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Inhibition of monoamine oxidase B by analogues of the adenosine A_{2A} receptor antagonist (*E*)-8-(3-chlorostyryl)caffeine (CSC)

Nevil Vlok,[a] Sarel F. Malan,[a] Neal Castagnoli, Jr.,[b] Jacobus J.Bergh[a] and 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, Blacksburg, VA 24061, USA*

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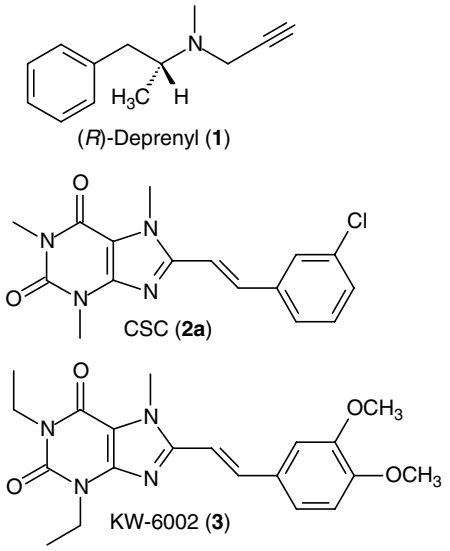
Abstract—The adenosine A_{2A} receptor has emerged as a possible target for the treatment of Parkinson's disease (PD). Evidence suggests that antagonism of the A_{2A} receptor not only improves the symptoms of the disease but may also protect against the underlying degenerative processes. We have recently reported that several known adenosine A_{2A} receptor antagonists (A_{2A} antagonists) also are moderate to very potent inhibitors of monoamine oxidase B (MAO-B). The most potent among these was (*E*)-8-(3-chlorostyryl)caffeine (CSC), a compound frequently used when examining the in vivo pharmacological effects of A_{2A} antagonists. Since MAO-B inhibitors are also thought to possess antiparkinsonian properties, dual targeting drugs that block both MAO-B and A_{2A} receptors may have enhanced therapeutic potential in the treatment of PD. In this study, we prepared selected analogues of CSC in an attempt to examine specific structural features that may be important for potent MAO-B inhibition. The results of a SAR study established that the potency of MAO-B inhibition by (*E*)-8-styrylcaffeinyl analogues depends upon the van der Waals volume (V_w), lipophilicity (π), and the Hammett constant (σ_m) of the substituents attached to C-3 of the phenyl ring of the styryl moiety. Potency also varies with substituents attached to C-4 with bulkiness (V_w) and lipophilicity (π) being the principal substituent descriptors.

1. Introduction

Due to their role in the metabolism of monoamine transmitters, monoamine oxidase A and B (MAO-A and -B) are of considerable pharmacological interest. Inhibitors of MAO provide a therapeutically useful approach for the treatment of neurological and psychiatric diseases. In particular, reversible MAO-A inhibitors are used as antidepressant and antianxiety drugs,[1] while selective inhibitors of MAO-B are in use and are under investigation for treatment of the symptoms or the underlying neurodegenerative processess leading to Parkinson's disease (PD)[2,3] and Alzheimer's disease.[4] The mechanism-based inactivator of MAO-B, (*R*)-deprenyl (**1**) (Scheme 1), is frequently used in combination with L-DOPA as dopamine replacement therapy in PD.[5] The beneficial effects of (*R*)-deprenyl may be dependent



Scheme 1. The structures of the MAO-B inactivator (*R*)-deprenyl (**1**) and the A_{2A} antagonists, CSC (**2a**) and KW-6002 (**3**).

Keywords: Monoamine oxidase B; Reversible inhibitors; Structure–activity relationship; SAR.
* Corresponding author. Tel.: +27 18 2992262; fax: +27 18 2994243; e-mail: fchjpp@puk.ac.za

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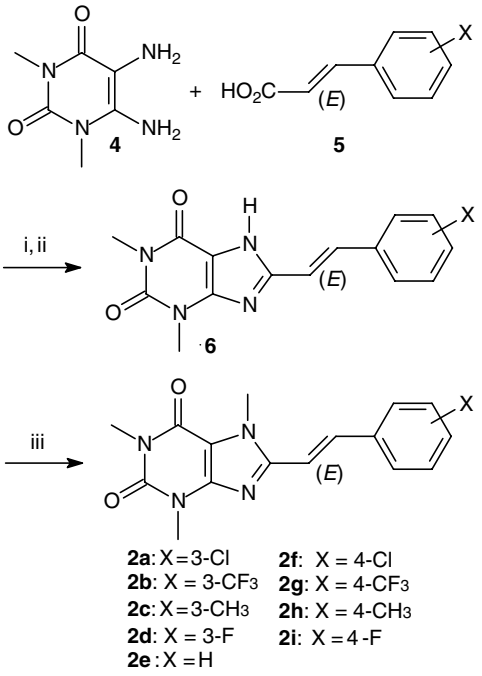
on the inhibition of the MAO-B catalyzed oxidation of dopamine in the CNS, consequently conserving the depleted supply of dopamine and delaying the need for levodopa therapy in patients diagnosed with early PD.[5] Inhibition of dopamine oxidation also results in the stoichiometric reduction of the production of hydrogen peroxide, which is thought to play a role in the etiology of neurodegenerative diseases such as PD.[6,7] (*R*)-Deprenyl is also reported to exert a neuroprotective effect by blocking apoptotic cell death[8,9] and may be clinically useful in postponing the emergence of symptoms that require the initiation of levodopa therapy in PD patients.[10] In contrast with reversible inhibitors, return of enzyme activity following treatment with inactivators such as (*R*)-deprenyl requires de novo synthesis of the MAO-B protein. Aside from the safety considerations associated with irreversible inhibitors, (*R*)-deprenyl is also metabolized to (*R*)-methamphetamine, a compound with vasopressor properties.[2] For these reasons, several studies are currently underway to develop safer inhibitors of MAO-B as an alternative to (*R*)-deprenyl.[11,12] In contrast to (*R*)-deprenyl, these inhibitors are designed to be reversible while retaining selectivity toward MAO-B.

