

# Chapter 4

## Enzymology

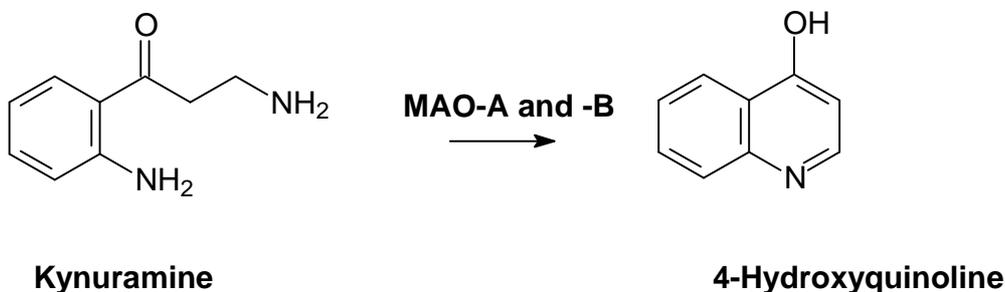
### 4.1 Introduction

Various methods are currently available for measuring the catalytic activity of the MAOs. These measurements are based on measuring the disappearance of reagents and/or the formation of products (Nicotra & Parvez, 1999). In this chapter, the 8-sulfanylcaffeine analogues, compounds **3a–e**, **4a–c** and **5a–b**, the 8-sulfinylcaffeine analogues, **6a–b**, and the 8-sulfonylcaffeine, **7**, which were synthesized in the previous chapter, will be investigated as inhibitors of human MAO-A and –B. The MAO inhibitory properties of these compounds will be investigated using a spectrofluorometric method. For this purpose kynuramine will be used as substrate. This assay is based on measuring the formation of 4-HQ, the fluorescent metabolite of kynuramine. MAO-A and –B oxidize kynuramine to yield 4-HQ, which may be measured fluorometrically at an excitation wavelength of 310 nm and an emission wavelength of 400 nm.

### 4.2 Biological evaluation to determine IC<sub>50</sub> values

#### 4.2.1 Introduction

As mentioned above, a spectrofluorometric method employing kynuramine as MAO substrate will be used to examine the MAO inhibitory properties of the test inhibitors. The inhibition potencies of the test inhibitors will be expressed as the corresponding IC<sub>50</sub> values. The IC<sub>50</sub> values are defined as the concentration of the inhibitor that produces 50% inhibition. For this assay, kynuramine will be used as a suitable substrate, which is non-fluorescent, with the formation of 4-HQ as metabolite, which is fluorescent.



**Figure 4.1.** MAO oxidation of kynuramine to yield 4-HQ.

To experimentally determine  $IC_{50}$  values, the initial MAO catalytic rates will be measured in the absence and presence of various concentrations of the test inhibitor. The catalytic rates will subsequently be graphed versus the logarithm of the inhibitor concentration to obtain a sigmoidal curve, and the  $IC_{50}$  value will be determined from this graph. If an inhibitor exhibits a low  $IC_{50}$  value, the inhibitor may be viewed as a potent inhibitor. In contrast, if an inhibitor exhibits a high  $IC_{50}$  value, the inhibitor may be viewed as a weak inhibitor.

#### **4.2.2 Chemicals and instrumentation**

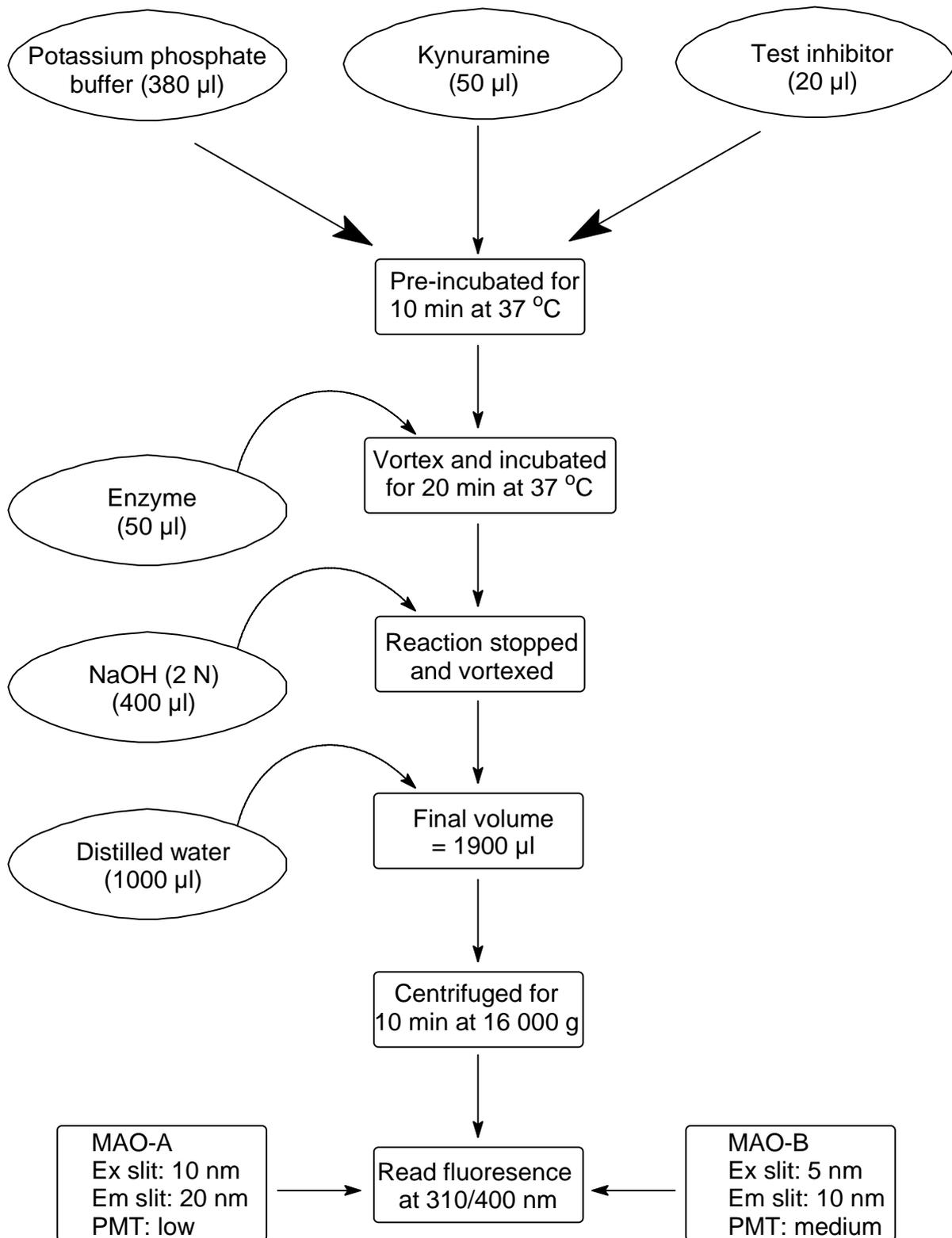
A Varian Cary Eclipse fluorescence spectrophotometer was used for the fluorometric measurements. Insect cell microsomes containing recombinant human MAO-A and MAO-B (5 mg/ml) and kynuramine.2HBr were obtained from Sigma-Aldrich. The Graphpad Prism 5 software package was used to construct the sigmoidal curves and to determine the  $IC_{50}$  values.

