1).[18,19] In this regard, substitution on the C8 position of caffeine yields structures which are high potency reversible MAO-B inhibitors. C8 substituents, which have been shown to be particularly effective, are the styryl and benzyloxy moieties. Examples of such structures are (E)-8-(3-chlorostyryl)caffeine (2) and 8-(3-bromobenzyloxy)caffeine (3).[18,19] These compounds are potent inhibitors of MAO-B with IC50 values 0.128 μM and 0.068 μM, respectively. To further explore the structure–activity relationships (SARs) of MAO-B inhibition by caffeine-derived compounds, the present study examines the MAO inhibitory properties of a series of caffeine analogues substituted on C8 with a variety of phenylalkyl groups. For this purpose, a homologous series (4a–f) containing the $-(CH_2)_n\text{-}C_6H_5$ ($n = 2$–7) moieties at C8 were synthesized and examined as inhibitors of human MAO-A and MAO-B. These homologues were designed to examine the effect of differing chain lengths of the C8 substituent on MAO-B inhibition activity and selectivity. In addition the $-(CH_2)_2\text{-}C_6H_{11}$ containing homologue (4g) was included to determine the requirement of a terminal phenyl ring for MAO-B inhibition. Two carbonyl containing homologues, compounds 4h and 4i, served to determine if hydrogen bonding potential of the C8 side chain may further improve MAO-B inhibitory activity. Literature reports that several amino acid residues in the MAO-B active site may be involved in such hydrogen bonding.

Examples of these are Gln206 and Tyr326.[20,21] Finally, the phenylpropenyl moiety (4j) was included to compare the MAO-B inhibition potency of a compound containing an isolated double bond to that of (E)-8-styrylcaffeine in which the vinyl group is conjugated with the caffeine ring.[18] These novel caffeine analogues are therefore a continuation of our efforts to discover highly potent and selective MAO-B inhibitors for PD therapy. As the results will show, longer alkyl side chains on C8 of the caffeine moiety yield highly potent and selective MAO-B inhibitors.

Materials and Methods

Unless otherwise noted, all starting materials were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were used without purification. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 150 MHz, respectively (Bruker, Karlsruhe, Germany). All NMR measurements were conducted in $CDCl_3$, and the chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the deuterated solvent. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet) or m (multiplet). High-resolution mass spectra (HRMS) were obtained on a Double focusing magnetic sector high resolution magnetic sector mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) in electrospray ionization (ESI) mode or with a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode. Melting points (mp) were determined on a Stuart SMP10 melting point apparatus (Stuart, Staffordshire, UK) and are uncorrected. To determine the purities of the synthesized compounds, high-performance liquid chromatography (HPLC) analyses were conducted as described previously.[15] HPLC grade acetonitrile (Merck, Darmstadt, Germany) and Milli-Q water (Millipore, Billerica, MA, USA) were used for the chromatography. For fluorescence spectrophotometry, a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was employed. The following were obtained from Sigma-Aldrich: microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg/ml), kynuramine dihydrobromide, (R)-deprenyl hydrochloride and toloxatone. Lazabemide hydrochloride was synthesized according to the patented method.[22]

Statistical methods

IC50 values were determined in triplicate and are expressed as mean ± standard deviation (SD). For the recovery of enzyme activity after the dilution and dialysis experiments, residual enzyme catalytic rates were determined in triplicate and are expressed as mean ± SD. The Kruskal–Wallis test with Dunn's post-hoc test was used to determine if statistical differences exist between the means of the residual enzyme rates recorded before and after dilution and dialysis. A P value of <0.05 is judged as being statistical significantly different. These analyses were carried out with the Prism 5 software package (GraphPad, San Diego, CA, USA).

The synthesis of caffeine analogues (4a–j)

1,3-Dimethyl-5,6-diaminouracil[23] (5, 10 mmol) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC; 13.4 mmol) were dissolved in 100 ml dioxane/H_2O (1 : 1), and the appropriate carboxylic acid (6, 10 mmol) was added. The pH of the resulting suspension was adjusted to 5 with HCl (2 M), and stirring was continued for an additional 2 h. The reaction mixture was neutralized with NaOH (1 M), cooled to 0°C and the precipitate was collected by filtration. The crude product was dissolved in 100 ml NaOH (1 M)/dioxane (1 : 1) and heated for 2 h under reflux. The reaction mixture was cooled to 0°C, acidified to a pH of 4 with 4 M aqueous hydrochloric acid and the precipitate was collected by filtration. The resulting 1,3-dimethyl-8-substituted-7*H*-xanthinyl analogues (**7**) were used in the subsequent reaction without further purification. Compound **7** (0.20 mmol) was dissolved in 5 ml N,N-dimethylformamide (DMF) and potassium carbonate (0.50 mmol) was added. To the resulting suspension, iodomethane (0.40 mmol) was added. Stirring was continued at 60°C for 60 min, the insoluble materials were removed by filtration and sufficient water (~100 ml) was added to the filtrate to precipitate the product (4), which was collected by filtration. Following crystallization from a methanol analytically pure samples of 4a–j were obtained.[24]

8-(2-Phenylethyl)caffeine (4a)

The title compound was prepared from 3-phenylpropanoic acid in a yield of 88%: mp 154–157°C (methanol). ^{1}H NMR ($CDCl_3$) δ 2.98 (t, 2H, J = 7.2 Hz), 3.05 (t, 2H, J = 7.2 Hz), 3.34 (s, 3H), 3.55 (s, 3H), 3.57 (s, 3H), 7.08 (d, 2H, J = 7.2 Hz), 7.18 (t, 1H, J = 7.2 Hz), 7.24 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 27.8, 28.9, 29.7, 31.3, 34.0, 107.1, 126.6, 128.3, 128.7, 139.9, 147.9, 151.6, 153.3, 155.2; ESI-HRMS *m/z*: calcd for $C_{16}H_{19}N_4O_2$ (MH^+), 299.1508, found 299.1502; Purity (HPLC): 99.7%.