(*E*)-8-(3-Chlorostyryl)caffeine (CSC) (**2a**) (Scheme 1) had been identified and employed as a selective inhibitor of the A_{2A} subtype of adenosine receptors.[13–15] However, it was recently found to inhibit MAO-B with a K_i value of 70 nM by a pathway that appears to be independent of its actions on the A_{2A} receptor.[16] Several analogues of CSC were prepared as part of an effort to define the structural requirements of this class of compounds to act as inhibitors of MAO-B.[17,18] The adenosine A_{2A} receptor antagonist (A_{2A} antagonist) properties of (*E*)-8-styrylcaffeinyl analogues[19] have been of special interest because A_{2A} antagonists are currently being investigated as possible therapeutic agents for the symptomatic treatment of motor deficits in PD.[20] One such compound, KW-6002 (**3**) (Scheme 1), is currently undergoing clinical trials for this purpose.[21,22] Results from recent studies in animal models of PD suggest that antagonism of the A_{2A} receptor may not only relieve the symptoms of the disease but may also protect against underlying neuronal degenerative processes.[23–25] Since MAO-B inhibitors also are considered to be antiparkinsonian agents, the possibility of designing drugs that act both as A_{2A} antagonists and inhibitors of MAO-B merits its consideration.

We have therefore chosen to examine additional analogues (**2b**–**i**, Scheme 2) of CSC in an attempt to identify specific structural features of CSC that may be responsible for its potency as an MAO-B inhibitor. Earlier studies suggested that structural features important for MAO-B inhibition are the *trans* configuration of the styryl moiety and 1,3,7-trimethyl substituents on of the xanthinyl ring system.[17] As part of the present SAR analysis, the compounds investigated in this study retained these features and only differed in the substituents on C-3 and C-4 of the styryl ring. The principal aim was to determine whether substitution of C-3 of the styryl ring with an electron-withdrawing group is essential



Scheme 2. Reagents and conditions: Synthetic pathway to (*E*)-8-styrylcaffeinyl analogues **2**. Key: (i) EDAC, dioxane/H_2O; (ii) NaOH (aq), reflux; (iii) CH_3I, K_2CO_3, DMF.

for potent MAO-B inhibition as suggested by the structure of CSC in an earlier study.[17] This study also reports a comparison between the enzyme–inhibitor dissociation constants (K_i values) obtained with two different MAO-B substrates.

2. Results

2.1. Chemistry

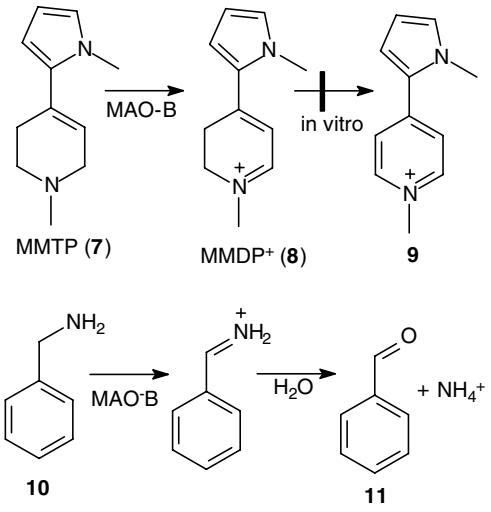
The procedures by which (*E*)-8-styrylcaffeinyl analogues (**2a**–**c** and **2e**–**i**) were synthesized are documented in the literature (Scheme 2).[13,14,19] Compound **2d** was not prepared in this study and the K_i value for the inhibition of MAO-B was obtained from the literature.[17] Because of the ease of synthesis and relatively good yields, we followed the procedure described by Suzuki et al.,[13] (Scheme 2). Acylation of 1,3-dimethyl-5,6-diaminouracil (**4**) with commercially available *trans*-cinnamic acid (**5e**) and 3-chloro- (**5a**), 3-trifluoromethyl- (**5b**), 3-methyl- (**5c**), 4-chloro- (**5f**), 4-trifluoromethyl- (**5g**), 4-methyl (**5h**) or 4-fluoro- (**5i**) *trans*-cinnamic acids in the presence of a carbodiimide reagent (1-ethyl-2-[3-(dimethylamino)propyl]-carbodiimide, EDAC) followed by treatment with sodium hydroxide resulted in the corresponding 1,3-dimethyl-(*E*)-8-styryl-7*H*-xanthinyl analogues (**6**). Without further purification, the precipitate obtained from this reaction was selectively 7N-methylated in the presence of an excess of iodomethane and potassium carbonate to yield the target compounds **2a**–**c** and **2e**–**i**. Following recrystallization from a suitable solvent the structures and purity of the compounds were verified by mass spectrometry, ^{1}H NMR, and ^{13}C NMR. For compounds **2a** and **2e** the physical data were compared

to the corresponding literature values as cited in Section 4. The *trans* geometry about the styryl π-bond was confirmed by proton–proton coupling constants which were in the range of 15.7–15.8 Hz for the olefinic proton signals.

2.2. Enzymology

The K_i values for the competitive inhibition of MAO-B by compounds **2a**–**c** and **2e**–**i** were estimated by two different assays. In the first instance, the extent by which various concentrations of the test inhibitor slowed the rate of the MAO-B catalyzed oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP) (**7**) to the corresponding dihydropyridinium metabolite ($MMDP^+$) (**8**) (Scheme 3) was measured spectrophotometrically.[26,27] $MMDP^+$ production was measured at 420 nm, a wavelength at which neither the substrate nor the test inhibitors absorb light. Because of the favorable chromophoric characteristics and in vitro chemical stability of $MMDP^+$, this assay is frequently used to estimate K_i values for the inhibition of both MAO-A and -B.[28,29] In order to prevent any contribution toward the oxidation of MMTP by MAO-A, the source of the MAO-B enzyme must either be devoid of any MAO-A activity or the oxidation of MMTP by MAO-A must be prevented by inactivating the enzyme with the selective mechanism-based inactivator clorgyline. Since we used the mitochondrial fraction isolated from baboon liver mitochondria, a source that has been shown to be devoid of MAO-A activity,[27] our assays were carried out in the absence of clorgyline pre-inactivation. The absence of MAO-A activity in the baboon liver mitochondrial fraction was confirmed, since pre-incubation of the enzyme with clorgyline (3×10^{-8} M) failed to decrease the rate at which MMTP is oxidized, while (*R*)-deprenyl (3×10^{-7} M) almost completely prevented oxidation (data not shown).