#### **4.2.3 Method**

All reactions were prepared in potassium phosphate buffer (100 mM at a pH of 7.4) and were carried out in triplicate. The reaction contained the following:

1. 380  $\mu$ l potassium phosphate buffer.
2. 50  $\mu$ l of the substrate (kynuramine) was added to yield final concentrations of 45  $\mu$ M and 30  $\mu$ M for MAO-A and -B, respectively.
3. 20  $\mu$ l of the test inhibitor. The stock solutions of the inhibitors were prepared in dimethyl sulfoxide (DMSO) and added to the enzyme reactions to yield inhibitor concentrations of 0, 0.003, 0.01, 0.1, 1, 10, and 100  $\mu$ M.
4. The reactions were pre-incubated for 10 min at 37 °C.
5. 50  $\mu$ l of recombinant human MAO-A or -B were added to the reactions to yield a concentration of 0.075 mg/ml of the enzymes.
6. The reactions were subsequently incubated at 37 °C for 20 min.
7. To terminate the reactions, 400  $\mu$ l of NaOH (2 N) was added followed by 1000  $\mu$ l of distilled water.
8. The reactions were then centrifuged at 16 000 g for 10 min.
9. The amount of fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 310 nm and an emission wavelength of 400 nm:

- The PMT voltage was set to low for MAO-A with an excitation slit width of 10 nm and an emission slit width of 20 nm.
  - The PMT voltage was set to medium for MAO-B with an excitation slit width of 5 nm and an emission slit width of 10 nm.
10. A calibration curve was prepared for each data set by measuring the fluorescence of increasing concentrations of 4-HQ (0.0469, 0.09375, 0.1875, 0.375, 0.75, 1.5  $\mu\text{M}$ ). Each of these calibration standards contained 4% (v/v) DMSO and potassium phosphate buffer to a final volume of 500  $\mu\text{l}$ . To each calibration standard were added 400  $\mu\text{l}$  of NaOH and 1000  $\mu\text{l}$  of distilled water.
11. Sigmoidal rate-concentration curves were constructed with the Graphpad Prism 5 software package and the  $\text{IC}_{50}$  values were calculated.

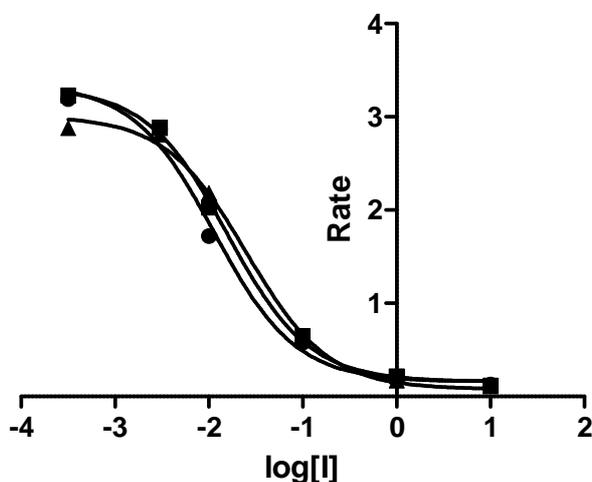


**Figure 4.2.** Summary of the protocol used to determine  $IC_{50}$  values.

## 4.3 Results

### 4.3.1 Sigmoidal rate-concentration curves obtained for the IC<sub>50</sub> value determinations

Sigmoidal rate-concentration curves were generated for the inhibition of MAO-A and –B by each of the test compounds. The rate of the MAO catalyzed oxidation of kynuramine was graphed versus the logarithms of the different concentrations of the inhibitor, and the concentration of the inhibitor which reduces the rate to half of the maximal value was calculated. As an example, the sigmoidal rate-concentration curve for the determination of the IC<sub>50</sub> for the inhibition of human MAO-B of the most potent compound (**3c**) is given in Fig. 4.3.

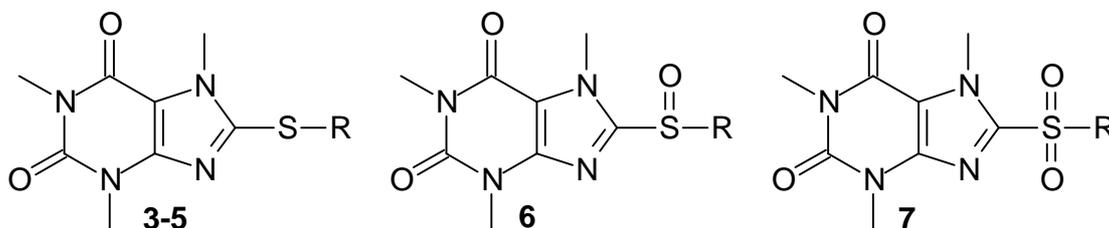


**Figure 4.3.** The sigmoidal rate-concentration curve of the initial rates of kynuramine oxidation by recombinant human MAO-B vs. the logarithm of the concentration of inhibitor **3c** (expressed in  $\mu\text{M}$ ).

### 4.3.2 IC<sub>50</sub> values

The IC<sub>50</sub> values for the inhibition of recombinant human MAO-A and –B by the test inhibitors are shown in table 4.1. As mentioned, a lower IC<sub>50</sub> value is an indication of a higher binding affinity of the inhibitor for the enzyme and a compound with a low IC<sub>50</sub> value is therefore a more potent inhibitor. Also shown is the selectivity index (SI) which can be defined as the selectivity of the inhibitor for the MAO-B isoform. The SI value is the ratio of the IC<sub>50</sub> value for the inhibition of MAO-A to the IC<sub>50</sub> value for the inhibition of MAO-B by a particular compound. The higher the SI, the more selective the inhibitor is toward the MAO-B isoform. The IC<sub>50</sub> values were determined in triplicate and were expressed as mean  $\pm$  standard deviation (SD).

**Table 4.1.** The IC<sub>50</sub> values for the inhibition of recombinant human MAO-A and –B by the 8-sulfanylcaffeine analogues, compounds **3a–e**, **4a–c** and **5a–b**, the 8-sulfinylcaffeine analogues, **6a–b**, and the 8-sulfonylcaffeine, **7**<sup>a</sup>.



| Compound  | -R   | MAO-A IC <sub>50</sub> (μM) | MAO-B IC <sub>50</sub> (μM) | SI <sup>P</sup> |
|-----------|--|-----------------------------|-----------------------------|-----------------|
| <b>3a</b> | -(CH <sub>2</sub> ) <sub>2</sub> -(3-Cl-C <sub>6</sub> H <sub>4</sub> )                | 5.66 ± 0.187                | 0.043 ± 0.0085              | 132             |
| <b>3b</b> | -(CH <sub>2</sub> ) <sub>2</sub> -(3-Br-C <sub>6</sub> H <sub>4</sub> )                | 5.70 ± 1.87                 | 0.040 ± 0.0017              | 143             |
| <b>3c</b> | -(CH <sub>2</sub> ) <sub>2</sub> -(3-CF <sub>3</sub> -C <sub>6</sub> H <sub>4</sub> )  | 141 ± 26.8                  | 0.017 ± 0.007               | 8294            |
| <b>3d</b> | -(CH <sub>2</sub> ) <sub>2</sub> -(3-CH <sub>3</sub> -C <sub>6</sub> H <sub>4</sub> )  | 12.0 ± 2.39                 | 0.051 ± 0.005               | 235             |
| <b>3e</b> | -(CH <sub>2</sub> ) <sub>2</sub> -(3-OCH <sub>3</sub> -C <sub>6</sub> H <sub>4</sub> ) | 48.1 ± 5.93                 | 0.125 ± 0.021               | 385             |
| <b>4a</b> | -(CH <sub>2</sub> ) <sub>3</sub> -C <sub>6</sub> H <sub>5</sub>                        | 6.48 ± 0.802                | 0.500 ± 0.041               | 13              |
| <b>4b</b> | -(CH <sub>2</sub> ) <sub>3</sub> -(3-Cl-C <sub>6</sub> H <sub>4</sub> )                | 3.53 ± 0.381                | 0.062 ± 0.008               | 57              |
| <b>4c</b> | -(CH <sub>2</sub> ) <sub>3</sub> -(4-Cl-C <sub>6</sub> H <sub>4</sub> )                | 0.708 ± 0.124               | 0.061 ± 0.004               | 12              |
| <b>5a</b> | -CH <sub>2</sub> -(3-Cl-C <sub>6</sub> H <sub>4</sub> )                                | 6.43 ± 1.55                 | 0.227 ± 0.024               | 28              |
| <b>5b</b> | -CH <sub>2</sub> -(3-Br-C <sub>6</sub> H <sub>4</sub> )                                | 37.9 ± 1.39                 | 0.199 ± 0.039               | 190             |
| <b>6a</b> | -CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>  | 250 ± 18.1                  | 131 ± 33.4                  | 2               |
| <b>6b</b> | -CH <sub>2</sub> -(4-F-C <sub>6</sub> H <sub>4</sub> )                                 | 166 ± 25.9                  | 11.8 ± 0.28                 | 14.1            |
| <b>7</b>  | -(CH <sub>2</sub> ) <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>                        | 240 ± 53.7                  | 5.11 ± 1.12                 | 47              |