8-(3-Phenylpropyl)caffeine (4b)

The title compound was prepared from 4-phenylbutanoic acid in a yield of 66%: mp 115–117°C (methanol). ^{1}H NMR ($CDCl_3$) δ 2.09 (qn, 2H, J = 7.5 Hz), 2.71 (q, 4H,

J = 7.5 Hz), 3.37 (s, 3H), 3.55 (s, 3H), 3.80 (s, 3H), 7.17 (m, 3H), 7.28 (t, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 26.0, 27.8, 28.7, 29.7, 31.6, 35.1, 107.3, 126.2, 128.4, 128.5, 140.9, 148.0, 151.7, 153.9, 155.3; ESI-HRMS *m/z*: calcd for $C_{17}H_{21}N_4O_2$ (MH^+), 313.1665, found 313.1659; Purity (HPLC): 99.4%.

8-(4-Phenylbutyl)caffeine (4c)

The title compound was prepared from 5-phenylpentanoic acid in a yield of 68%: mp 131–133°C (methanol). ^{1}H NMR ($CDCl_3$) δ 1.69–1.79 (m, 4H), 1.79 (t, 2H, J = 7.2 Hz), 1.77 (t, 2H, J = 7.2 Hz), 3.37 (s, 3H), 3.54 (s, 3H), 3.85 (s, 3H), 7.15 (m, 3H), 7.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 26.7, 27.1, 27.8, 29.7, 30.9, 31.7, 35.5, 107.3, 125.9, 128.3, 128.4, 141.8, 148.0, 151.7, 154.1, 155.3; ESI-HRMS *m/z*: calcd for $C_{18}H_{23}N_4O_2$ (MH^+), 327.1821, found 327.1812; Purity (HPLC): 99.2%.

8-(5-Phenylpentyl)caffeine (4d)

The title compound was prepared from 6-phenylhexanoic acid in a yield of 44%: mp 114–116°C (methanol). ^{1}H NMR ($CDCl_3$) δ 1.41 (qn, 2H, J = 7.5 Hz), 1.66 (qn, 2H, J = 7.9 Hz), 1.75 (qn, 2H, J = 7.5 Hz), 2.61 (t, 2H, J = 7.5 Hz), 2.69 (t, 2H, J = 7.9 Hz), 3.38 (s, 3H), 3.54 (s, 3H), 3.87 (s, 3H), 7.15 (m, 3H), 7.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 26.8, 27.5, 27.8, 28.3, 29.7, 31.0, 31.7, 35.7, 107.3, 125.8, 128.3, 128.4, 142.3, 148.0, 151.7, 154.3, 155.3; ESI-HRMS *m/z*: calcd for $C_{19}H_{25}N_4O_2$ (MH^+), 341.1978, found 341.1974; Purity (HPLC): 99.0%.

8-(6-Phenylhexyl)caffeine (4e)

The title compound was prepared from 6-phenylheptanoic acid in a yield of 44%: mp 114–116°C (methanol). ^{1}H NMR ($CDCl_3$) δ 1.41 (m, 4H), 1.65 (qn, 2H, J = 7.5 Hz), 1.75 (qn, 2H, J = 7.5 Hz), 2.62 (t, 2H, J = 7.9 Hz), .2.71 (t, 2H, J = 7.9 Hz), 3.40 (s, 3H), 3.57 (s, 3H), 3.89 (s, 3H), 7.17 (d, 2H, J = 7.9 Hz), 7.27 (t, 3H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 26.7, 27.4, 27.7, 28.7, 29.0, 29.6, 31.1, 31.6, 35.7, 107.1, 125.6, 128.2, 128.3, 142.4, 147.9, 151.6, 154.3, 155.2; APCI-HRMS *m/z*: calcd for $C_{20}H_{27}N_4O_2$ (MH^+), 355.2134, found 355.2134; Purity (HPLC): 99.4%.

8-(7-Phenylheptyl)caffeine (4f)

The title compound was prepared from 6-phenyloctanoic acid in a yield of 44%: mp 114–116°C (methanol). ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H), 1.54 (qn, 2H, J = 7.2 Hz), 1.65 (qn, 2H, J = 7.2 Hz), 2.52 (t, 2H, J = 7.9 Hz), .2.63 (t, 2H, J = 7.9 Hz), 3.31 (s, 3H), 3.48 (s, 3H), 3.81 (s, 3H), 7.09 (m, 3H), 7.18 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 26.7, 27.5, 27.7, 28.9, 29.0, 29.1, 29.6, 31.3, 31.6, 35.8, 107.1, 125.5, 128.1, 128.3, 142.6, 147.9, 151.6, 154.4, 155.2; APCI-HRMS *m/z*: calcd for $C_{21}H_{29}N_4O_2$ (MH^+), 369.2291, found 369.2289; Purity (HPLC): 99.2%.

8-(2-Cyclohexylethyl)caffeine (4g)

The title compound was prepared from 3-cyclohexylpropanoic acid in a yield of 78%: mp 133–134°C (methanol). ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 2H), 1.20 (m, 4H), 1.57–1.75 (m, 7H), 2.70 (t, 2H, J = 7.9 Hz), 3.37 (s, 3H), 3.54 (s, 3H), 3.88 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 24.4, 26.2, 26.5, 27.8, 29.7, 31.6, 33.0, 34.9, 37.5, 107.3, 148.0, 151.7, 154.8, 155.3; ESI-HRMS *m/z*: calcd for $C_{16}H_{25}N_4O_2$ (MH^+), 305.1978, found 305.1977; Purity (HPLC): 99.8%.

