Synthesis and evaluation of chromone derivatives as inhibitors of monoamine oxidase

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Abstract

Based on reports that chromone compounds are good potency inhibitors of monoamine oxidase (MAO), the present study evaluates the effect of substitution with flexible side chains on the 3 position on MAO inhibition potency. Fifteen chromone derivatives were synthesised by reacting aromatic and aliphatic amines and alcohols with chromone 3-carboxylic acid in the presence of carbonyldiimidazole (CDI). This yielded chromane-2,4-dione and ester chromone derivatives. Generally, the esters exhibited weak MAO inhibition, while the chromane-2,4-dione derivatives showed promise as selective MAO-B inhibitors with IC_{50} values in the micromolar range. Compound **14b**, 3-[(benzylamino)methylidene]-3,4-dihydro-2H-1-benzopyran-2,4-dione, was the most potent MAO-B inhibitor with an IC_{50} value of 638 μ M. This compound was shown to be a reversible and competitive MAO-B inhibitor with a K_i of 94 μ M. In conclusion, the effect of chain elongation and introduction of flexible substituents on position 3 of chromone were explored and the results showed that aminomethylidene substitution is preferable over ester substitution. Good potency MAO-B inhibitors may act as leads for the design and development of therapy for Parkinson's disease where these agents reduce the central metabolism of dopamine.

Graphical abstract

Chromane-2,4-dione derivatives show promise as selective MAO-B inhibitors.

Keywords Chromone · Chromandione · Monoamine oxidase · MAO · Inhibitor · Competitive · Parkinson's disease

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Introduction

Parkinson's disease is the second most common neurodegenerative disorder affecting the brain and is the most common progressive neurodegenerative movement disorder [1–3]. Pathologically, Parkinson's disease is characterised by the death of the neurons in the substantia nigra and the presence

of proteinaceous deposits known as Lewy bodies, resulting in the characteristic motor symptoms [4]. Due to its distinctive pathology, Parkinson's disease is primarily characterised by motor symptoms, although non-motor symptoms frequently appear especially in later stages of the disease [5]. Current treatment strategies are mainly aimed at restoring striatal dopamine activity with levodopa as the gold standard of therapy. Levodopa is administered in combination with carbidopa, an aromatic L-amino acid decarboxylase (AADC) inhibitor which prevents peripheral levodopa metabolism [1, 6]. Other dopaminergic therapies include dopamine agonists, which stimulate dopamine receptors, monoamine oxidase B (MAO-B) inhibitors and catechol-O-methyltransferase (COMT) inhibitors which reduce dopamine degradation [6]. Several potential neuroprotective or disease-modifying agents have been evaluated in clinical trials, but none have been conclusively proven to be neuroprotective in Parkinson's disease [7, 8]. As discussed below, however, a mechanistic rationale and preclinical evidence exist for a potential neuroprotective effect by MAO-B inhibitors.

MAO is an enzyme distributed extensively in higher eukaryotes and in mammals and is present in two isoforms, MAO-A and MAO-B [9]. The MAOs contain flavin adenine dinucleotide (FAD) as a cofactor and are bound to the outer mitochondrial membrane. The MAOs catalyse the oxidative deamination of various biogenic and dietary monoamines, and abnormal MAO levels may be associated with several disease states such as depression and Parkinson's disease [9]. Since MAO-A and MAO-B are products of distinctive genes, they differ with respect to their amino acid sequence, three-dimensional structure, tissue distribution as well as substrate and inhibitor specificity [10–12]. MAO-A preferentially deaminates serotonin, while MAO-B shows preference for benzylamine and phenethylamine as substrates. Both MAO isoforms deaminate dopamine equally well [9]. Since inhibition of the MAOs can potentially modulate neurotransmitter levels (for example dopamine) in the brain that are actively involved in the pathogenesis of some disease states [13, 14], MAO inhibitors have found application in the therapy of disorders such as Parkinson's disease [15, 16]. In this respect, MAO-B inhibitors are effective as symptomatic treatment by compensating for diminished striatal dopamine levels; however, arresting disease progression remains the ultimate goal [8, 14].