<sup>a</sup> All values are expressed as the mean ± SD of triplicate determinations.

<sup>b</sup> The selectivity index is the selectivity for the MAO-B isoform and is given as the ratio of the IC<sub>50</sub>(MAO-A)/IC<sub>50</sub>(MAO-B).

As shown in table 4.1, 8-sulfanylcaffeine analogues, compounds **3a–e**, **4a–c** and **5a–b**, the 8-sulfinylcaffeine analogues, **6a–b**, and the 8-sulfonylcaffeine, **7**, evaluated in this study were inhibitors of MAO-A and –B. The following general observations can be made from the IC<sub>50</sub> values in table 4.1:

- Compound **3c** was shown to be the most potent inhibitor of MAO-B with an  $IC_{50}$  value of 0.017  $\mu$ M. This compound was, however, a weak inhibitor of MAO-A with an  $IC_{50}$  value of 141  $\mu$ M. Compound **3c** also showed the highest degree of isoform selectivity with a selectivity index of 8294, which indicates that it is a much more potent inhibitor of MAO-B compared to MAO-A. Compound **3c** is therefore a potent selective MAO-B inhibitor.
- Compound **4c** was shown to be the most potent inhibitor of MAO-A with an  $IC_{50}$  value of 0.708  $\mu$ M.
- The least potent inhibitor for MAO-A and -B was compound **6a** with  $IC_{50}$  values of 250  $\mu$ M and 131  $\mu$ M, respectively. This compound also showed the lowest degree of isoform selectivity with a selectivity index of 2 which indicates that it is a non-selective inhibitor of MAO.

To gain insight into the SAR of MAO inhibition by the test inhibitors, the following comparisons and conclusions may be made:

a) Firstly, the MAO-A and MAO-B inhibition potencies of the test inhibitors may be compared.

Table 4.1 shows that the SI values of the test inhibitors range from 2 to 8294. Since all the SI values are  $> 1$ , it may be concluded that the inhibitors evaluated in this study are MAO-B selective. As mentioned above, compound **3c** represents a particularly selective inhibitor with an SI value of 8294. The results also show that the test inhibitors are in general highly potent MAO-B inhibitors with ten of the thirteen analogues examined, exhibiting  $IC_{50}$  values in the submicromolar range. These are notably the 8-[(phenylethyl)sulfanyl]caffeines (**3a-e**), the 8-[(phenylpropyl)sulfanyl]caffeines (**4a-c**), and the 8-(benzylsulfanyl)caffeines (**5a-b**). The 8-sulfanylcaffeines (**6a-b**) and 8-sulfonylcaffeine (**7**) were comparatively weak MAO-B inhibitors with  $IC_{50}$  values  $> 5 \mu$ M. Only one compound, **4c**, exhibited an  $IC_{50}$  value (0.708  $\mu$ M) for the inhibition of MAO-A in the submicromolar range. It is interesting to note that the 8-[(phenylethyl)sulfanyl]caffeines (**3a-e**), the 8-[(phenylpropyl)sulfanyl]caffeines (**4a-c**), and the 8-(benzylsulfanyl)caffeines (**5a-b**) also are more potent MAO-A inhibitors than the 8-sulfanylcaffeines (**6a-b**) and 8-sulfonylcaffeine (**7**). Compounds **3-5** exhibited  $IC_{50}$  values for the inhibition of MAO-A of 0.708–141  $\mu$ M while **6-7** exhibited  $IC_{50}$  values of 166–250  $\mu$ M. From this analysis it may be concluded that:

- The test inhibitors are selective for the MAO-B isoform.
- The 8-[(phenylethyl)sulfanyl]caffeines (**3a-e**), the 8-[(phenylpropyl)sulfanyl]caffeines (**4a-c**), and the 8-(benzylsulfanyl)caffeines (**5a-b**) are highly potent MAO-B inhibitors.

- The 8-sulfinylcaffeines (**6a–b**) and 8-sulfonylcaffeine (**7**) are comparatively weak MAO-B inhibitors.
- The only highly potent MAO-A inhibitor identified was **4c** with an IC<sub>50</sub> value of 0.708 μM.
- While only moderately potent MAO-A inhibitors, the 8-[(phenylethyl)sulfanyl]caffeines (**3a–e**), the 8-[(phenylpropyl)sulfanyl]caffeines (**4a–c**), and the 8-(benzylsulfanyl)caffeines (**5a–b**) also are more potent MAO-A inhibitors than the 8-sulfinylcaffeines (**6a–b**) and 8-sulfonylcaffeine (**7**).

b) The MAO-A and –B inhibition potencies of the 8-[(2-phenylethyl)sulfanyl]caffeine analogues that are substituted on the phenyl ring may be compared to the IC<sub>50</sub> value of the unsubstituted homologue, 8-[(2-phenylethyl)sulfanyl]caffeine analogue (**2a**) (Table 4.2).

**Table 4.2.** A comparison of the IC<sub>50</sub> values for the inhibition of MAO-B by the unsubstituted homologue, 8-[(2-phenylethyl)sulfanyl]caffeine analogue (**2a**), with those homologues containing substituents at C-3 of the phenyl ring.

| Compound  | -R               | MAO-B IC <sub>50</sub> (μM) | Ratio H/R <sup>a</sup> |
|-----------|------------------|-----------------------------|------------------------|
| <b>2a</b> | H                | 0.223                       | -                      |
| <b>3a</b> | Cl               | 0.043                       | 5.2                    |
| <b>3b</b> | Br               | 0.040                       | 5.6                    |
| <b>3c</b> | CF <sub>3</sub>  | 0.017                       | 13.1                   |
| <b>3d</b> | CH <sub>3</sub>  | 0.051                       | 4.4                    |
| <b>3e</b> | OCH <sub>3</sub> | 0.125                       | 1.8                    |

<sup>a</sup>The ratio of IC<sub>50</sub>(**2a**)/IC<sub>50</sub>(**3a–e**)