In the second approach, we utilized the selective MAO-B substrate benzylamine (**10**) (Scheme 3) to measure K_i values. Benzylamine is arguably the most thoroughly characterized substrate of MAO-B and is frequently used in inhibition[30] and mechanistic[31,32] studies involving this enzyme. When using purified MAO-B as enzyme source, which is relatively free from background interference, the concentration of the α-carbon oxidation product, benzaldehyde (λ_{max} = 250 nm), may be measured spectrophotometrically.[33] In contrast, background absorption in the near-UV wavelength range when using the mitochondria as enzyme source is too high to measure benzaldehyde concentrations by spectrophotometry. Even protein precipitation and subsequent centrifugation (the last two steps during a discontinuous assay) of the incubations do not solve this problem. For this reason we chose to measure the extent of benzylamine oxidation by an HPLC-UV assay. Figure 1 presents an example of the chromatographic tracings routinely observed during this study. The incubation time of the enzyme catalyzed reaction was chosen to be 8 min since the benzylamine oxidation was found to be linear (Fig. 2) for at least 10 min at all substrate concentrations used (125–750 μM). Also in order to select appropriate substrate concentrations for the inhibition assays, the K_m for benzylamine oxidation by baboon liver MAO-B was estimated. Considering the similarity of the steady-state kinetic parameters (K_m and V_{max}) between baboon and human MAO-B for tetrahydropyridinyl substrates,[27] we expected that the K_m values for benzylamine oxidation by the two enzymes would also be similar. Consistent with this anticipation, the K_m value for benzylamine oxidation by baboon liver MAO-B was estimated to be 616 ± 23 μM (Fig. 3), while the reported value with human MAO-B is 500 ± 0.1 μM.[33]



Scheme 3. The MAO-B catalyzed oxidation of MMTP (**7**) and benzylamine (**10**) to the corresponding dihydropyridinium product **8** and benzaldehyde (**11**), respectively. The in vitro oxidation of **8** to the pyridinium species (**9**) is not observed.

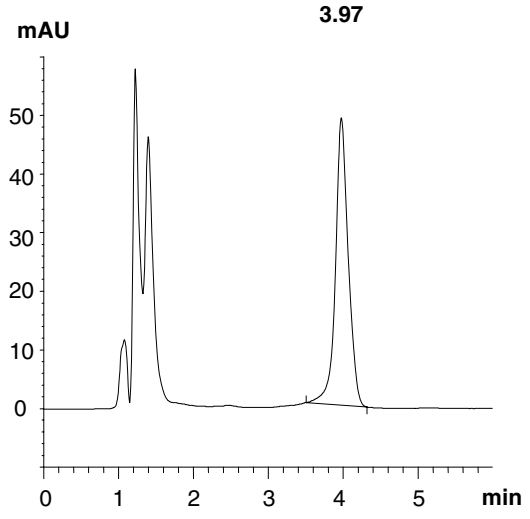


Figure 1. An HPLC-UV tracing showing the presence of benzaldehyde (retention time of 3.97 min) in an incubation of benzylamine (750 μM) with baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). Following an 8-min incubation at 37 °C, the reaction was terminated by precipitating the mitochondrial protein with perchloric acid. After centrifugation, 50 μL of the supernatant was injected into the HPLC and the effluent was monitored at a wavelength of 250 nm.

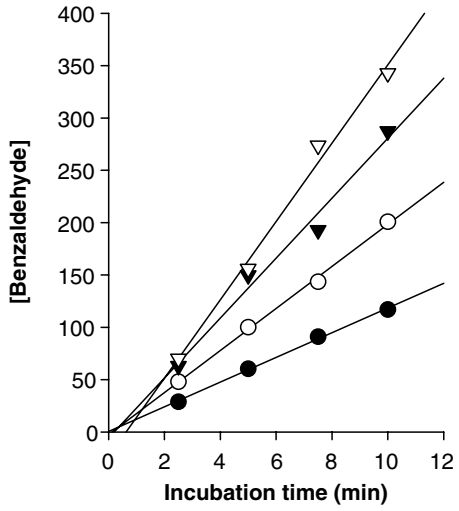


Figure 2. Linearity in the oxidation of benzylamine by baboon liver MAO-B (0.15 mg/mL of the mitochondrial preparation). The concentration of benzaldehyde produced was measured by the HPLC analysis following termination of the enzyme catalyzed reaction at time points of 2.5, 5, 7.5 or 10 min. The concentration of benzaldehyde produced is expressed as nmoles/mg mitochondrial protein. The concentrations of benzylamine used in this study were 125 μM (filled circles), 250 μM (open circles), 500 μM (filled triangles), and 750 μM (open triangles).

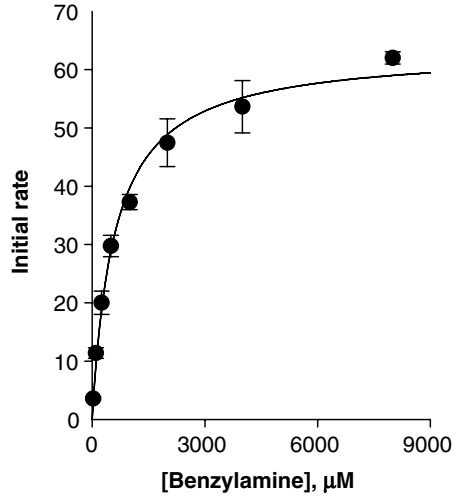


Figure 3. Determination of the K_m value of benzylamine oxidation by baboon liver MAO-B. The concentration of benzaldehyde produced was measured by the HPLC analysis following an 8-min incubation with 0.15 mg/mL baboon liver mitochondria at 37 °C. A K_m value of 616 ± 23 μM was estimated by fitting the data to the Michaelis–Menten equation using a nonlinear least-squares fitting routine. All measurements were conducted in triplicate and the benzylamine concentration in the incubations ranged from 25 to 8000 μM. The initial rates are expressed as nmol mg protein^{-1} min^{-1} of benzaldehyde formed.