As mentioned above, a mechanistic rationale exists for a potential neuroprotective effect by MAO-B inhibitors. The MAO catalytic cycle produces hydrogen peroxide as a by-product which, via the Fenton reaction, is a precursor to harmful reactive oxygen species that may lead to oxidative damage and exacerbate neurodegeneration [14]. Thus, the inhibition of the MAO enzymes may reduce the formation of hydrogen peroxide and act as potential neuroprotectants [17]. Furthermore, MAO-B levels in several brain regions

increase with age in humans, thus providing an additional rationale for the use of MAO-B inhibitors in Parkinson's disease with the aim of reducing oxidative neuronal damage [18]. Three MAO-B inhibitors are currently approved for use in Parkinson's disease. These are the mechanism-based inhibitors, rasagiline and selegiline, and the reversible inhibitor safinamide [9, 19]. These inhibitors are specific for the MAO-B isoform since MAO-A inhibition may result a potentially fatal hypertensive crisis when taken with certain food [20, 21]. For the design and development of new MAO-B inhibitors, isoform specificity is an important consideration.

Several heterocyclic scaffolds have been considered for the design of MAO inhibitors. These include coumarins, chalcones as well as chromones [22, 23]. Chromone (4*H*-1-benzopyran-4-one, **1**) is an isomer of coumarin (**2**) and forms part of the flavone backbone (Fig. 1). Chromone derivatives occur in nature, and a large range of pharmacological activities such as immune stimulation [24, 25], antioxidant [26], anti-HIV [27], anticancer [28], biocidal [29], wound healing [30], antibacterial and antifungal activities [31] have been reported for these compounds. Due to their presence in plants, chromones are part of the human diet and generally exhibit low mammalian toxicity [28]. The chromone ring system is therefore considered to be a privileged scaffold due to its range of pharmacological and biological effects as well as the low risk of toxicity associated with chromone compounds [32].

The MAO inhibitory potential of chromone derivatives has been illustrated. For example, series of C6- and C7-substituted chromone derivatives were found to be potent, reversible MAO-B inhibitors. Compounds **3–6** are examples of high potency MAO-B inhibitors from these studies [22, 33, 34]. Although some of these chromones also exhibited IC₅₀ values in the nM range for the inhibition of MAO-A, these compounds are specific inhibitors of the MAO-B isoform.

Interestingly, C5 substitution of chromone yields poor MAO-B inhibition when compared to the C6- and C7-substituted derivatives (e.g. **7**) [35]. Also of interest is the finding that chromone 3-carboxylic acid (**8**) is a potent and specific MAO-B inhibitor (IC₅₀ = 0.048 μM), while the presence of the carboxylic acid group in position 2 of the 4-pyrone nucleus (**9**) results in a loss of activity [36–38]. Similarly, phenylcarboxamide substitution on position 3 of the 4-pyrone nucleus results in potent MAO-B inhibition, with compounds **10** and **11** exhibiting IC₅₀ values of 0.40 and 0.063 μM, respectively, while related 2-phenylcarboxamide substitution generally results in poor activity [37–39].

From the above discussion it is thus clear that chromones are suitable for the design of MAO-B-specific inhibitors. The MAO inhibitory activity of the chromones

Fig. 1 The structures of chromone (1), coumarin (2) and chromone derivatives 3–11 referred to in the text

has been validated, and this study will contribute by examining the MAO inhibition potential of C3-substituted chromones. Although the MAO inhibitory activity of rigid C3-substituted phenylcarboxamide derivatives has previously been investigated, the effect of chain elongation and the introduction of a more flexible substituent in this position have not been explored [37–39]. As discussed above, flexible substituents on positions 6 and 7, and rigid amide functions on position 3 have resulted in potent MAO inhibitors [33, 34, 37–39]. It is thus postulated that the introduction of a flexible ester- or amide-containing substituents on position 3 of chromone will result in potent, specific MAO inhibition. This study therefore attempted to synthesise chromone derivatives (12 and 13) where flexible substituents will be attached via ester and amide functions on position 3 of chromone (Fig. 2). The chromone derivatives were synthesised by reacting aromatic and aliphatic alcohols and amines with chromone 3-carboxylic acid (8). As will be shown, this yielded the anticipated ester chromone derivatives (12), while the amide derivatives 13 were not obtained. Instead, the reactions of amines with chromone 3-carboxylic acid yielded chromane-2,4-diones (14).