The results in table 4.2 show that substitution on the phenyl ring of **2a** leads to a considerable enhancement of its MAO-B inhibition potency, with all of the substituted homologues exhibiting more potent MAO-B inhibition than **2a**. The IC<sub>50</sub> values recorded for these homologues (**3a–e**) ranged from 0.017–0.125 μM, making them twofold to 13-fold more potent MAO-B inhibitors than the lead compound, **2a**. For comparison, the reversible MAO-B selective inhibitor, lazabemide, exhibits an IC<sub>50</sub> value of 0.091 μM under the same conditions (unpublished data from our laboratory). Interestingly, both alkyl (CF<sub>3</sub>, CH<sub>3</sub> and OCH<sub>3</sub>) and halogen (Cl and Br) substitution lead to highly potent MAO-B inhibition. It may therefore be concluded that substitution on C-3 is a general strategy to enhance the MAO-B inhibition potency of 8-[(phenylethyl)sulfanyl]caffeine (**2a**). A similar analysis for the inhibition of MAO-A shows that substitution on the phenyl ring of **2a** (IC<sub>50</sub> = 20.5 μM) with Cl (IC<sub>50</sub> = 5.66 μM),

Br ( $IC_{50} = 5.7 \mu\text{M}$ ) and  $\text{CH}_3$  ( $IC_{50} = 12 \mu\text{M}$ ) leads to an enhancement in MAO-A inhibition potency. In contrast, substitution on the phenyl ring of **2a** with  $\text{CF}_3$  ( $IC_{50} = 141 \mu\text{M}$ ) and  $\text{OCH}_3$  ( $IC_{50} = 48.1 \mu\text{M}$ ) leads to a reduction of the MAO-A inhibition potency.

c) The MAO-A and -B inhibition potencies of the 8-[(phenylpropyl)sulfanyl]caffeine analogues (**4b–c**), that are substituted on the phenyl ring, may be compared to the  $IC_{50}$  value of the unsubstituted homologue, 8-[(phenylpropyl)sulfanyl]caffeine (**4a**).

**Table 4.3.** A comparison of the  $IC_{50}$  values for the inhibition of MAO-B by the unsubstituted homologue, 8-[(phenylpropyl)sulfanyl]caffeine (**4a**), with those homologues containing substituents on the phenyl ring.

| Compound  | -R   | MAO-B $IC_{50}$ ( $\mu\text{M}$ ) | Ratio H/R <sup>a</sup> |
|-----------|------|-----------------------------------|------------------------|
| <b>4a</b> | H    | 0.500                             | -                      |
| <b>4b</b> | 3-Cl | 0.062                             | 8.1                    |
| <b>4c</b> | 4-Cl | 0.061                             | 8.2                    |

<sup>a</sup>The ratio of  $IC_{50}(\mathbf{4a})/IC_{50}(\mathbf{4b-c})$

The results in table 4.3 show that substitution on the phenyl ring of **4a** leads to a considerable enhancement of its MAO-B inhibition potency, with both the substituted homologues exhibiting more potent MAO-B inhibition than **4a**. The  $IC_{50}$  values recorded for these homologues (**4b** and **4c**) are  $0.062 \mu\text{M}$  and  $0.061 \mu\text{M}$ , respectively. The substituted homologues are therefore at least eightfold more potent MAO-B inhibitors than the unsubstituted compound, **4a**. It may therefore be concluded that substitution on C-3 and C-4 with chlorine is a general strategy to enhance the MAO-B inhibition potency of 8-[(phenylpropyl)sulfanyl]caffeine (**4a**). It is interesting to note that substitution on the phenyl ring of **4a** ( $IC_{50} = 6.48 \mu\text{M}$ ) with chlorine also leads to an enhancement in MAO-A inhibition potency. The C-3 and C-4 substituted homologues exhibited  $IC_{50}$  values for the inhibition of MAO-A of  $3.53 \mu\text{M}$  and  $0.708 \mu\text{M}$ , respectively. In fact, **4c** was the most potent MAO-A inhibitor among the compounds examined, with an  $IC_{50}$  value of  $0.708 \mu\text{M}$ .

d) The MAO-A and -B inhibition potencies of the 8-(benzylsulfanyl)caffeine analogues (**5a–b**) examined in the present study were compared to the  $IC_{50}$  values of the 8-(benzylsulfanyl)caffeines previously studied (**2b–e**) (Booyesen *et al.*, 2011) (Table 4.4).

**Table 4.4.** Comparison of the IC<sub>50</sub> values for the inhibition of MAO-B by the 8-(benzylsulfanyl)caffeine (**5a–b**) analogues examined in the present study, to the IC<sub>50</sub> values of the 8-(benzylsulfanyl)caffeinines previously studied (**2b–e**) (Booyesen *et al.*, 2011).

| Compound  | -R   | MAO-B IC <sub>50</sub> (μM) |
|-----------|------|-----------------------------|
| <b>5a</b> | 3-Cl | 0.227                       |
| <b>5b</b> | 3-Br | 0.199                       |
| <b>2b</b> | 4-Cl | 0.192                       |
| <b>2c</b> | 4-Br | 0.167                       |
| <b>2d</b> | H    | 1.86                        |
| <b>2e</b> | 4-F  | 0.348                       |

The results show that the IC<sub>50</sub> values of the 8-(benzylsulfanyl)caffeine (**5a–b**) analogues examined in the present study are similar to the IC<sub>50</sub> values of the 8-(benzylsulfanyl)caffeinines previously studied (**2b–e**). For example, the chlorine substituted homologues **5a** and **2b** exhibit IC<sub>50</sub> values of 0.227 μM and 0.192 μM, respectively. Also, the bromine substituted homologues **5b** and **2c** exhibit IC<sub>50</sub> values of 0.199 μM and 0.167 μM, respectively. It is noteworthy that both C-3 and C-4 substitution on the phenyl ring of 8-(benzylsulfanyl)caffeine enhances the MAO-B inhibition potency of 8-(benzylsulfanyl)caffeine (**2d**). It may therefore be concluded that substitution on C-3 and C-4 are general strategies to enhance the MAO-B inhibition potency of 8-(benzylsulfanyl)caffeine (**2d**). It is interesting to note that, substitution on C-3 of the phenyl ring of **2d** (IC<sub>50</sub> = 8.22 μM) with chlorine and bromine, yields compounds which are weaker MAO-A inhibitors compared to substitution on C-4. The homologues substituted with chlorine (**5a**) and bromine (**5b**) on C-3 of the phenyl ring exhibited IC<sub>50</sub> values for the inhibition of MAO-A of 6.43 μM and 37.9 μM, respectively, while the homologues substituted with chlorine (**2b**) and bromine (**2c**) on C-4 of the phenyl ring exhibited IC<sub>50</sub> values of 2.76 μM and 2.61 μM, respectively.

e) A comparison of the MAO-A and –B inhibition potencies of the the 8-sulfanylcaffeine analogues, compounds **3a–e**, **4a–c** and **5a–b** was made with those of the 8-sulfinylcaffeine analogues, **6a–b**, and the 8-sulfonylcaffeine, **7**.

The results in table 4.1 show that the 8-[(phenylethyl)sulfanyl]caffeinines (**3a–e**), the 8-[(phenylpropyl)sulfanyl]caffeinines (**4a–c**), and the 8-(benzylsulfanyl)caffeinines (**5a–b**) exhibit IC<sub>50</sub> values in the submicromolar range for the inhibition of MAO-B (0.017–0.5 μM). In contrast, the

8-sulfinylcaffeines (**6a–b**) and 8-sulfonylcaffeine (**7**) are weak MAO-B inhibitors with  $IC_{50}$  values ranging from 5.11–131  $\mu$ M. It may therefore be concluded that the 8-sulfanylcaffeines are more potent MAO-B inhibitors than the 8-sulfinylcaffeines and 8-sulfonylcaffeines. The 8-[(phenylethyl)sulfanyl]caffeines (**3a–e**), the 8-[(phenylpropyl)sulfanyl]caffeines (**4a–c**), and the 8-(benzylsulfanyl)caffeines (**5a–b**) also are more potent MAO-A inhibitors than the 8-sulfinylcaffeines (**6a–b**) and 8-sulfonylcaffeine (**7**). The 8-sulfanylcaffeines **3–5** exhibited  $IC_{50}$  values for the inhibition of MAO-A of 0.708–141  $\mu$ M while the 8-sulfinylcaffeines (**6a–b**) and 8-sulfonylcaffeine (**7**) exhibited  $IC_{50}$  values of 166–250  $\mu$ M. The 8-sulfanylcaffeines are therefore also more potent MAO-A inhibitors than the 8-sulfinylcaffeines and 8-sulfonylcaffeines.

f) A comparison of the MAO-B inhibition potencies was made between the the 8-sulfanylcaffeine analogues, compounds **3a–e**, **4a–c** and **5a–b**.