2.3. Inhibition and SAR studies

All of the (*E*)-8-styrylcaffeinyl analogues (**2a**–**c** and **2e**–**i**) tested were found to be inhibitors of MAO-B. As demonstrated by example with (*E*)-8-(3-trifluoromethylstyryl)caffeine (**2b**) (Fig. 4), the lines of the Lineweaver–Burk plots intersected, indicating that the mode of inhibition was competitive. The K_i values for the inhibition of MAO-B are presented in Table 1. The lead compound for this study, CSC (**2a**), was confirmed[16] to be a very potent inhibitor with a K_i value of 148 nM for the inhibition of benzylamine oxidation by MAO-B. The similarity in range of this value with that obtained with MMTP as substrate (128 nM) is an indication of the reliability of the K_i estimations and also suggests that specific alterations in experimental conditions (such as

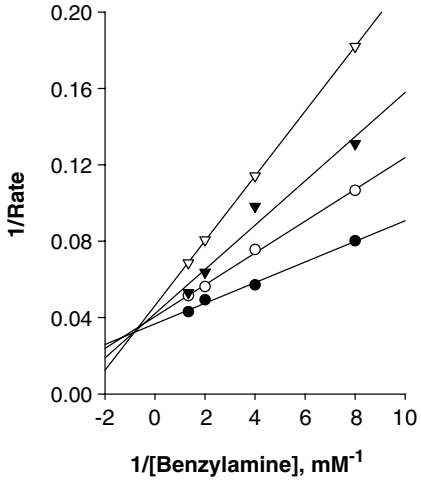
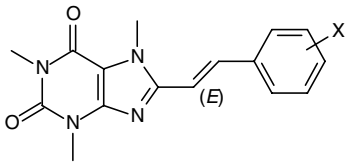


Figure 4. Lineweaver–Burk plots of the oxidation of benzylamine by baboon liver MAO-B in the absence (filled circles) and presence of various concentrations of **2b** (open circles, 0.1 μM; filled triangles, 0.2 μM; open triangles, 0.4 μM). The concentration of the baboon liver mitochondrial preparation was 0.15 mg/mL and the rates are expressed as nmol mg protein^{-1} min^{-1} of benzaldehyde formed.

Table 1. The K_i values for the inhibition of MAO-B by (*E*)-8-styrylcaffeinyl analogues. The values of the selected physiochemical parameters used in the SAR study are also listed[36,37]



Compound	X	K_i value (nM)[a]		V_w[d]	π[e]	σ[e]
		Substr **7**[b]	Substr **10**[c]			
2a	3-Cl	70,[f] 128	148	1.07	0.71	0.37
2b	3-CF_3	133	188	1.11	0.88	0.43
2c	3-CH_3	1431	1526	1.01	0.56	−0.07
2d	3-F	400[g]	—	0.36	0.14	0.34
2e	H	2704,[g] 2864	2316	0.08	0.00	0.00
2f	4-Cl	260	187	1.07	0.71	0.23
2g	4-CF_3	245	238	1.11	0.88	0.54
2h	4-CH_3	367	373	1.01	0.56	−0.17
2i	4-F	1559	1374	0.36	0.14	0.06

[a] The enzyme source used was MAO-B from baboon liver mitochondria.
[b] MMTP served as enzyme substrate.
[c] Benzylamine served as enzyme substrate.
[d] Values obtained from Ref. 37.
[e] Values obtained from Ref. 36.
[f] K_i value obtained from Ref. 16.
[g] K_i value obtained from Ref. 17.

change of substrate or analytical technique) do not affect the estimated values to a large extent. In accordance with this view, for the other analogues (**2b–c** and **2e–i**) tested, the differences between the K_i values estimated by the two different techniques are within the range expected for experimental error (Table 1). The correlation between the K_i values obtained with the two different techniques was found to be 0.98 (Fig. 5).

Qualitative inspection of the results in Table 1 suggests that the potency of MAO-B inhibition by (*E*)-8-styrylcaffeinyl analogues bearing substituents on C-3 of the phenyl ring (**2a–d**) depends upon the electronic characteristics of the substituent since those analogues with electron-withdrawing groups (**2a–b** and **2d**) are significantly more potent as inhibitors than the unsubstituted analogue (**2e**) and the analogue bearing an electron-donating methyl group (**2c**). In contrast, the inhibition potency by analogues bearing substituents on C-4 of the phenyl ring (**2f–i**) appears to be dependent upon the size of the substituent since inhibitors with relatively bulky substituents (**2f–h**) are more potent than the unsubstituted analogue (**2e**) and the analogue bearing a C-4 fluorine substituent (**2i**). In order to quantify these apparent relationships between MAO-B inhibitory activity and the physiochemical properties of the substituents, a Hansch-type SAR study[34–36] was carried out by stepwise multiple linear regression analysis. Five parameters were used to describe each substituent. The van der Waals volume (V_w)[37] and Taft steric parameter (E_s)[36] were used as descriptors of bulkiness, while the lipophilicities of the substituents were described by the Hansch constant (π).[38] The classical Hammett (σ_m or σ_p) and Swain–Lupton (F) constants[39] served as electronic parameters. All physiochemical values of the substituents were obtained from standard compilations.[36,37] The analogues were divided into two groups – those bearing substituents on C-3 of the phenyl ring (**2a–d**) and those with substituents on C-4 of the phenyl ring (**2f–i**). The unsubstituted (*E*)-8-styrylcaffeinyl analogue (**2e**) was considered a member of both groups. Results of the statistical analysis for the two groups are shown in Tables 2 and 3, respectively. These tabulated results were generated using only the K_i values obtained with MMTP (**7**) as substrate.