8-(3-Oxo-3-phenylpropyl)caffeine (4h)

The title compound was prepared from 4-oxo-4-phenylbutanoic acid in a yield of 59%: mp 191–194°C (methanol). ^{1}H NMR ($CDCl_3$) δ 3.11 (t, 2H, J = 7.4 Hz), 3.36 (s, 3H), 3.46 (s, 3H), 3.58 (t, 2H, J = 7.4 Hz), 4.01 (s, 3H), 7.46 (t, 2H, J = 7.5 Hz), 7.57 (t, 1H, J = 7.5 Hz), 7.98 (d, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 20.7, 27.8, 29.6, 31.7, 35.6, 107.4, 128.1, 128.7, 133.4, 136.4, 147.9, 151.7, 153.2, 155.3, 198.1; ESI-HRMS *m/z*: calcd for $C_{17}H_{19}N_4O_3$ (MH^+), 327.1457, found 327.1469; Purity (HPLC): 92.2%.

8-(4-Oxo-4-phenylbutyl)caffeine (4i)

The title compound was prepared from 5-oxo-5-phenylpentanoic acid in a yield of 62%: mp 145–146°C (methanol). ^{1}H NMR ($CDCl_3$) δ 2.20 (qn, 2H, J = 7.15 Hz), 2.83 (t, 2H, J = 7.5 Hz), 3.11 (t, 2H, J = 6.4 Hz), 3.37 (s, 3H), 3.48 (s, 3H), 3.93 (s, 3H), 7.44 (t, 2H, J = 7.5 Hz), 7.55 (t, 1H, J = 7.2 Hz), 7.92 (d, 2H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 21.6, 26.0, 27.9, 29.6, 31.8, 37.0, 107.5, 127.9, 128.7, 133.3, 136.7, 147.9, 151.7, 153.5, 155.3, 199.2; ESI-HRMS *m/z*: calcd for $C_{18}H_{21}N_4O_3$ (MH^+), 341.1614, found 341.1606; Purity (HPLC): 98.9%.

8-((2E)-3-Phenylprop-2-en-1-yl)caffeine (4j)

The title compound was prepared from trans-styrylacetic acid in a yield of 76%: mp 169–171°C (methanol). ^{1}H NMR ($CDCl_3$) δ 3.38 (s, 3H), 3.57 (s, 3H), 3.93 (s, 3H), 3.69 (d, 2H, J = 6.4 Hz), 6.28 (m, 1H), 6.44 (d, 1H, J = 15.8 Hz), 7.22 (m, 1H), 7.29 (t, 2H, J = 7.5 Hz), 7.32 (d, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 27.9, 29.8, 30.9, 31.9, 107.7, 122.5, 126.3, 127.9, 128.6, 133.3, 136.3, 148.0, 151.7, 155.3; ESI-HRMS *m/z*: calcd for $C_{17}H_{19}N_4O_2$ (MH^+), 311.1508, found 311.1493; Purity (HPLC): 99.2%.

IC50 value determinations

The IC50 values for the inhibition of the recombinant human MAO-A and MAO-B were determined according to

a previously reported protocol.[25] Potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) served as solvent for the enzyme reactions (500 µl final volume). The reactions contained the MAO-A/B mixed substrate kynuramine (45 µM for MAO-A and 30 µM for MAO-B) and the test inhibitors (0.003–100 µM). The test inhibitors were dissolved in dimethyl sulfoxide (DMSO) and were added to the reactions to yield a final concentration of 4% DMSO. Similar reactions were also carried out in the absence of inhibitor and also using the reference MAO inhibitors, toloxatone and lazabemide, as test inhibitors. MAO-A or MAO-B (0.0075 mg protein/ml) were added, and the reactions were incubated for 20 min at 37°C. The reactions were terminated with the addition of 400 µl NaOH (2 N) and 1000 µl water, and the concentrations of 4-hydroxyquinoline, the MAO-catalysed oxidation product of kynuramine, were measured by fluorescence spectrophotometry ($\lambda_{ex} = 310$; $\lambda_{em} = 400$ nm).[26] For this purpose, linear calibration curves (4-hydroxyquinoline: 0.047–1.5 µM) were constructed. The resulting MAO catalytic rates were fitted to the one site competition model incorporated into the Prism 5 software package. The IC50 values were determined in triplicate from the resulting sigmoidal concentration–inhibition curves and are expressed as mean ± SD.

Recovery of enzyme activity after dilution

Potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) served as solvent for these studies. Compound 4f (IC50(MAO-B) = 0.086 µM) at concentrations equal to 10 × IC50 and 100 × IC50 was preincubated with recombinant human MAO-B (0.75 mg/ml) for 30 min at 37°C. (R)-Deprenyl (IC50(MAO-B) = 0.079 µM) were similarly preincubated with MAO-B at a concentration equal to 10 × IC50.[25] Control incubations were conducted in the absence of inhibitor, and DMSO (4%) was added as cosolvent to all preincubations. The reactions were diluted 100-fold with the addition of kynuramine to yield final concentrations of 4f equal to 0.1 × IC50 and 1 × IC50, and of (R)-deprenyl equal to 0.1 × IC50. The final concentration of kynuramine was 30 µM, and the final concentration of MAO-B was 0.0075 mg/ml. The reactions were incubated for a further 20 min at 37°C, terminated and the residual rates of 4-hydroxyquinoline formation were measured as described above. The residual enzyme catalytic rates were expressed as mean ± SD of triplicate determinations.