Fig. 2 The general structures of chromone derivatives 12–14 that were considered for this study, and the synthesis of ester chromone derivatives (12a–e) and 3-aminomethylidene-2,4-chromandiones (14a–j). Reagents and conditions **a** CDI, DMF, 60 °C, 2 h; **b** R–OH, DMF, rt, overnight; **c** R–NH₂, DMF, rt, overnight

Results

Chemistry

The aim of this study was to synthesise ester (**12**) and amide (**13**) derivatives of chromone. The synthesis of these compounds was attempted using standard literature procedures with the synthetic strategy employed for both series involving a coupling reaction of chromone 3-carboxylic acid (**8**) with an equimolar quantity of an alcohol or amide in the presence of carbonyldiimidazole (CDI) as coupling reagent (Fig. 2) [40]. In total, 15 compounds were synthesised in poor to moderate yields (13–70%). All synthesised compounds were characterised by NMR and infrared spectroscopy and mass spectrometry. For all compounds, the mass data correlated well with the calculated mass values. Purity of the compounds was determined by HPLC and was between 79 and 100%. Decomposition of the ester derivatives was observed over time. Melting points generally correlated with reported literature values; however, differences in the melting points of the chromane-2,4-diones (**14**) when compared to the literature values are most likely due to differences in the ratios of the *E/Z*-isomers [41].

NMR assignments were based on interpretation of both 1D NMR (^{13}C , ^1H , DEPT) and 2D NMR (COSY, HSQC, HMBC) spectra, while NMR data of known compounds were also considered. The results of the attempted synthesis of amide derivatives **13** were initially surprising, as analogous amide derivatives were obtained using related methodology [38, 42]. However, in a study done by Cagide and co-workers [39], similar results to this study were obtained using PyBOP as coupling reagent in the presence of *N,N*-diisopropylethylamine (DIPEA), while Gaspar and co-workers used PyBOP or BOP as coupling agent, also in the presence of DIPEA [38]. Under acidic conditions (POCl_3 in DMF), however, the target carboxamide derivatives could be obtained [39]. The formation of the chromane-2,4-diones versus the carboxamide derivatives is thus dependant on the

experimental conditions, and activation of the carboxylic acid probably precedes nucleophilic attack by the amine on C2. Careful consideration of the NMR data revealed that the desired amide derivatives were in fact not synthesised, but that the products that were obtained were the structurally related chromane-2,4-diones **14**. In the ^1H NMR spectra for example, the presence of a methine proton (H-9) that occurs as a doublet coupling to the adjacent imino proton provided evidence that the keto-enamines were obtained and not the desired amides (where both H-2 and the NH signals are expected to appear as singlets). A crystal structure was obtained for compound **14j** (Fig. 3) and provided further confirmation that this was indeed the case.

A reaction mechanism for the formation of the chromane-2,4-dione derivatives, based on literature reports for related systems, is illustrated in Fig. 4 [39, 43]. It is postulated that the carboxylic acid is activated by CDI as expected. Reaction of the amine however does not result in the displacement of imidazole, but leads to ring opening of the pyrone ring, followed by ring closure through lactonisation with loss of imidazole.

NMR data further revealed that the chromane-2,4-diones exist as mixtures of isomers. In the proton spectra there are two distinct NH signals as well as two signals for H-9 and generally also for H-5. Double signals of almost all carbons also occur, but are especially clear for C-4, C-2 and C-9. A literature survey revealed that these compounds exist as mixtures of *E*- and *Z*-isomers, with one isomer predominating (Fig. 5) [41, 44–47]. In the ^1H NMR spectrum of compound **14i** for example, the two NH signals are present at 13.57 and 11.91 ppm for the major and minor products, respectively. It is postulated that the more downfield signal corresponds to the *E*-isomer, where the NH forms a short, strong intramolecular hydrogen bond with the C(4)=O group. The NH signal for the *Z*-isomer (thus at 11.91 ppm) also forms a hydrogen bond with the ester carbonyl (C2), but it has been reported that this bond is significantly weaker [41, 44]. On the other hand, the H-9 proton of the minor

Fig. 3 ORTEP drawing representing the crystal structure of the *E*-isomer of compound **14j**

Fig. 4 Proposed mechanism for aminomethylidene-2,4-chromandione formation (adapted from the literature) [39, 43]

Fig. 5 The *E*- and *Z*-isomers of the chromane-2,4-diones obtained in this study

isomer (presumably the *Z*-isomer) is found more downfield (9.71 ppm) than that of the major *E*-isomer (9.61 ppm).