The present study shows that C-3 substitution with chlorine (**5a**;  $IC_{50}$  = 0.227  $\mu$ M) and bromine (**5b**;  $IC_{50}$  = 0.199  $\mu$ M) act as potent MAO-B inhibitors. The 8-[(phenylethyl)sulfanyl]caffeine analogues are, however, significantly more potent MAO-B inhibitors than the corresponding 8-(benzylsulfanyl)caffeines. For example, the 8-[(phenylethyl)sulfanyl]caffeine analogues substituted with chlorine (**3a**;  $IC_{50}$  = 0.043  $\mu$ M) on the C-3 position of the phenyl ring are fivefold more potent than the corresponding C-3 (**5a**;  $IC_{50}$  = 0.227  $\mu$ M) chlorine substituted 8-(benzylsulfanyl)caffeine. Similarly, the 8-[(phenylethyl)sulfanyl]caffeine analogue substituted with bromine (**3b**;  $IC_{50}$  = 0.040  $\mu$ M) on the C-3 position of the phenyl ring is fivefold more potent than the corresponding C-3 (**5b**;  $IC_{50}$  = 0.199  $\mu$ M) bromine substituted 8-(benzylsulfanyl)caffeine. It may therefore be concluded that, the 8-[(phenylethyl)sulfanyl]caffeines are more active inhibitors than the 8-(benzylsulfanyl)caffeines.

This study also examined the MAO-B inhibitory properties of a limited series of 8-[(phenylpropyl)sulfanyl]caffeine analogues (**4a–c**). The results show that these compounds are potent inhibitors of MAO-B with  $IC_{50}$  values of 0.061–0.500  $\mu$ M. Those homologues substituted with chlorine on the C-3 and C-4 positions of the phenyl ring, compounds **4b–c**, were found to be exceptionally potent inhibitors with  $IC_{50}$  values of 0.061  $\mu$ M and 0.062  $\mu$ M, respectively. These compounds are slightly less active than the corresponding 8-[(phenylethyl)sulfanyl]caffeine homologues, compounds **3a** ( $IC_{50}$  = 0.043  $\mu$ M) and **3b** ( $IC_{50}$  = 0.040  $\mu$ M). It may therefore be concluded that, the 8-[(phenylethyl)sulfanyl]caffeines are more active inhibitors than the 8-[(phenylpropyl)sulfanyl]caffeines.

## **4.4 Hansch-type structure activity relationship studies**

### **4.4.1 Introduction**

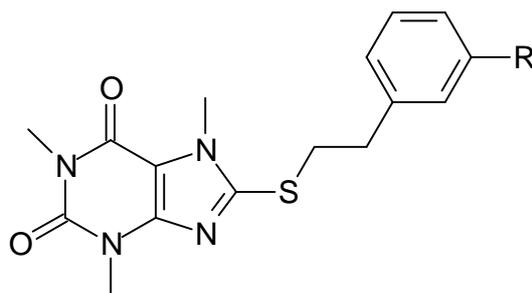
The results of the MAO-B inhibition studies have shown that substitution on C-3 of the phenyl ring enhances the MAO-B inhibition potency of 8-[(phenylethyl)sulfanyl]caffeine (**2a**). For example, the IC<sub>50</sub> values recorded for the C-3 substituted homologues, **3a–e**, ranged from 0.017–0.125 μM, making them twofold to 13-fold more potent MAO-B inhibitors than the lead compound, **2a** (IC<sub>50</sub> = 0.223 μM). To investigate the relationship between the MAO-B inhibition potencies and the properties of the substituents on C-3 of the phenyl ring, a Hansch-type QSAR study was carried out. Five parameters were used to describe the physicochemical properties of the C-3 substituents. The bulkiness of the substituents is described by the Van der Waals volume ( $V_w$ ) and the Taft steric parameter ( $E_s$ ) whereas the lipophilicities are described by the Hansch constant ( $\pi$ ). The electronic properties of the substituents are described by the Hammett constant ( $\sigma_m$ ) and the Swain-Lupton constant ( $F$ ) (Hansch & Leo, 1995). With a Hansch-type QSAR study the logarithm of the measured activities ( $\log IC_{50}$ ) of the inhibitors are correlated with the substituent descriptor values using a linear ( $y = ax + c$ ) or multivariate linear ( $y = ax_1 + bx_2 + c$ ) equation. In these equations,  $y$  equals the  $\log IC_{50}$  while  $x_1$ ,  $x_2$  equals the  $V_w$ ,  $E_s$ ,  $\pi$ ,  $\sigma_m$  and  $F$  values. To determine linearity, the correlation coefficient ( $R^2$ ) was determined. When  $R^2$  is close to 1, it can be concluded that there is a good correlation between the inhibitor's potency and a particular physicochemical property of the substituent. The validity of the correlation may also be judged by the statistical  $F$  value. The statistical  $F$  value must be higher than the calculated  $F_{max}$  value for the correlation to be significant (Livingstone & Salt, 2005). As a general rule of thumb, 5 compounds are needed for each term in the linear equation.

### **4.4.2 Results**

These studies were carried out with the inhibition data obtained with recombinant human MAO-B as enzyme source and the analysis was restricted to the six 8-[(phenylethyl)sulfanyl]caffeine analogues for which inhibition data are available. These include the 8-[(phenylethyl)sulfanyl]caffeine (**2a**), examined in a previous study (Booyesen *et al.*, 2011) and the 8-[(phenylethyl)sulfanyl]caffeine analogues (**3a–e**) examined in this study. Linear regression analyses were carried out with the Statistica 10 software package (StatSoft).

It should be noted that no correlation between the physicochemical parameters of the substituents and the MAO-A inhibition potencies were observed. Thus only the results obtained with the MAO-B inhibition potencies will be discussed.

**Table 4.5.** The values of the physicochemical parameters used for the Hansch-type QSAR study.



| Compd. | Substituents     | IC <sub>50</sub><br>( $\mu$ M) | Log<br>IC <sub>50</sub> | $\sigma_m$ | F    | $\pi$ | V <sub>w</sub> | E <sub>s</sub> |
|--------|------------------|--------------------------------|-------------------------|------------|------|-------|----------------|----------------|
| 2a     | H                | 0.223                          | -0.652                  | 0          | 0    | 0     | 0.08           | 0              |
| 3a     | Cl               | 0.043                          | -1.367                  | 0.37       | 0.42 | 0.71  | 1.07           | -0.97          |
| 3b     | Br               | 0.040                          | -1.398                  | 0.39       | 0.45 | 0.86  | 1.32           | -1.16          |
| 3c     | CF <sub>3</sub>  | 0.017                          | -1.770                  | 0.43       | 0.38 | 0.88  | 1.11           | -2.4           |
| 3d     | CH <sub>3</sub>  | 0.051                          | -1.292                  | -0.07      | 0.01 | 0.56  | 1.01           | -1.24          |
| 3e     | OCH <sub>3</sub> | 0.125                          | -0.903                  | 0.12       | 0.29 | -0.02 | 1.49           | -0.55          |