Dialysis

The reversibility of the MAO-B inhibition by 4f was investigated by dialysis, employing Slide-A-Lyzer® dialysis cassettes (Thermo Scientific, Waltham, MA, USA) with a molecular weight cut-off of 10 000 and a sample volume capacity of 0.5–3 ml. MAO-B (0.03 mg/ml) and 4f, at a concentration equal to 4 × IC50, were preincubated for 15 min at 37°C. These reactions were conducted in potassium phosphate buffer (100 mM, pH 7.4) containing 5% sucrose to final volume of 0.8 ml, and DMSO (4%) was added as cosolvent. As negative and positive controls, MAO-B was also preincubated in the absence of inhibitor and presence of a concentration equal to 4 × IC50 of the irreversible inhibitor, (R)-deprenyl (IC50(MAO-B) = 0.079 µM).[25] The reactions were subsequently dialysed at 4°C in 80 ml of outer buffer (100 mM potassium phosphate, pH 7.4, 5% sucrose). The outer buffer was replaced with fresh buffer at 3 h and 7 h after the start of dialysis. At 24 h after dialysis was started, the reactions were diluted twofold with the addition of kynuramine (dissolved in potassium phosphate buffer, 100 mM, pH 7.4, made isotonic with KCl), and the residual MAO-B activities were measured as described above. The final concentration of kynuramine in these reactions was 50 µM while the final inhibitor concentrations were equal to twofold their IC50 values for the inhibition of MAO-B. For comparison, undialysed mixtures of MAO-B with 4f were maintained at 4°C over the same time period. These reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean ± SD.

Construction of Lineweaver–Burk plots

Lineweaver–Burk double reciprocal plots (1/V vs. 1/(S)) were constructed to examine the mode of MAO-B inhibition by compound 4f. For this purpose, a plot was constructed in the absence of the test inhibitor while five plots were each constructed in the presence of different concentrations of the test inhibitor. The concentrations of 4f for these studies were one-fourth × IC50, one-half × IC50, three-fourth × $IC5_0$, 1 × IC50 and 1 and one-fourth × IC50. For each Lineweaver–Burk plot, six different concentrations (15–250 µM) of kynuramine were employed. All enzymatic reactions were carried out as described for the determination of IC50 values above with the exception that the concentration of recombinant human MAO-B employed was 0.015 mg/ml. The rates of the MAO-B-catalysed formation of 4-hydroxyquinoline were measured by fluorescence spectrophotometry as described above. Linear regression analysis was performed using Prism 5, and the K_i value was estimated from the *x*-axis intercept ($-K_i$) of a replot of the slopes of the Lineweaver–Burk plots versus inhibitor concentration.

Results

Synthesis of caffeine analogues

The syntheses of the caffeine derivatives 4a–j were accomplished using the literature procedure (Figure 2).[24] 1,3-

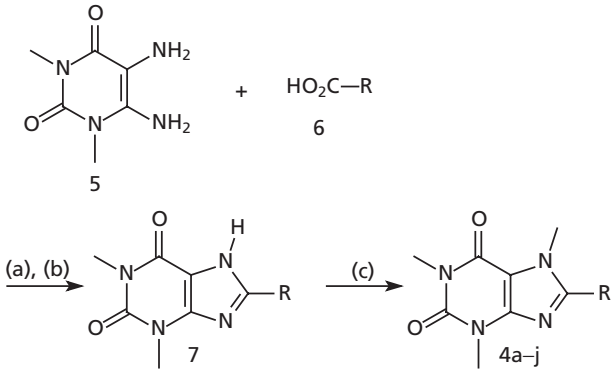


Figure 2 Synthetic pathway to caffeine derivatives 4a–j. Reagents and conditions: (a) EDAC dioxane/H_2O, rt; (b) dioxane/NaOH (aq), reflux; (c) CH_3I, K_2CO_3, DMF, 60°C.

Dimethyl-5,6-diaminouracil[23] (5) was reacted with a carboxylic acid derivative (6) in the presence of the carbodiimide dehydrating reagent, EDAC. The amide intermediate thus obtained was treated with aqueous sodium hydroxide to yield the 7*H*-xanthinyl analogues (7). Without further purification, the crude xanthine was 7*N*-methylated with an excess of iodomethane to yield the target caffeine derivatives 4a–j. Following crystallization, the structures of all compounds were verified by mass spectrometry, ^{1}H NMR and ^{13}C NMR, and the purities were estimated by HPLC analyses.

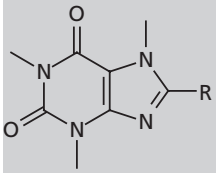
MAO inhibitory potencies of the caffeine analogues

For the purpose of this study, recombinant human MAO-A and MAO-B were used to examine the MAO inhibitory properties of the phenylalkylcaffeines, 4a–j. The MAO-A/B mixed substrate, kynuramine, served as enzyme substrate. Kynuramine is oxidized by the MAOs to ultimately yield 4-hydroxyquinoline, which can be readily monitored by fluorescence spectrophotometry ($\lambda_{ex} = 310$ nm; $\lambda_{em} = 400$ nm). This protocol is free from interference of the test inhibitors and kynuramine since these species are non-fluorescent under the measurement conditions.[26]