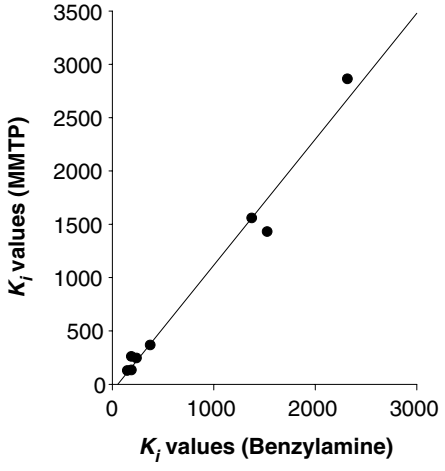


Figure 5. Correlation of enzyme–inhibitor dissociation constants (K_i values) of **2a–c** and **2e–i** obtained with two different MAO-B substrates, benzylamine (**10**) and MMTP (**7**). The correlation coefficient was found to be 0.98.

For analogues substituted at C-3 of the phenyl ring (Table 2) the only single substituent parameters that showed a meaningful correlation with the logarithm of the K_i values (expressed in μM) were the Hammett electronic parameter (σ_m) and the Swain–Lupton F constant. Regression analysis of $\log K_i$ with σ_m and F exhibited moderate correlations with R^2 values of 0.86 and 0.80, respectively. The statistical F values were found to be 18.9 and 12.3 for the two correlations (a higher F value indicates a better fit)[40] with confidence levels of 97.8% and 96.1%, respectively. All other single-parameter correlations with the $\log K_i$ values exhibited poorer statistical correlations. Correlation may be improved by addition of an additional substituent parameter to the regression analysis. Two-parameter fits with V_w and σ_m or with π and σ_m yielded correlation coefficients of 0.99 and 0.98, respectively. For these correlations, the probabilities that V_w and π are zero are 3.3% and 9%, respectively. Therefore, the best mathematical description of

Table 2. Correlations of the MAO-B inhibition constants ($\log K_i$) of (*E*)-8-styrylcaffeinyl analogues bearing substituents on C-3 of the styryl ring (**2a–e**) with steric, electronic, and hydrophobic substituent parameters[a]

Parameter	Slope	y-intercept	R^2	F[b]	Significance[c]
σ_m	−2.44 ± 0.56	0.21 ± 0.17	0.86	18.9	0.022
F	−2.40 ± 0.68	0.29 ± 0.22	0.80	12.3	0.039
V_w	−0.83 ± 0.56	0.29 ± 0.47	0.42	2.2	0.23
E_s	0.42 ± 0.30	0.11 ± 0.39	0.39	1.9	0.26
π	−1.17 ± 0.64	0.23 ± 0.36	0.53	3.3	0.16
$V_w + \sigma_m$	−0.49 ± 0.09	0.49 ± 0.07	0.99	109	0.033
	−2.10 ± 0.19				0.0079
$\pi + \sigma_m$	−0.61 ± 0.20	0.39 ± 0.10	0.98	41.2	0.090
	−1.98 ± 0.32				0.025

[a] The $\log K_i$ values (expressed in μM) obtained with substrate **7** (MMTP) were used in the linear regression analysis.

[b] The F test statistic relates the mean squares due to regression to the error variance. Higher F values indicate a better fit and a regression equation with an F value higher than the critical F value may be judged as significant. Critical F values may be calculated as described recently.[40]

[c] The significance is the fractional probability that the coefficient of the added variable is zero.

Table 3. Correlations of the MAO-B inhibition constants ($\log K_i$) of (*E*)-8-styrylcaffeinyl analogues bearing substituents on C-4 of the styryl ring (**2e–i**) with steric, electronic, and hydrophobic substituent parameters[a]

Parameter	Slope	y-intercept	R^2	F[b]	Significance[c]
σ_p	−0.86 ± 0.92	−0.08 ± 0.25	0.22	0.86	0.42
F	−0.72 ± 1.17	−0.02 ± 0.38	0.11	0.38	0.58
V_w	−1.03 ± 0.04	0.55 ± 0.04	0.99	608	0.0002
E_s	0.45 ± 0.17	0.26 ± 0.22	0.70	7.0	0.077
π	−1.28 ± 0.15	0.39 ± 0.08	0.96	75.2	0.0032
$V_w + \sigma_p$	−1.00 ± 0.03	0.55 ± 0.03	0.99	542	0.0012
	−0.11 ± 0.06				0.21

[a–c] See Table 2 for footnotes.

binding affinity ($\log K_i$) of C-3-substituted (*E*)-8-styrylcaffeinyl analogues to MAO-B is:

$$\begin{aligned}\mathrm{Log}K_i = & -2.10(\pm 0.19)\sigma_m - 0.49(\pm 0.09)V_w \\ & + 0.49(\pm 0.07) \\ & (F = 109 \text{ and } R^2 = 0.99).\end{aligned}$$

The negative signs of both the σ_m (-2.10 ± 0.19) and the V_w (-0.49 ± 0.09) parameter coefficients indicate that the potency ($\log K_i$) by which (*E*)-8-styrylcaffeinyl analogues inhibit MAO-B may be enhanced by substitution with larger C-3 functional groups with electron-withdrawing characteristics. Since the Hansch constant (π) is also negatively correlated (-0.61 ± 0.20; Table 2) with $\log K_i$, C-3 lipophilic substituents also appear to enhance MAO-B inhibition potency.

For analogues substituted at C-4 of the phenyl ring (Table 3) the single-parameter fit that showed the best correlations with the $\log K_i$ values were the van der Waals volume V_w and the Hansch constant (π) of the substituents. The regression analysis yielded R^2 values of 0.99 and 0.96 with a confidence level of >99.6% for the two correlations. The mathematical description of the binding affinity ($\log K_i$) of C-4 substituted (*E*)-8-styrylcaffeinyl analogues to MAO-B may therefore be presented as:

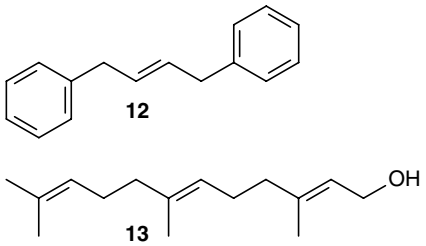
$$\begin{aligned}\mathrm{Log}K_i = & -1.03(\pm 0.04)V_w + 0.55(\pm 0.04) \\ & (F = 608 \text{ and } R^2 = 0.99).\end{aligned}$$