In the ^{13}C NMR spectrum of **14i**, the signal for the C-4 carbonyl at 182.4 ppm for the major (*E*-isomer) is shifted downfield compared to the signal of the minor *Z*-isomer at 178.8 ppm. The opposite is observed for the C-2 carbonyl, where the signal for the minor *Z*-isomer is more downfield (165.3 ppm) compared to the signal for the major *E*-isomer which is present at 163.3 ppm. This is due to the possibility that C-4 carbonyl oxygen is hydrogen bonded to the NH proton in the *E*-isomer, whereas C-2 carbonyl oxygen is hydrogen bonded to the NH for the minor *Z*-isomer. The carbonyl that undergoes hydrogen bonding therefore occurs further downfield than its non-hydrogen-bonded counterpart [41, 44]. Since similar observations were made for all the 3-chromane-2,4-diones synthesised in this study, it is proposed that the *E*-isomer is the major isomer. According to the literature further evidence for the *E*-isomer as major product is provided by molecular calculations where the energy of formation of the *E*-keto-enamine is lower than that of the *Z*-keto-enamine form [41].

NMR assignments were based on chemical shifts, integration, multiplicities, coupling constants, correlations in

HMBC, HSQC and COSY spectra as well as DEPT data. The assignments and observed HMBC correlations of the major isomer of compound **14i** are shown in Table S1 (supplementary) as an example. The most characteristic difference between the ^1H NMR spectra of these chromane-2,4-diones (**14**) and those of the ester derivatives (**12**) is the presence of H-9 which appears as a doublet coupling to the adjacent imino proton. The NH signals further also occur quite far downfield due to intramolecular hydrogen bonding with C(4)=O (major isomer) or C(2)=O (minor isomer). The shifts of the aromatic protons of ring A of these derivatives were similar to those of the ester derivatives and were assigned as follows for compound **14i**: δ_{H} : 8.05 (H-5), 7.63–7.56 (H-7), 7.32–7.23 (H-6 and H-8). The ^{13}C NMR spectrum for compound **14i** showed the presence of 30 signals, thus providing further evidence of the presence of two isomers. Analysis of the ^{13}C spectrum together with the DEPT 135 spectra indicated the presence of six quaternary carbons and nine CH carbons (for each isomer). Chemical shifts and observed 2D correlations were used to further assign carbon signals (Table S1, supplementary). Similar correlations were observed in all cases for the minor *Z*-isomer.

Ratios of the *E*:*Z* mixtures may be calculated using the integral intensities of the H-9 signals. Interestingly, Traven and co-workers noted that the rate of equilibration between these isomers strongly depends on solvent polarity, where for 3-(*p*-tolyliminomethyl)chromane-2,4-dione equilibrium is almost immediately established after dissolution in methanol, 4 h after dissolution in DMSO and 24 h after dissolution in chloroform [41]. Thus, if a different solvent was used to dissolve these compounds and if they were observed over time, the observed ratios of these isomers could change. In order to investigate the effect of temperature on the ratios

of isomers, variable temperature NMR experiments were performed. The results with compound **14b** are shown in Fig. S1 (supplementary). Based on these results it appears that changes in temperature from -10 to 45 °C do not alter the observed ratios of the *E*- and *Z*-isomers.

Monoamine oxidase inhibition: IC_{50} values

The compounds synthesised in this study were evaluated as inhibitors of MAO-A and MAO-B. Recombinant human MAO-A and MAO-B enzymes were used for this purpose. MAO activity was measured by making use of a fluorometric assay with kynuramine as the enzyme–substrate. Kynuramine is a non-selective substrate for both MAO-A and MAO-B and is oxidised to yield 4-hydroxyquinoline, a metabolite that fluoresces in alkaline media [48, 49]. By measuring MAO activity in the absence and presence of different concentrations of the test inhibitors, sigmoidal plots of enzyme activity versus the logarithm of inhibitor concentration were constructed from which the IC_{50} values were estimated. Figure 6 gives examples of representative sigmoidal plots.

The results of the MAO inhibition studies are given in Tables 1 and 2. Selectivity index (SI) values provide an indication of the selectivity for the inhibition of MAO-B over the MAO-A isoform. From Table 1 it is apparent that the ester derivatives are generally weak MAO-A and MAO-B inhibitors with IC_{50} values > 9 μ M. Comparing the MAO-A and MAO-B potencies of compound **12a** (phenyl substituted) and **12b** (benzyl substituted), it is evident that the benzyl derivative is more potent than the phenyl derivative. Thus, chain elongation from phenyl to benzyl improved MAO inhibition activity. A similar trend is observed when the activity of compound **12c** is compared to that of **12a**.