The results of the MAO inhibitory studies are given in Table 1. As shown, the caffeine analogues 4a–j are inhibitors of both MAO-A and MAO-B. With the exception of compound 4h, all of the homologues are selective for the MAO-B isoform with selectivity index (SI) values ranging from 2 to 79. Among the compounds examined, 4d is the most selective MAO-B inhibitor with a SI value of 79. Compared with the MAO-B selective inhibitor, lazabemide (SI = 2219), these selectivities for the MAO-B isoform are moderate. The previously studied 8-(3-bromobenzyloxy)caffeine (3) is reported to possess a SI value of 13.8, which also makes it a moderately selective inhibitor the MAO-B isoform.[19] Three homologues, 4d–f, display IC50 values for the inhibition of MAO-B in the submicromolar range. In fact, 4f (IC50 = 0.086 μM), the most potent MAO-B inhibitor among the compounds evaluated, may be considered as a highly potent MAO-B inhibitor and equipotent to the reference inhibitor lazabemide (IC50 = 0.091 μM) (Figure 3). Compound 4f also inhibited MAO-A (IC50 = 3.01 μM), although 35-fold weaker compared with its inhibitory potency towards MAO-B. In spite of this, 4f may still be considered as a moderately potent MAO-A inhibitor since it is slightly more potent than the reference inhibitor toloxatone (IC50 = 3.92 μM). It is interesting to note that 4f also was the most potent MAO-A inhibitor among the compounds evaluated. This suggests that structural modifications made to the caffeine analogues, which result in enhanced MAO-B inhibition potency, in general, also enhances MAO-A inhibition potency. As shown below, increasing the alkyl chain length of the C8 substituent is an example of such a structural modification. Among homologues 4a–f, which contain the $-(CH_2)_n$-C_6H_5 ($n = 2$–7) moieties at C8, interesting SARs are apparent. The general order of MAO-B inhibition potency of these homologues are 4f ($n = 7$) > 4e ($n = 6$) > 4d ($n = 5$) > 4c ($n = 4$) > 4a ($n = 2$) > 4b ($n = 3$). This result demonstrates that increasing the alkyl chain length of the C8 substituent is associated with enhanced MAO-B inhibition potency. The only exception is the increase of the chain length by one methylene unit from 4a to 4b, which leads to a twofold reduction in MAO-B inhibition potency. The general order of MAO-A inhibition potency of these homologues are 4f ($n = 7$) > 4e ($n = 6$) > 4d ($n = 5$) > 4b ($n = 3$) > 4c ($n = 4$) > 4a ($n = 2$). For the inhibition of MAO-A, the same trend exists as observed for the inhibition of MAO-B, where increasing the alkyl chain length of the C8 substituent enhances MAO-A inhibition potency. The only exception is found with 4b (IC50 = 117 μM) and 4c (IC50 = 125 μM), which exhibit equipotent inhibitory potencies towards MAO-A. As mentioned above, increasing the alkyl chain length of the C8 substituent is thus a structural modification that leads to enhanced inhibition of both MAO-A and MAO-B.

The $-(CH_2)_2$-C_6H_{11} containing homologue (4g) was also found to be a MAO inhibitor with IC50 values of 6.59 μM and 73.3 μM for the inhibition of MAO-B and MAO-A, respectively. A comparison of the MAO inhibitory potencies of 4g with its phenyl-substituted homologue 4a shows that 4g is a superior inhibitor of both isoforms than 4a. These data suggests that, as a terminal moiety on the C8 substituent of caffeines, the cyclohexyl moiety is more suitable than the phenyl ring for MAO inhibition. A possible explanation for this observation is that the higher degree of lipophilicity of the cyclohexyl ring compared with the phenyl ring facilitates more productive Van der Waals interactions with the

Table 1 The IC50 values for the inhibition of recombinant human MAO-A and MAO-B by compounds 4a–j, and the reference inhibitors lazabemide and toloxatone. The selectivity index (SI) values are also provided



	R	IC50 (μM)[a] MAO-A	MAO-B	SI[b]
4a	$-(CH_2)_2-C_6H_5$	172 ± 26.5	26.0 ± 5.57	7 ± 0.41
4b	$-(CH_2)_3-C_6H_5$	117 ± 0.071	43.3 ± 6.20	3 ± 0.39
4c	$-(CH_2)_4-C_6H_5$	125 ± 22.1	3.25 ± 0.176	38 ± 8.9
4d	$-(CH_2)_5-C_6H_5$	51.6 ± 2.37	0.656 ± 0.0048	79 ± 4.2
4e	$-(CH_2)_6-C_6H_5$	19.2 ± 0.74	0.442 ± 0.015	43 ± 0.33
4f	$-(CH_2)_7-C_6H_5$	3.01 ± 0.24	0.086 ± 0.003	35 ± 3.7
4g	$-(CH_2)_2-C_6H_{11}$	73.3 ± 10.0	6.59 ± 1.54	11 ± 4.2
4h	$-(CH_2)_2-CO-C_6H_5$	10.4 ± 0.767	187 ± 7.21	0.06 ± 0.002
4i	$-(CH_2)_3-CO-C_6H_5$	216 ± 5.73	46.9 ± 7.65	5 ± 0.64
4j	$-(CH_2)-CH=CH-C_6H_5$	68.4 ± 1.94	33.1 ± 4.50	2 ± 0.22
	Lazabemide	202 ± 26	0.091 ± 0.015	2219 ± 703
	Toloxatone	3.92 ± 0.015	–	–

MAO, monoamine oxidase. [a]All values are expressed as the mean ± SD of triplicate determinations. [b]The SI is the selectivity for the MAO-B isoform and is given as the ratio of IC50(MAO-A)/IC50(MAO-B). The values are reported as mean ± SD.