As observed with the analogues substituted at the C-3 position, the negative sign of the V_w parameter coefficient (-1.03 ± 0.04) suggests that the potency ($\log K_i$) of MAO-B inhibition may be enhanced by substitution with bulky C-4 functional groups. On the other hand, the electronic contribution of the C-4 substituents to $\log K_i$ appears to be negligible since in a two-parameter fit with V_w and σ_p, the probability of the term containing σ_p being zero is 21%. The same argument applies to the other electronic parameter (*F*) considered in this study (data not shown). There is also a meaningful correlation ($R^2 = 0.96$; $F = 75$) between the Hansch constants (π) of the C-4 substituents and the $\log K_i$ values (Table 3). Hydrophobic C-4 functional groups appear to favor inhibition potency since the sign of the π parameter coefficient (-1.28 ± 0.15) is negative. Again there does not seem to be an electronic contribution of the C-4 substituents to $\log K_i$ since in a two-parameter fit of $\log K_i$ with π and σ_p or of $\log K_i$ with π and *F* the probability of the terms containing σ_p and *F* being zero are 13.9% and 86.8%, respectively (data not shown).

3. Discussion

Recent descriptions of the crystal structures of human recombinant MAO-B co-crystallized with several propargylamine inactivators[41,42] have led to the proposal that the substrate must transverse an 'entrance cavity' in order to gain access to the 'substrate cavity'. The 'gate' separating the two cavities appears to be the side chain of Ile-199, which may exhibit different rotamer conformations which allows for the fusion of the two cavities in order to accommodate larger inhibitors such as the reversible inhibitor 1,4-diphenyl-2-butene (**12**) (Scheme 4).[43] For this inhibitor the first phenyl ring extends into the entrance cavity space where it is in contact with specific hydrophobic amino acid residues (Phe-103, Ile-199 and Ile 316), whereas the second phenyl ring is in contact with Tyr-398 and Tyr 435 in the substrate cavity space. The latter two amino acids together with the FAD co-factor, which exists in a bent and twisted conformation, form an aromatic cage for the substrate amine recognition. Since compounds **2a**–**i** and 1,4-diphenyl-2-butene are similar in size, it may be expected that the phenyl ring of **2a**–**i** projects into the entrance cavity in a similar manner as that observed for 1,4-diphenyl-2-butene, while the polar functional groups of the caffeine ring are in contact with the flavin within the substrate cavity. A similar binding mode is observed for the reversible MAO-B inhibitor, *trans*,*trans*-farnesol (**13**) (Scheme 4),[29] which also spans both the entrance and substrate cavities with the polar OH moiety in close contact with the flavin. Extension and binding of the styryl moiety into the entrance cavity may be responsible for the high potency of certain (*E*)-8-styrylcaffeinyl analogues as MAO-B inhibitors. In accordance with this hypothesis, caffeine was found to be a very weak competitive inhibitor of MAO-B.[16]

In the present study, we have observed that C-3 and C-4 substitution of the phenyl ring has a considerable effect on the potency with which (*E*)-8-styrylcaffeinyl analogues inhibit MAO-B. This may be in part due to specific interactions between the substituents and amino acid side chains in the entrance cavity. Since the entrance cavity is lined by the side chains of hydrophobic amino acids, these interactions are likely to be van der Waals forces. In accordance with this view, the SAR study indicates that increasing inhibition potency correlates with increasing hydrophobicity and size of the C-3 and C-4 substituents. Interestingly it appears that electron-withdrawing substituents at C-3 enhances MAO-B inhibition of the analogues tested in this study. A possible explanation may be that electron-withdrawing functionalities at C-3, but not at C-4, cause partial positive charge localization in the phenyl ring which in turn may lead to electrostatic interactions with electronegative regions within the entrance cavity. With electron-withdrawing functionalities at C-4 a similar charge localization may be diminished through delocalization via the styryl double bond to the xanthine ring system.



Scheme 4. The structures of the reversible MAO-B inhibitors 1,4-diphenyl-2-butene (**12**) and *trans*,*trans*-farnesol (**13**).

4. Experimental

Caution. MMTP (**7**) 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.[44]

4.1. Chemicals and instrumentation

All starting materials not described elsewhere were obtained from Sigma–Aldrich and were used without purification. The oxalate salt of MMTP (**7**)[45] and 1,3-dimethyl-5,6-diaminouracil (**4**)[46] were prepared as previously reported. Because of chemical instability compound **4** was used within 3 h of preparation. Melting points (mp) were determined by differential scanning calorimetry (DSC) on a Shimadzu DSC-50 instrument or on a Gallenkamp melting point apparatus (capillary method). All melting points are uncorrected. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. Proton (^{1}H) spectra were recorded in $CDCl_3$ at a frequency of 300 MHz and carbon (^{13}C) spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the $CDCl_3$. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Direct insertion electron impact ionization (EIMS) and high-resolution mass spectra (HRMS) were obtained on a VG 7070E mass spectrometer. HPLC analyses were performed with an Agilent 1100 HPLC system equipped with a variable wavelength detector and a Macherey–Nagel CC 125/4 Nucleosil C18 column (4.6 × 100 mm, 5 μm). UV–vis spectra were recorded on a Milton-Roy Spectronic 1201 spectrophotometer.