**Table 4.6.** Correlations of the recombinant human MAO-B inhibition potencies (logIC<sub>50</sub>) of 8-[(phenylethyl)sulfanyl]caffeine (**2a**) and the 8-[(phenylethyl)sulfanyl]caffeine analogues (**3a–e**) with steric, electronic and hydrophobic descriptors of the substituents at C-3 of the phenyl ring<sup>a</sup>.

| Parameter      | Slope              | y-intercept        | R <sup>2</sup> | F      | Significance <sup>b</sup> |
|----------------|--------------------|--------------------|----------------|--------|---------------------------|
| $\sigma_m$     | -1.284 $\pm$ 0.643 | -0.965 $\pm$ 0.185 | 0.499          | 3.97   | 0.116                     |
| F              | -1.176 $\pm$ 0.775 | -0.927 $\pm$ 0.246 | 0.365          | 2.303  | 0.204                     |
| $\pi$          | -0.899 $\pm$ 0.174 | -0.782 $\pm$ 0.109 | 0.869          | 26.546 | 0.0067                    |
| V <sub>w</sub> | -0.411 $\pm$ 0.347 | -0.814 $\pm$ 0.384 | 0.260          | 1.404  | 0.302                     |
| E <sub>s</sub> | 0.470 $\pm$ 0.073  | -0.735 $\pm$ 0.094 | 0.912          | 41.270 | 0.003                     |

|                  |                                  |                |       |        |                |
|------------------|----------------------------------|----------------|-------|--------|----------------|
| $\sigma_m + \pi$ | -0.163 ± 0.531<br>-0.838 ± 0.282 | -0.779 ± 0.124 | 0.873 | 10.316 | 0.779<br>0.059 |
| $\sigma_m + E_s$ | -0.398 ± 0.311<br>0.407 ± 0.084  | -0.719 ± 0.088 | 0.943 | 24.725 | 0.291<br>0.017 |
| $F + \pi$        | -0.159 ± 0.495<br>-0.852 ± 0.246 | -0.764 ± 0.135 | 0.873 | 10.348 | 0.769<br>0.040 |
| $F + E_s$        | -0.397 ± 0.299<br>0.423 ± 0.076  | -0.682 ± 0.095 | 0.944 | 25.462 | 0.276<br>0.011 |
| $\pi + V_w$      | -0.832 ± 0.191<br>-0.150 ± 0.160 | -0.664 ± 0.168 | 0.899 | 13.338 | 0.022<br>0.410 |
| $V_w + E_s$      | -0.075 ± 0.149<br>0.449 ± 0.091  | -0.681 ± 0.150 | 0.919 | 16.906 | 0.650<br>0.016 |

<sup>a</sup>The logarithm of the IC<sub>50</sub> values (expressed in μM) was used in the linear regression analysis.

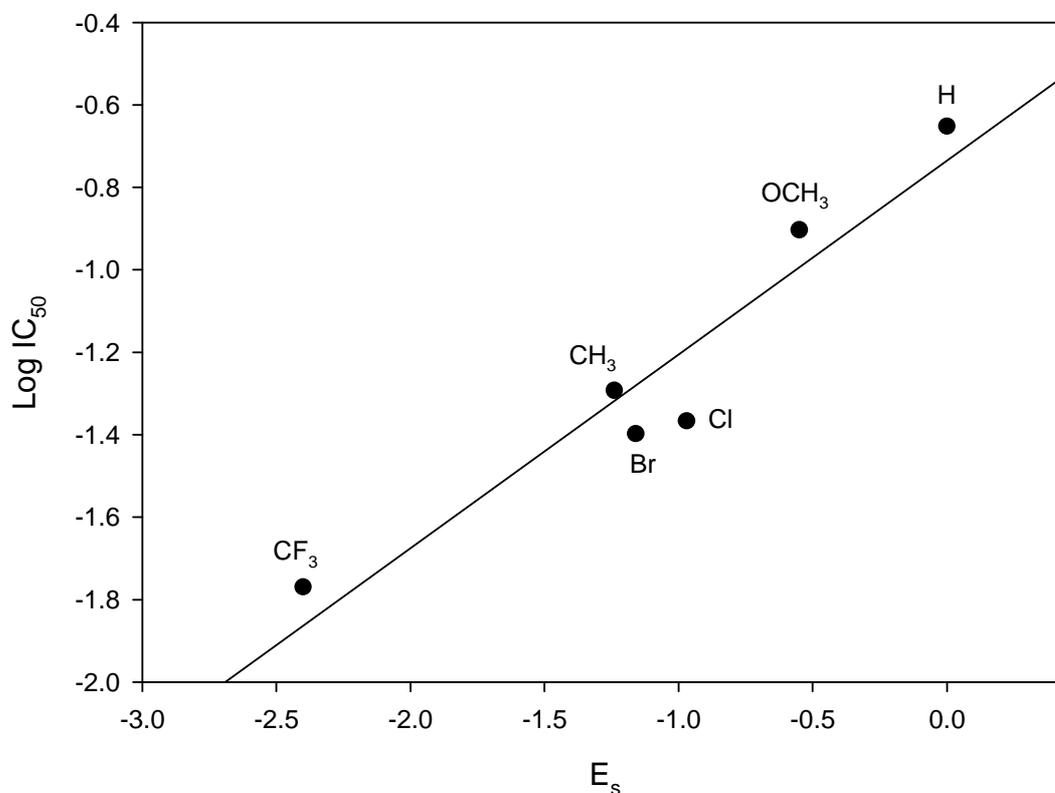
<sup>b</sup>The significance is the fractional probability that the coefficient of the added variable is zero.

The results indicate that the E<sub>s</sub> parameter has the best correlation with the logIC<sub>50</sub> values with an R<sup>2</sup> value of 0.912 and an F value of 41.27 (F<sub>max</sub> = 35.15). Note the R<sup>2</sup> value is relatively close to 1, which means that there is a very good correlation between the inhibition potencies of the 8-[(phenylethyl)sulfanyl]caffeine analogues and bulkiness of the C-3 substituent on the phenyl ring. Since the calculated F value is higher than the F<sub>max</sub> value, the correlation may be judged as statistically significant. The best mathematical description for the binding affinity of the 8-[(phenylethyl)sulfanyl]caffeine analogues to recombinant human MAO-B is therefore:

$$\text{Log IC}_{50} = 0.470(\pm 0.073)E_s - 0.735(\pm 0.094)$$

$$R^2 = 0.912 \text{ and } F = 41.27$$

The equation is graphically represented in figure 4.4:



**Figure 4.4.** Correlation of the  $\text{logIC}_{50}$  values for the inhibition of recombinant human MAO-B by the 8-[(phenylethyl)sulfanyl]caffeine analogues with the Taft steric parameter ( $E_s$ ).

As illustrated in the graph, potent MAO-B inhibition may be obtained with sterically large C-3 substituents on the phenyl ring of 8-[(phenylethyl)sulfanyl]caffeine. The trifluoromethyl group is an example of such a substituent. Small substituents, such as the hydrogen atom, which has only a small steric bulk, in contrast, yields the weakest MAO-B inhibition. Therefore a potent MAO-B inhibition correlates with a large steric substituent on C-3 of 8-[(phenylethyl)sulfanyl]caffeine.