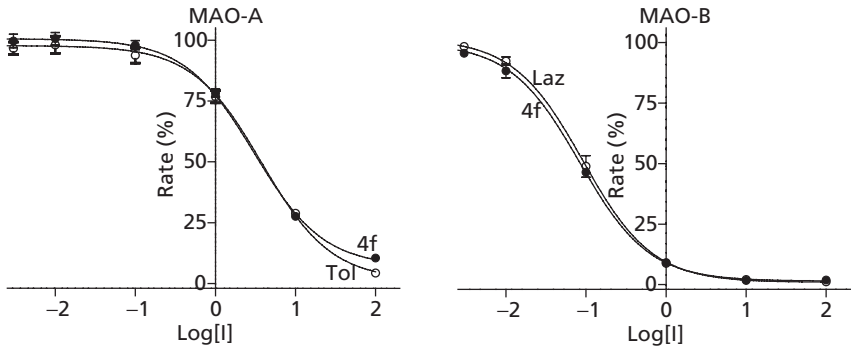


Figure 3 The sigmoidal dose–response curves (filled circles) of the monoamine oxidase (MAO)-A and MAO-B catalytic rates in the presence of compound 4f (concentration expressed in μM). For comparison, the dose-response curves (open circles) for the inhibition of MAO-A and MAO-B by toloxatone (Tol) and lazabemide (Laz), respectively, are also provided. The values are given as mean ± standard deviation (SD) of triplicate determinations.

MAO active sites. Since the active sites of both MAO-A and MAO-B are lined mostly by hydrophobic residues, lipophilic moieties of ligands are expected to interact productively with hydrophobic patches in the MAO active sites.[27,28] This proposal is supported by the observation that increasing the alkyl chain length of the C8 substituent enhances MAO inhibition potency, since an increasing alkyl chain length would also enhance the lipophilicities of the caffeine-derived inhibitors.

The two carbonyl containing homologues, compounds 4h and 4i, were found to be relatively weak MAO-B inhibitors with IC50 values of 187 μM and 46.9 μM, respectively. These MAO-B inhibition potencies are the weakest recorded for the series, which suggests that the introduction of a carbonyl group in the C8 side chain does not enhance binding to MAO-B, and potential hydrogen bonding with these carbonyl groups does not contribute to productive interactions with the MAO-B active site. While 4i was a

weak MAO-A inhibitor (IC50 = 216 μM), 4h proved to be the second most potent MAO-A inhibitor of the series with an IC50 value of 10.4 μM. These data suggest that for 4h, the carbonyl group in the C8 side chain interacts productively with the MAO-A active site. It is, however, unclear why 4i acts as a weak MAO-A inhibitor. Further investigation of the molecular interactions of 4i with MAO-A would be of interest.

The phenylpropenyl-substituted homologue 4j was found to be a moderately potent MAO-A and MAO-B inhibitor with IC50 values of 68.4 μM and 33.1 μM, respectively. These activities are more potent than those recorded for the corresponding $n = 3$ homologue 4b. This suggests that the introduction of a C–C double bond into the C8 side chain is beneficial for MAO inhibition. Compared with the MAO-B inhibition potency of (E)-8-styrylcaffeine (IC50 = 2.7 μM), 4j is, however, a 12-fold weaker inhibitor.[18] This result demonstrates that substitution on C8 of caffeine with the styryl moiety is more favourable for MAO-B inhibition than the phenylpropenyl moiety. A possible explanation for this behaviour is that the coplanar characteristic of the caffeine and styryl phenyl ring promotes binding of (E)-8-styrylcaffeine to the MAO-B active site, which is reported to be a relatively narrow and flat cavity.[28] In contrast, structures such as 4j, where the caffeine and side chain phenyl ring are not coplanar, may be less suitable for fitting into the narrow MAO-B active site cavity.

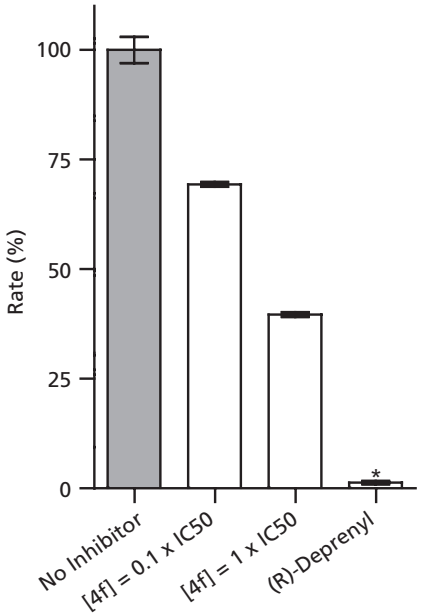


Figure 4 The reversibility of monoamine oxidase (MAO)-B inhibition of by compound 4f. MAO-B was preincubated with 4f at 10 × IC50 and 100 × IC50 for 30 min and then diluted to 0.1 × IC50 and 1 × IC50. The residual enzyme activities were subsequently measured. For comparison, MAO-B was similarly treated with (R)-deprenyl at 10 × IC50 and subsequently diluted to 0.1 × IC50. *Statistical significantly different from the mean of No inhibitor. The values are given as mean ± standard deviation (SD) of triplicate determinations.