4.2. General procedure for the preparation of (*E*)-8-styrylcaffeinyl analogues (2a–i)

The preparations of **2a**–**c** and **2e**–**i** were accomplished using the procedure (Scheme 2) described by Suzuki et al.[13] To a solution of 1,3-dimethyl-5,6-diaminouracil (**4**, 2.7 mmol) and 1-ethyl-2-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDAC; 3.9 mmol) in 20 mL dioxane–H_2O (1:1) was added the appropriately substituted *trans*-cinnamic acid (**5**, 2.9 mmol). The pH of the suspension was adjusted to 5 with 2 M aqueous hydrochloric acid and stirring was continued for an additional 2 h. Following neutralization with 1 M aqueous sodium hydroxide the reaction was cooled to 0 °C and the precipitate was collected by filtration. The crude product in 30 mL aqueous sodium hydroxide (1 M)–dioxane (1:1) was heated under reflux for 45 min. After cooling to 0 °C, the solution was acidified to a pH of 4 with 4 M aqueous hydrochloric acid and the precipitate was collected by filtration. The resulting 1,3-methyl-(*E*)-8-styryl-7*H*-xanthinyl analogues (**6**) were used in the subsequent reaction without further purification. Iodomethane (0.32 mmol) was added to a stirred suspension of **6** (0.16 mmol) and potassium carbonate (0.25 mmol) in 5 mL DMF. After stirring at 50 °C for 60 min, the insoluble materials were removed by filtration and sufficient water was added to the filtrate to precipitate the product (**2**) that was collected by filtration. Following recrystallization from a mixture of methanol–ethyl acetate (9:1) analytically pure samples of **2** were obtained. For previously reported **2a** and **2e** we found the melting points to be 212 and 222 °C [from methanol–ethyl acetate (9:1)], while the reported melting points are 205 and 220–222 °C, respectively.[19] NMR and MS data also correlated to those described. The characterization of previously unreported compounds (**2b**–**c** and **2f**–**i**) is summarized below.

(*E*)-*8*-(*3-Trifluoromethylstyryl*)*caffeine* (**2b**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-3-trifluoromethylcinnamic acid in a yield of 35%: mp 243.7 °C (238 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 3.40 (s, 3H), 3.61 (s, 3H), 4.08 (s, 3H), 6.96 (d, 1H, J = 15.8 Hz), 7.51 (m, 1H), 7.59 (d, 1H, J = 7.8 Hz), 7.72 (d, 1H, J = 6.32 Hz), 7.80 (s, 1H), 7.81 (d, 1H, J = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 27.94 (CH_3), 29.73 (CH_3), 31.61 (CH_3), 108.22 (C), 113.02 (CH), 122.07 (C), 123.72 (CH), 125.81 (CH), 129.48 (CH), 130.51 (CH), 131.32 (C), 131.76 (C), 136.45 (CH), 148.53 (C), 149.19 (C), 151.67 (C), 155.28 (C); EIMS *m*/*z* 364 ($M^{\cdot+}$); HRMS calcd. 364.1147, exp. 364.1136.

(*E*)-*8*-(*3-Methylstyryl*)*caffeine* (**2c**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-3-methylcinnamic acid in a yield of 24%: mp 216.1 °C (219 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 3H), 3.37 (s, 3H), 3.59 (s, 3H), 4.02 (s, 3H) 6.86 (d, 1H, J = 15.8 Hz), 7.15 (d, 1H, J = 7.0 Hz), 7.26 (m, 1H), 7.34 (d, 2H, J = 7.9 Hz), 7.74 (d, 1H, J = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 21.33 (CH_3), 27.86 (CH_3), 29.69 (CH_3), 31.45 (CH_3), 107.82 (C), 110.96 (CH), 124.58 (CH), 127.89 (CH), 128.78 (CH), 130.32 (CH), 135.41 (C), 138.43 (CH), 138.55 (C), 148.52 (C), 149.99 (C), 151.65 (C), 155.17 (C); EIMS *m*/*z* 310 ($M^{\cdot+}$); HRMS calcd. 310.1430, exp. 310.1415.

(*E*)-*8*-(*4-Chlorostyryl*)*caffeine* (**2f**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-4-chlorocinnamic acid in a yield of 17%: mp 226.9 °C (232 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 3.37 (s, 3H), 3.58 (s, 3H), 4.02 (s, 3H), 6.84 (d, 1H, J = 15.7 Hz), 7.34 (d, 2H, J = 8.5 Hz), 7.47 (d, 2H, J = 8.5 Hz), 7.71 (d, 1H, J = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 27.89 (CH_3), 29.70 (CH_3), 31.49 (CH_3), 107.99 (C), 111.71 (CH), 128.45 (CH), 129.17 (CH), 133.97 (C), 135.28 (C), 136.79 (CH), 148.50 (C), 149.55 (C), 151.63 (C), 155.19 (C); EIMS *m*/*z* 330 ($M^{\cdot+}$); HRMS calcd. 330.0884, exp. 330.0877.

(*E*)-*8*-(*4-Trifluoromethylstyryl*)*caffeine* (**2g**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-4-trifluoromethylcinnamic acid in a yield of 64%: mp 230.2 °C (235 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 3.35 (s, 3H), 3.57 (s, 3H), 4.04 (s, 3H), 6.95 (d, 1H, J = 15.8 Hz), 7.62 (m, 4H), 7.77 (d, 1H, J = 15.7 Hz); ^{13}C NMR ($CDCl_3$) δ 27.87 (CH_3), 29.67

(CH_3), 31.50 (CH_3), 108.14 (C), 113.52 (CH), 125.76 (CH), 127.38 (CH), 130.68 (C), 131.12 (C), 136.28 (CH), 138.83 (C), 148.42 (C), 149.07 (C), 151.55 (C), 155.16 (C); EIMS *m/z* 364 ($M^{\cdot+}$); HRMS calcd. 364.1147, exp. 364.1136.

(*E*)-*8*-(*4-Methylstyryl*)*caffeine* (**2h**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-4-methylcinnamic acid in a yield of 80%: mp 205.5 °C (208 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H), 3.37 (s, 3H), 3.58 (s, 3H), 4.00 (s, 3H) 6.81 (d, 1H, J = 15.7 Hz), 7.17 (d, 2H, J = 8.0 Hz), 7.43 (d, 2H, J = 8.1 Hz), 7.73 (d, 1H, J = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 21.37 (CH_3), 27.83 (CH_3), 29.67 (CH_3), 31.40 (CH_3), 107.74 (C), 110.14 (CH), 127.27 (CH), 129.61 (CH), 132.71 (C), 138.25 (CH), 139.79 (C), 148.52 (C), 150.13 (C), 151.64 (C), 155.13 (C); EIMS *m/z* 310 ($M^{\cdot+}$); HRMS calcd. 310.1430, exp. 310.1428.