Fig. 6 Sigmoidal plots for the inhibition of MAO-B by **14a** (open circles), **14b** (filled circles) and **14c** (triangles). Each data point represents a mean $\pm$ SD of triplicate determinations

Table 1 IC_{50} values for the inhibition of MAO-A and MAO-B by ester chromone derivatives (**12**)

	R	IC_{50} (μ M) ^a		SI ^b
		MAO-A	MAO-B	
12a		66.7 ± 2.06	27.3 ± 0.946	2.4
12b		26.8 ± 0.560	14.7 ± 0.416	1.8
12c		21.6 ± 8.72	14.8 ± 1.09	1.5
12d		18.5 ± 8.85	12.0 ± 0.400	1.5
12e		18.6 ± 3.27	9.74 ± 0.123	1.9

^aAll values are expressed as the mean $\pm$ SD of triplicate determinations

^bSelectivity index (SI) = $IC_{50}(\text{MAO-A})/IC_{50}(\text{MAO-B})$

4-Chlorophenyl substitution (**12c**) has improved MAO inhibition activity compared to unsubstituted phenyl side chain (**12a**). This trend is also observed when comparing compound **12e**, a 4-chlorobenzyl-substituted derivative, to compound **12b**. Comparing the 4-chlorophenyl (**12c**) with 3-chlorophenyl substitution (**12d**), it is clear that the position of the chlorine does not significantly affect MAO inhibition activity. It may therefore be concluded that elongation of the side chain from phenyl to benzyl and substituting with a chlorine on the 3'- or 4'-position enhance MAO inhibition activity of the ester derivatives.

From Table 2 it may be concluded that the chromane-2,4-diones are selective inhibitors of MAO-B with IC_{50} values in the micromolar range. Generally, the chromane-2,4-diones are more potent MAO-B inhibitors than the ester derivatives. (They are however weaker inhibitors of MAO-A.) Compound **14b** is the most potent MAO-B inhibitor with an IC_{50} value of 0.638 μ M, while **14d** is the most selective MAO-B inhibitor with a SI value of 218. MAO-B inhibition increases with side chain elongation from compound **14a** to **14b** (phenyl to benzyl) and is similar for compounds **14a** and **14c**, but from compounds **14d** to **14e**, as further chain elongation occurs, MAO-B inhibitory activity decreases. Comparing the MAO-A and MAO-B inhibition potencies of compounds **14f**, **14g** and **14h**, it is evident that chain

Table 2 IC₅₀ values for the inhibition of MAO-A and MAO-B by 3-aminomethylidene-2,4-chromandiones (**14**)

	R	IC ₅₀ (μM) ^a		SI ^b
		MAO-A	MAO-B	
14a		79.6 ± 30.2	0.947 ± 0.125	84
14b		77.9 ± 25.5	0.638 ± 0.287	122
14c		101 ± 3.49	0.897 ± 0.166	113
14d		312 ± 89.1	1.43 ± 0.103	218
14e		155 ± 19.1	142 ± 47.2	1.1
14f		72.1 ± 6.53	3.08 ± 0.279	23
14g		288 ± 61.8	NI ^c	–
14h		NI ^c	NI ^c	–
14i		73.1 ± 20.4	38.7 ± 1.73	1.9
14j		41.6 ± 2.29	16.6 ± 1.04	2.5

^aAll values are expressed as the mean ± SD of triplicate determinations

^bSelectivity index (SI) = IC₅₀(MAO-A)/IC₅₀(MAO-B)

^cNo inhibition at a maximum tested concentration of 100 μM

elongation results in loss of activity. Interestingly, compounds **14g** and **14h** do not inhibit MAO-B. This may be due to lengthening of the side chain in combination with the addition of a sterically large chlorine. Comparing the MAO inhibition activities of compounds **14a–c** with those of compounds **14f–h**, it may be concluded that chlorine substitution reduces MAO-B inhibition potency. The compounds with pyridine-containing side chains (compounds **14i** and **14j**) both show poor MAO inhibition; however, with chain elongation from pyridyl (**14i**) to ethylpyridyl (**14j**) a twofold increase in MAO inhibition is observed. The results further show that the chromane-2,4-diones **14** are more potent