The reversibility of MAO-B inhibition

Caffeine analogues have previously been shown to act as reversible inhibitors of human MAO-B.[29] As mentioned in the introduction, reversible inhibition has the advantage that MAO activity is recovered when treatment is terminated and the inhibitor is cleared from the tissues. In contrast, after the termination of treatment with an irreversible inhibitor, de novo protein synthesis is required for enzyme activity to recover. To examine the possibility that the most potent MAO-B inhibitor of the present series, compound 4f, is a reversible inhibitor, the reversibility of the interaction of 4f with MAO-B was investigated. Since none of the compounds examined were potent MAO-A inhibitors, the reversibility of MAO-A inhibition was not further studied. To investigate the reversibility of inhibition, the recoveries of the MAO-B catalytic activity after dilution of enzyme-inhibitor complexes were examined. Compound 4f was combined and preincubated with MAO-B at concentrations of 10 × IC50 and 100 × IC50 for 30 min. The reactions were subsequently diluted 100-fold to yield inhibitor concentrations of 0.1 × IC50 and 1 × IC50. As shown in Figure 4, after dilution of the inhibitor to 0.1 × IC50 and 1 × IC50, the MAO-B catalytic activities are recovered to 69 and 39%, respectively, of the control value. For comparison, after similar treatment of MAO-B with the irreversible inhibitor (R)-deprenyl (at 10 × IC50) and dilution of the resulting complexes to 0.1 × IC50, MAO-B activity is not recovered (1.5% of control). It may thus be concluded that 4f interacts reversibly with MAO-B. For reversible enzyme inhibition, the enzyme activities are expected to recover to levels of approximately 90 and 50%, respectively, after dilution of the inhibitor to 0.1 × IC50 and 1 × IC50. It is interesting to note that, after dilution, MAO-B catalytic activity is not recovered to 90 and 50%, respectively, as expected. This result suggests that the binding of 4f may possess a quasi-reversible or tight-binding component. Although further investigation is necessary to clarify this point, it is noteworthy that the side chains of several residues lining the MAO-B active site are flexible. The side chain of Ile199, in particular, may adopt at least two alternate conformations.[28] It is thus conceivable that upon binding of certain inhibitors (such as 4f), a conformational change of the MAO-B active site may occur, which results in slow dissociation of the inhibitor from the active site. Such a scenario may result in tight-binding of the inhibitor.

The reversibility of MAO-B inhibition by 4f was also investigated by measuring the recoveries of MAO-B activity

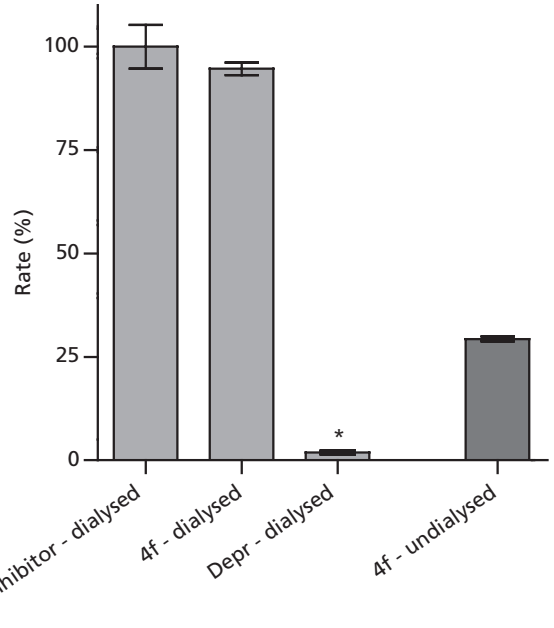


Figure 5 Reversibility of the inhibition of monoamine oxidase (MAO)-B by 4f. MAO-B and 4f (at 4 × IC50) were preincubated for 15 min, dialysed for 24 h and the residual enzyme activity was measured (4f–dialysed). MAO-B was similarly preincubated in the absence (No inhibitor–dialysed) and presence of the irreversible inhibitor, (R)-deprenyl (Depr–dialysed), and dialysed. For comparison, the residual MAO activity of undialysed mixtures of MAO-B with 4f is also shown (4f–undialysed). *Statistical significantly different from the mean of No inhibitor–dialysed. The values are given as mean ± standard deviation (SD) of triplicate determinations.

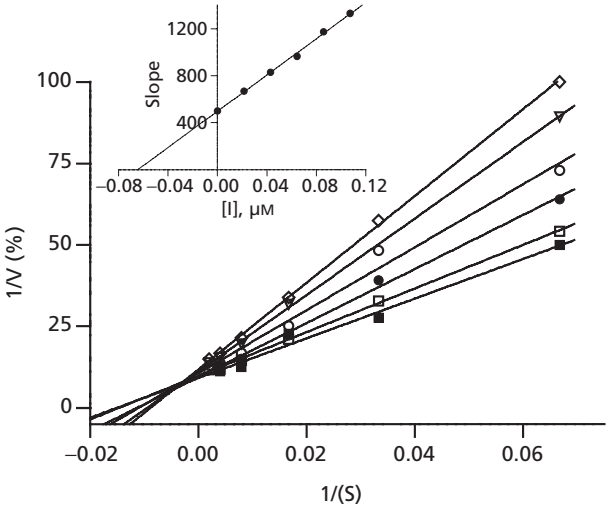


Figure 6 Lineweaver–Burk plots for the inhibition of monoamine oxidase (MAO)-B in the absence (filled squares) and presence of various concentrations of 4f. The inset is a plot of the slopes of the double reciprocal plots versus inhibitor concentration.

after dialysis of enzyme-inhibitor mixtures. MAO-B and 4f, at a concentration of 4 × IC50, were preincubated for a period of 15 min and subsequently dialysed for 24 h. The results show that MAO-B inhibition by 4f is almost completely reversed after 24 h of dialysis (Figure 5). After dialysis, MAO-B activity is recovered to a level of 95% of the control value (recorded in the absence of inhibitor). In contrast, the MAO-B activity in undialysed mixtures are 29% of the control value. This result is consistent with a reversible interaction between MAO-B and 4f. After similar preincubation and dialysis of samples containing MAO-B and the irreversible inhibitor, (R)-deprenyl, the enzyme activities are not recovered with MAO-B activity at a level of only 1.9% of the control value.