(*E*)-*8*-(*4-Fluorostyryl*)*caffeine* (**2i**) was prepared from 1,3-dimethyl-5,6-diaminouracil (**4**) and *trans*-4-fluorocinnamic acid in a yield of 55% yield: mp 242.26 °C (245 °C by capillary method); ^{1}H NMR ($CDCl_3$) δ 3.36 (s, 3H), 3.58 (s, 3H), 4.02 (s, 3H), 6.78 (d, 1H, J = 15.8 Hz), 7.13 (m, 2H), 7.52 (m, 2H), 7.72 (d, 1H, J = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 27.85 (CH_3), 29.67 (CH_3), 31.44 (CH_3), 107.87 (C), 110.95 (CH), 128.99 (CH), 129.10 (CH), 131.75 (C), 136.93 (CH), 148.48 (C), 149.71 (C), 151.61 (C), 155.15 (C), 161.71 (C); EIMS *m/z* 314 ($M^{\cdot+}$); HRMS calcd. 314.1179, exp. 314.1174.

4.3. MAO-B activity measurements and inhibition studies

Mitochondria were isolated from baboon liver tissue as described by Salach and Weyler[47] and stored at −70 °C. The mitochondrial isolate was suspended in sodium phosphate buffer (100 mM, pH 7.4, containing 50% glycerol, w/v) and the protein concentration was determined by the method of Bradford.[48] For the inhibition studies on MAO-B we utilized the MAO-A and -B mixed substrate MMTP (K_m = 60.9 μM for baboon liver MAO-B)[26] and the MAO-B selective substrate benzylamine (K_m = 616 ± 23 μM for baboon liver MAO-B). Since baboon liver mitochondria are devoid of MAO-A activity, inactivation of this enzyme was unnecessary in the studies where MMTP was utilized as substrate.[27] A typical incubation (500 μL final volume in 100 mM sodium phosphate buffer, pH 7.4) contained MMTP (30–120 μM) or benzylamine (125–750 μM), the mitochondrial isolate (0.15 mg protein/mL), and various concentrations of the test inhibitors. Stock solutions of the inhibitors were prepared in DMSO and were added to the incubation mixtures to yield a final DMSO concentration of 4% (v/v). DMSO concentrations higher than 4% are reported to inhibit MAO-B.[11] The samples were incubated at 37 °C and the incubation times were 15 min for MMTP and 8 min for benzylamine. For these time periods the MAO-B catalyzed production of $MMDP^+$ and benzaldehyde was linear with time.[27] The reactions were terminated by the addition of 10 μL of 70% perchloric acid and the samples were centrifuged at 16,000*g* for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B generated products, $MMDP^+$ (where MMTP served as substrate) and benzaldehyde (where benzylamine served as substrate), were measured. $MMDP^+$ formation was monitored spectrophotometrically at a wavelength of 420 nm (ε = 25,000 M^{-1})[27], while benzaldehyde concentrations were measured by the HPLC analysis (see Chemicals and instrumentation). In the latter case, a reversed-phase C18 column was used with the mobile phase consisting of 60% distilled water [containing 0.6% (v/v) glacial acetic acid and 1% (v/v) triethylamine], 30% methanol, and 10% acetonitrile and of a flow rate of 1 mL/min. A volume of 50 μL of the supernatant was injected into the HPLC system and the elution of benzaldehyde (3.97 min) was monitored at a wavelength of 250 nm. Quantitative estimations of benzaldehyde were made by means of a linear calibration curve ranging from 6.25 to 50 μM. Initial rates were expressed as nmoles of product ($MMDP^+$ or benzaldehyde) formed per mg mitochondrial protein per min. Competitive K_i values were determined by calculating the initial rates of substrate oxidation in the absence and presence of varying concentrations of the inhibitors and constructing Lineweaver–Burk plots with increasing concentrations of the inhibitor. The slopes of the Lineweaver–Burk plots were plotted as a function of the inhibitor concentration and the K_i values were determined from the abscissa intercept (intercept = $-K_i$). Linear regression analysis was performed using the SigmaPlot software package (Systat Software Inc.).

4.4. Linearity of benzylamine oxidation with time

In order to select a suitable incubation time for benzylamine with baboon liver MAO-B, we determined the time interval during which the MAO-B catalyzed benzaldehyde formation remains linear under our assay conditions. Various concentrations of benzylamine (125–750 μM) were incubated with the mitochondrial isolate (0.15 mg protein/mL) at 37 °C for various periods of time (2.5–10 min). The same incubation conditions were followed as described above. For the purpose of this study no inhibitor was included in the reaction incubations. Following termination of the reactions by the addition of 10 μL perchloric acid, the concentrations of the MAO-B generated benzaldehyde were measured by the HPLC analysis. Graphs of benzaldehyde concentration as a function of time were constructed.

4.5. K_m determination of benzylamine for baboon liver MAO-B

The steady-state rate of benzylamine oxidation by baboon liver MAO-B was measured by the HPLC analysis as outlined above. In order to estimate a K_m value for the oxidation of benzylamine, rates were measured at eight different substrate concentrations spanning at least two orders of magnitude (25–8000 μM). The enzyme concentration utilized was 0.15 mg baboon liver mitochondrial protein/mL and all incubations were carried out at 37 °C for a time period of 8 min. Benzaldehyde concentrations were measured with the aid of a linear calibration curve prepared over a range of 1.5 to

50 μM. The kinetic data (initial rates as a function of substrate concentration) were fitted to the Michaelis–Menten equation using a nonlinear least-squares fitting routine incorporated into the SigmaPlot software package. This determination was carried out in triplicate and the K_m value was expressed as mean ± standard deviation.

4.6. SAR studies

Values for the substituent parameters σ_m, σ_p, F, π, and E_s were obtained from Hansch and Leo[36], and those for the Van der Waals volume (V_w) were obtained from Van de Waterbeemb and Testa.[37] Stepwise multiple linear regression analysis of the $\log K_i$ values as a function of the substituent parameters was carried out with Statistica software package (StatSoft Inc.). In order to estimate the significance of the regression equations, the F statistic was employed. An F value higher than the critical F value was judged to be significant and critical F values were calculated as described recently.[40] The critical F value (F_{max}) for 95% significance for models constructed from five $\log K_i$ values (Tables 2 and 3) and which contains one parameter (out of a possible five: V_w, E_s, π, σ_m, and$F$) was calculated to be 34.29 while a model containing two parameters has an F_{max} value of 170.59.

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