MAO-B inhibitors than the ester chromone derivatives **12**. For example, the phenyl-substituted compound, **14a**, is approximately 28-fold more potent than **12a**. Similarly, the chromane-2,4-diones **14b** and **14f** are also significantly more potent MAO-B inhibitors than their corresponding ester derivatives **12b** and **12c**. It should be noted that the 3-aminomethylidene-2,4-chromandiones are represented by inseparable mixtures of *E*- and *Z*-isomers, and the MAO inhibition potencies recorded are that of the mixtures. The observation that chromane-2,4-diones are weak inhibitors of MAO-A is in agreement with a recent report of the MAO inhibition properties of four 3-[(phenylamino)methylidene]chromane-2,4-dione derivatives [39]. These compounds did not inhibit human MAO-A at a maximal tested concentration of 10 μM. The IC₅₀ values reported for the inhibition of human MAO-B by **14a** (0.268 μM) and **14f** (0.065 μM), however, differ significantly from the values recorded in the present study. The reason for this discrepancy is unclear, but may be related to different experimental approaches to measuring MAO activity.

Monoamine oxidase inhibition: reversibility

The reversibility of the MAO-B inhibition by compound **14b** was examined using dialysis [49]. Compound **14b** was selected since it is the most potent MAO-B inhibitor of this study. None of the study compounds were potent MAO-A inhibitors, and therefore reversibility of MAO-A inhibition was not examined. For these studies MAO-B and compound **14b** (at 4 × IC₅₀) were combined and preincubated for 15 min. As controls, the MAO-B enzyme was similarly preincubated in the absence of inhibitor (negative control) and presence of the irreversible MAO-B inhibitor, (*R*)-deprenyl (positive control). The preincubated mixtures were subsequently dialysed for 24 h and diluted twofold to yield a final inhibitor concentration of 2 × IC₅₀. The residual MAO-B activity of the diluted samples was measured and is shown in Fig. 7. Non-dialysed mixtures of MAO-B and the test inhibitor were maintained over the same time period (24 h) for comparison. The results show that the inhibition of MAO-B by compound **14b** is completely reversed after 24 h of dialysis. After dialysis of mixtures containing **14b** and MAO-B, the MAO-B catalytic activity was recovered to 115% of the negative control value (100%). This suggests that compound **14b** is a reversible inhibitor of MAO-B. After dialysis of the positive control samples containing (*R*)-deprenyl and MAO-B, catalytic activity is not recovered with only 3% activity remaining. As anticipated, inhibition of MAO-B by **14b** persists in non-dialysed samples with the residual activity at 41%.

A set of Lineweaver–Burk plots were also constructed to determine the mode of MAO-B inhibition of **14b**. Four Lineweaver–Burk plots were constructed with the first plot

Fig. 7 The reversibility of inhibition of MAO-B by compound **14b**. MAO-B was preincubated and dialysed in the absence of inhibitor (NI—dialysed) and the presence of inhibitor **14b** (**14b**—dialysed) and (*R*)-deprenyl (depr—dialysed). The residual MAO-B activities were recorded and are given as the mean $\pm$ standard deviation. The residual activities of non-dialysed mixtures of MAO-B and **14b** were also recorded (**14b**—not dialysed)

Fig. 8 Lineweaver–Burk plots of the inhibition of MAO-B by **14b**. The inset is a plot of the slopes of the Lineweaver–Burk plots versus inhibitor concentration. Key: no inhibitor (filled squares); inhibitor concentration = $\frac{1}{2} \times IC_{50}$ (circles), $1 \times IC_{50}$ (triangles), $\frac{1}{4} \times IC_{50}$ (diamonds)

prepared in the absence of inhibitor, while the remaining three plots were prepared in the presence of different concentrations of **14b** ($\frac{1}{2} \times IC_{50}$, $1 \times IC_{50}$; $\frac{1}{4} \times IC_{50}$). Kynuramine at eight different concentrations (15–250 μM) was used as substrate. The results are given in Fig. 8 and show

that the lines are linear and intersect at a single point just to the left of the y-axis. This suggests that **14b** interacts competitively with the MAO-B enzyme. From a replot of the slopes of the Lineweaver–Burk plots versus inhibitor concentration, the K_i value is estimated at 0.94 μM .