Competitive mode of MAO-B inhibition

As shown above, compound 4f is a reversible MAO-B inhibitor. To examine whether the mode of inhibition of MAO-B is competitive, sets of Lineweaver–Burk plots were constructed. For this purpose, the MAO-B catalytic activity was recorded in the absence and presence of five different concentrations of 4f employing six substrate concentrations (15–250 μM). The resulting Lineweaver–Burk plots are shown in Figure 6. For the inhibition of MAO-B, the Lineweaver–Burk plots are linear and intersect at a single point on the *y*-axis. This suggests that 4f most likely interacts competitively with MAO-B and is further support for a reversible mode of inhibition. From the replot of the slopes of the Lineweaver–Burk plots versus the inhibitor concentrations, a K_i value of 0.064 μM is estimated for the inhibition of MAO-B by 4f.

Discussion

Caffeine acts as a useful scaffold for the design of MAO inhibitors. Previous studies have shown that substitution of caffeine on C8 with a variety of moieties yields highly potent MAO inhibitors.[18,19] In this regard, the selection of the C8 moiety has a significant effect on the potency of MAO-B inhibition as well as the selectivity over the MAO-A isoform. For example, styryl substitution on C8 yields highly selective MAO-B inhibitors while benzyloxy-substituted caffeines possess affinities for both MAO-A and MAO-B. Phenoxy substitution, in contrast, results in weak MAO-B inhibitory activity.[30] The present study shows that, while shorter phenylalkyl substituents ($-(CH_2)_n$-C_6H_5, $n = 2$–4) on C8 of caffeine results in moderate MAO-B inhibition, substitution with longer phenylalkyl substituents yields highly potent MAO-B inhibitors. In this regard, substitution of caffeine with the $-(CH_2)_n$-C_6H_5 ($n = 5$–7) moieties yields inhibitors 4d–4f with IC50 values in the submicromolar range. These inhibitors also display

relatively good selectivities (SI = 35–79) for the MAO-B isoform. The observation that substitution with longer phenylalkyl substituents results in potent MAO-B inhibition may be explained, at least in part, by considering the architecture of the MAO-B active site. The MAO-B active site is reported to consist of two cavities, an entrance cavity and a substrate cavity.[28] Substrate and inhibitor molecules must pass through the entrance cavity to reach the substrate cavity where amine oxidation takes place. While small molecules bind within the substrate cavity of MAO-B, larger structures such as 1,4-diphenyl-2-butene and *trans*,*trans*-farnesol (Figure 1) span both entrance and substrate cavities. In order for larger molecules to be able to bind to MAO-B, the side chain of residue Ile199 rotates out of its normal conformation to allow for the fusion of the two cavities.[31] Larger inhibitors thus interact with residues and water molecules in both the substrate and entrance cavities while smaller molecules interact with only the substrate-binding cavity. Modelling studies have shown that substituents on C8 of caffeine most likely bind within the entrance cavity, which is reported to be a hydrophobic space.[19] The caffeine moiety is located in the substrate cavity where the carbonyl oxygens may interact with polar amino acid residues in proximity to the flavin adenine dinucleotide cofactor. The possibility therefore exists that extended phenylalkyl substituents may protrude deeper into the entrance cavity thereby maximizing the formation of productive Van der Waals interactions with the hydrophobic entrance cavity. This is expected to lead to enhanced binding affinity and MAO-B inhibition. An explanation for the observation that substitution with longer alkylphenyl substituents also enhances MAO-A inhibition is not apparent. In certain instances, compounds with extended structures may not bind well to MAO-A.[27] The reason for this is that, in the MAO-A active site, the residue corresponding to Ile199 in MAO-B is Phe208. The increased size of the Phe side chain compared with the side chain of Ile prevents it from rotating from the MAO-A active site cavity into an alternative conformation.[27] The binding of certain inhibitors may thus by hindered by Phe208 in the MAO-A active site. The possibility that the caffeine analogues examined here may undergo potential unfavourable interactions with Phe208 may, at least partly, explain why this series are MAO-B selective inhibitors. Possible reasons why the two carbonyl containing homologues, compounds 4h and 4i, act as weak MAO-B inhibitors are also not apparent. One possibility is that the introduction of a sp^2 carbon may restrict the flexibility of the C8 side chain, thus hindering it from adopting the conformation required for maximal interaction with the MAO-B entrance cavity.

Conclusion

The present study shows that substitution of caffeine on C8 with extended side chains yields structures with potent MAO-B inhibitory activities. This behaviour is exemplified by 4f ($K_i = 0.064$ μM), which possesses the 7-phenylheptyl moiety on the C8 position. Compound 4f is not only a high potency MAO-B inhibitor, but displays relatively good selectivity for MAO-B over the MAO-A isoform. A high degree of selectivity is a desirable attribute for anti-parkinsonian drugs since MAO-A inhibition is associated with potentially serious side effects. Compound 4d is also a notable inhibitor since it displayed the highest degree of selectivity for MAO-B while possessing relatively potent MAO-B inhibitory activity. The results also document that 4f is a reversible MAO-B inhibitor, which from a safety point of view is a desirable property. In contrast to irreversible inhibitors, de novo protein synthesis is not required for enzyme activity to recover after termination of treatment with a reversible inhibitor. It may thus be concluded that 4f is a promising lead for the development of reversible and selective MAO-B inhibitors, which may be used in the treatment of Parkinson's disease.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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