## **4.5 Reversibility studies**

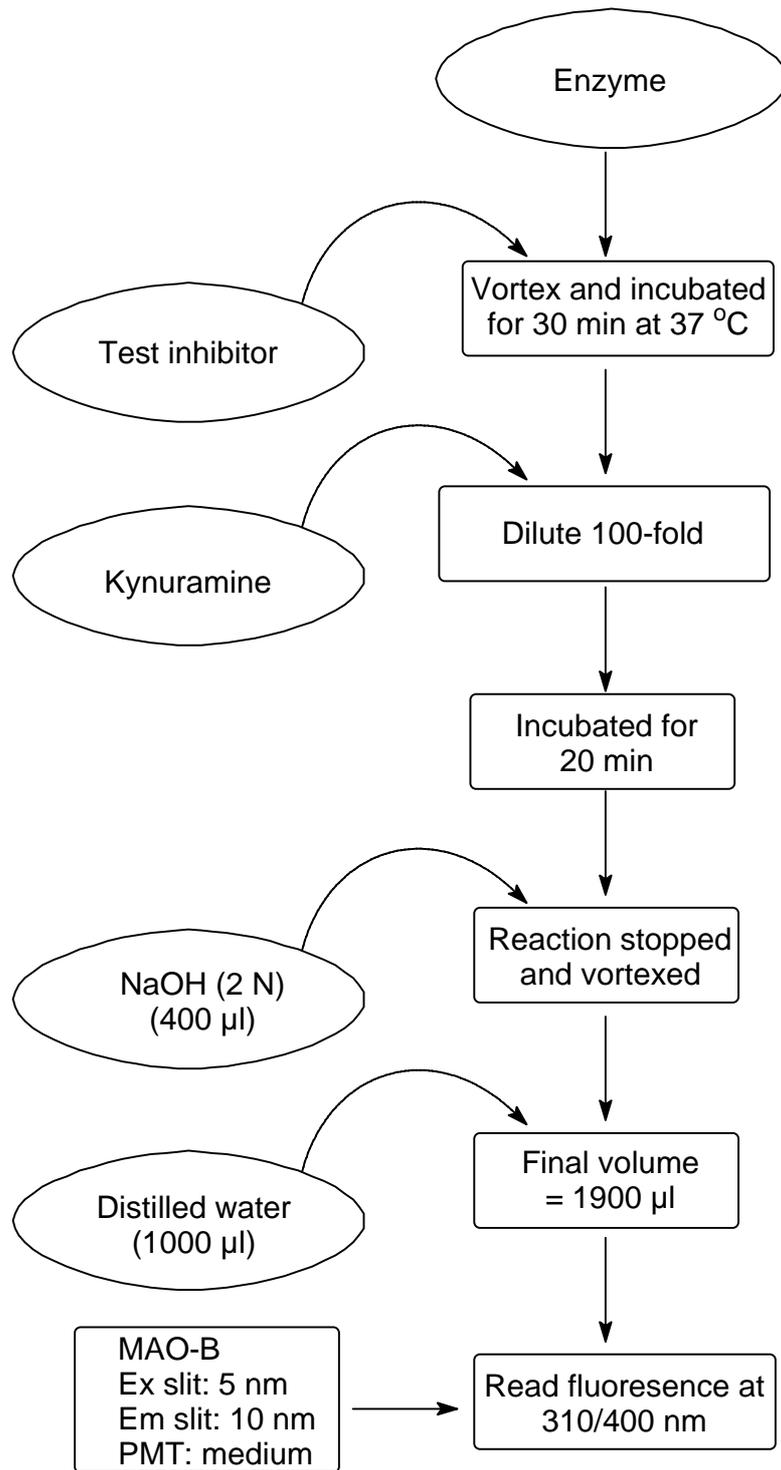
### **4.5.1 Introduction**

Reversibility studies determine whether an inhibitor binds covalently (irreversibly) or non-covalently (reversibly) to an enzyme. As mentioned earlier, it is preferable for an inhibitor to bind reversibly to MAO-B since reversible inhibitors are considered to be safer than irreversible inhibitors. The principal reason for this is that, with reversible inhibitors, enzyme activity is regained as soon as the drug is withdrawn and cleared from the tissues. After termination of treatment with irreversible inhibitors, the rate of enzyme activity is slow and variable. Compound **3c**, the most potent inhibitor towards MAO-B, was selected as representative inhibitor to determine the reversibility of inhibition in this study. The reversibility of inhibition was examined using the dilution method. For this purpose kynuramine served as substrate and the formation of its MAO-catalyzed oxidation product, 4-HQ, was measured by spectrofluorometry.

### **4.5.2 Method**

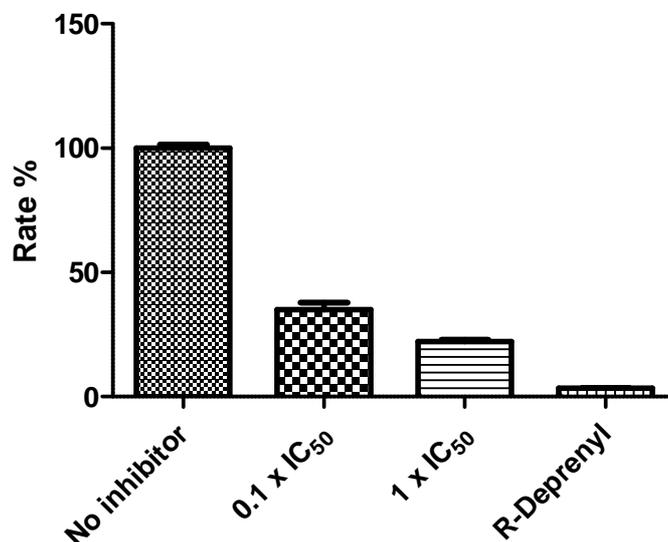
1. Stock solutions of inhibitor **3c** ( $IC_{50}$  for MAO-B = 0.017  $\mu$ M) were prepared in DMSO.
2. Compound **3c** was preincubated with a concentration of 0.75 mg/ml recombinant human MAO-B for 30 min at 37 °C. The concentrations of **3c** employed were equal to  $10 \times IC_{50}$  and  $100 \times IC_{50}$ , respectively, and each reaction (50  $\mu$ l) contained 4% DMSO. The reaction solvent was potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl).
3. A control preincubation with MAO-B was also carried out as above in the absence of inhibitor.
4. The reactions were subsequently diluted 100-fold with the addition of kynuramine to yield final concentrations of kynuramine equal to 30  $\mu$ M, and of MAO-B equal to 0.0075 mg/ml. The final reaction volume was 500  $\mu$ l and the final concentrations of **3c** were  $0.1 \times IC_{50}$  and  $1 \times IC_{50}$ .
5. The reactions were incubated for a further 20 min at 37 °C and terminated with the addition of 400  $\mu$ l of NaOH (2N) followed by 1000  $\mu$ l of distilled water.
6. The reactions were then centrifuged at 16 000 g for 10 min.
7. The amount of fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 310 nm and an emission wavelength of 400 nm:

- The PMT voltage was set to medium with an excitation slit width of 5 nm and an emission slit width of 10 nm.
8. A calibration curve was prepared by measuring the fluorescence of increasing concentrations of 4-HQ (0.0469, 0.09375, 0.1875, 0.375, 0.75, 1.5  $\mu\text{M}$ ). Each of these calibration standards contained 4% (v/v) DMSO and potassium phosphate buffer to a final volume of 500  $\mu\text{l}$ . To each calibration standard were added 400  $\mu\text{l}$  of NaOH and 1000  $\mu\text{l}$  of distilled water.
  9. For comparison, (R)-deprenyl ( $\text{IC}_{50} = 0.079 \mu\text{M}$ ) was similarly preincubated with MAO-B at  $10 \times \text{IC}_{50}$  and diluted 100-fold with the addition of kynuramine to yield a final concentration of (R)-deprenyl equal to  $0.1 \times \text{IC}_{50}$  (Petzer *et al.*, 2012).
  10. The data was subsequently plotted (histogram) and interpreted.



**Figure 4.5.** Summary of the method used to examine the reversibility of inhibition.

### 4.5.3 Results



**Figure 4.6.** Reversibility of inhibition of MAO-B by **3c**. The enzyme was preincubated with **3c** at  $10 \times IC_{50}$  and  $100 \times IC_{50}$  for 30 min and then diluted to  $0.1 \times IC_{50}$  and  $1 \times IC_{50}$ , respectively. As control, MAO-B was also preincubated with (R)-deprenyl at  $10 \times IC_{50}$  and subsequently diluted to  $0.1 \times IC_{50}$ . The residual enzyme activities were subsequently measured.

The reversibility of MAO-B inhibition by the most potent inhibitor, compound **3c**, was investigated by measuring the degree of enzyme recovery after dilution of the enzyme-inhibitor complex. After the MAO-B was pre-incubated with the inhibitor at  $10 \times IC_{50}$  and  $100 \times IC_{50}$ , the reaction was diluted to  $0.1 \times IC_{50}$  and  $1 \times IC_{50}$ , respectively. The results indicate that the MAO-B catalytic activities are recovered to levels of approximately 35% and 22%, respectively. For reversible enzyme inhibition, the enzyme activities are expected to recover to levels of approximately 90% and 50%, respectively, after 100-fold dilution of the preincubations containing inhibitor concentrations of  $10 \times IC_{50}$  and  $100 \times IC_{50}$ . After preincubation of MAO-B with the irreversible inhibitor (R)-deprenyl (at  $10 \times IC_{50}$ ), and dilution of the resulting complex to  $0.1 \times IC_{50}$ , MAO-B activity is not recovered (3.0% of control). These data indicate that **3c** interacts reversibly with MAO-B. Interestingly, after dilution, the enzyme activities are not recovered to 90% and 50%, respectively, as expected. This result suggests that the binding of **3c** may possess a quasi-reversible or tight-binding component. This behaviour was also observed with other 8-[(phenylethyl)sulfanyl]caffeines.

## **4.6 Lipophilicity**

Lipophilicity can be defined as the tendency of a compound to partition into a nonpolar lipid matrix versus an aqueous matrix and has been correlated to many other properties such as solubility and permeability. The lipophilicity estimates may thus be used to predict if a compound will penetrate the BBB (Kerns & Di, 2008). The octanol/water partition coefficients (P) of selected compounds were estimated using the “shake flask method”:

In a 5 ml glass vessel, 2 ml of each of the n-octanol and water phases (both phases were mutually saturated) were placed followed by the analyte to yield a final analyte concentration of 1 mM. The vessels were shaken by hand for 5 min and centrifuged at 4,000 g for 10 min. The n-octanol phase was diluted 20-fold into neat n-octanol and the absorbance of the resulting solution was recorded at an absorbance maximum of 292 nm. The concentrations of the analytes in the n-octanol phase were determined by employing the molar extinction coefficients recorded for each analyte in n-octanol: **3c**, 17669 M<sup>-1</sup>; **4a**, 19170 M<sup>-1</sup>; **4b**, 18380 M<sup>-1</sup>; **4c**, 19695 M<sup>-1</sup>. Without further dilution, the concentration of the analyte in the water phase was determined by HPLC analysis. For this purpose, a Venusil XBP C18 column (4.60 x 150 mm, 5 μm) was used and the mobile phase consisted of 75% acetonitrile and 25% Milli-Q water at a flow rate of 1 mL/min. A volume of 20 μL of the water phases were injected into the HPLC system and the eluent was monitored at a wavelength of 292 nm. To quantify the analytes, linear calibration curves were constructed by similarly analyzing known amounts of the analytes (0.05–10 μM) in 50% acetonitrile. These studies were carried out in triplicate for each analyte and the LogP values are expressed as mean ± SD.

**Table 4.7.** LogP values.

| <b>Compound</b> | <b>LogP values</b> |
|-----------------|--------------------|
| <b>3c</b>       | 3.14 ± 0.019       |
| <b>4a</b>       | 3.30 ± 0.030       |
| <b>4b</b>       | 3.76 ± 0.051       |
| <b>4c</b>       | 3.59 ± 0.059       |

The results of the LogP determinations are shown in table 4.7. As shown, all of the evaluated compounds may be viewed as lipophilic since they display LogP values in excess of 3.14. As a general guide, LogP values ranging from 0–3 are optimal for passive diffusion permeability and

such compounds are expected to display good oral bioavailability and BBB permeation, but according to Lipinski's rules, LogP values that are less than 5 indicate that compounds will not exhibit good oral bioavailability and BBB permeation. Therefore, although the LogP values for the compounds tested here are not in the optimal range, the LogP values of the selected 8-sulfanylcaffeine analogues may still be viewed as appropriate for good oral bioavailability and BBB permeation.

#### **4.7 Conclusion**

This chapter describes the MAO inhibitory properties of a series of synthetic 8-sulfanylcaffeine analogues. These analogues are the:

- 8-[(phenylethyl)sulfanyl]caffeines (**3a–e**),
- 8-[(phenylpropyl)sulfanyl]caffeines (**4a–c**),
- 8-(benzylsulfanyl)caffeines (**5a–b**),
- 8-sulfinylcaffeines (**6a–b**)
- and 8-sulfonylcaffeine (**7**)

The results showed that the 8-[(phenylethyl)sulfanyl]caffeines (**3a–e**) are highly potent MAO-B inhibitors, and that substitution on the phenyl ring of **2a**, the lead compound, leads to an enhancement of MAO-B inhibition potency. The most potent inhibitor of this study, the 3-CF<sub>3</sub> substituted homologue (**3c**), is approximately 13-fold more potent as a MAO-B inhibitor than **2a**, and exhibits a higher degree of selectivity for MAO-B. The results of a Hansch-type structure-activity relationship study showed that the MAO-B inhibition potencies of the 8-[(phenylethyl)sulfanyl]caffeine analogues correlated best with the Taft steric parameter ( $E_s$ ). This correlation suggests that MAO-B inhibition potency may be enhanced with placement of sterically bulky C3 substituents on the phenyl ring of 8-[(phenylethyl)sulfanyl]caffeine.

This study also shows that 8-[(phenylethyl)sulfanyl]caffeine analogues are significantly more potent as MAO-B inhibitors than the corresponding 8-(benzylsulfanyl)caffeines (**5a–b**). They, however, were found to be comparatively weak MAO-A inhibitors.

Interestingly 8-[(phenylpropyl)sulfanyl]caffeine analogues (**4a–c**) are also highly potent MAO-B inhibitors with comparable potencies to those of the 8-[(phenylethyl)sulfanyl]caffeines. Compounds **4a–c** are, however, less selective for MAO-B. In fact, **4c** is the most potent MAO-A inhibitor among the investigated compounds, and the only inhibitor with an IC<sub>50</sub> value in the

submicromolar range. Although DA is metabolized by both MAO-A and -B in the human brain, the inhibition of MAO-A is associated with potentially dangerous side effects, and highly selective MAO-B inhibitors may therefore be more desirable for the treatment of Parkinson's disease. Based on this analysis, 8-[(phenylethyl)sulfanyl]caffeines may be better suited as antiparkinsonian drugs than 8-[(phenylpropyl)sulfanyl]caffeines, since several 8-[(phenylethyl)sulfanyl]caffeines are highly potent ( $IC_{50} < 0.05 \mu\text{M}$ ) MAO-B inhibitors with SI values in excess of 1000.

The 8-sulfinylcaffeines (**6a-b**) and 8-sulfonylcaffeine (**7**), on the other hand, exhibited comparatively weak MAO-B inhibition and are thus not suited for the design of MAO inhibitors.