Discussion and conclusion

This study attempted to synthesise ester (**12a–e**) and amide (**13**) derivatives of chromone by reacting chromone 3-carboxylic acid with aromatic and aliphatic alcohols and amines in the presence of CDI. While the target esters were successfully synthesised, the reaction of chromone 3-carboxylic acid with amine compounds yielded the chromane-2,4-diones, which was not entirely unexpected (**14a–j**). Qualitative analysis using 1D and 2D NMR and X-ray crystallography assisted in structure elucidation of the chromane-2,4-diones. The results of the MAO inhibition studies showed that the ester derivatives are weak MAO inhibitors, while several of the chromane-2,4-diones exhibited good MAO-B inhibition potencies. The most potent MAO-B inhibitor is compound **14b** with an IC_{50} value of 0.638 μM . This compound is much less potent as an MAO-A inhibitor with an IC_{50} of 77.9 μM . It was further established that **14b** is a reversible MAO-B inhibitor. Compared to the reversible MAO-B selective inhibitor, lazabemide ($IC_{50} = 0.091 \mu M$), which was evaluated under identical experimental conditions, compound **14b** is however a lower potency MAO inhibitor [50]. Similarly, the MAO-B inhibition potency of **14b** is significantly lower compared to previously reported C6- and C7-substituted chromones [22, 33, 34]. This is exemplified by chromones **3–5**, which are at least an order of magnitude more potent MAO-B inhibitors compared to **14b**.

Molecular docking is considered to be a useful tool for predicting potential binding orientations and interactions of ligands in the MAO active site [51–53]. Based on the crystal structures of MAO-B in complex with reversible inhibitors, it may be expected that chromane-2,4-diones will bind to the active site with the chromane-2,4-dione moiety in proximity to the FAD, the most polar region. Here the chromane-2,4-dione moiety can establish hydrogen bonding with amino acid residues and water molecules. The 3-aminomethylidene side chain is expected to extend into the entrance cavity, which is reported to be lined by mostly non-polar residues. To illustrate this, the *trans* and *cis* isomers of **14b** were docked into the active site of MAO-B (PDB: 2Z5X) [54] with the CDOCKER module of Discovery Studio 3.1 (Accelrys) according to the reported protocol [49]. The results are given in Fig. 9 and show that both isomers indeed bind with the chromane-2,4-dione moiety placed in the substrate cavity. Interestingly, the binding orientations of the *trans* and *cis* isomers are very similar. While conventional

Fig. 9 Overlay of the predicted binding orientations of the *trans* (purple) and *cis* (yellow) isomers of **14b** in the MAO-B active site

hydrogen bonding is not observed, a pi–pi stacking interaction is seen with Tyr-398, as well as a pi-sulphur interaction with Cys-172 (*cis* isomer). Pi-alkyl interactions between the chromane-2,4-dione and Leu-171 (both isomers) and Ile-198 and Cys-172 (*trans* isomer) are formed, while the side chain phenyl ring is stabilised by pi-alkyl interactions with Ile-199 and Ile-316 (both isomers). Ile-199 is of further significance by establishing carbon-hydrogen bonding with a benzylic H of the side chain and the C2 carbonyl oxygen of the chromane-2,4-dione (both isomers). The phenolic oxygen of Tyr-326 also forms a carbon-hydrogen bond with the benzylic H (both isomers). This analysis suggests that both isomers of **14b** are able to bind and interact with MAO-B, with the binding modes and interactions being very similar. It may be concluded that both isomers contribute to the observed MAO-B inhibition.

An analysis of the structure–activity relationships (SARs) for MAO-B inhibition revealed some interesting trends. While the ester derivatives are weak MAO-B inhibitors, for the chromane-2,4-diones substitution with the benzyl group appears to be appropriate for MAO-B inhibition among the compounds evaluated. In this regard, larger substituents or further substitution with chlorine on the side chain phenyl results in decreased activity. It is noteworthy that substitution with the benzyl moiety (e.g. **14b**) yielded the highest potency MAO-B inhibition among the chromane-2,4-diones. This structural element is present in many experimental MAO inhibitors as well as in the clinically used agent, safinamide. Since MAO inhibition of these compounds is modest, further structural modifications will be required to optimise these compounds for further development as potent, reversible inhibitors of MAO-B. As mentioned, an interesting observation is that MAO-B inhibition decreases with side chain elongation from compound **14b** to **14c** and **14d**, with a large decrease in potency as further chain

elongation occurs to yield **14e**. Considering the results of the molecular docking study, it may be concluded that steric conflicts may occur between the 3-aminomethylidene side chain and the MAO-B entrance cavity, as the length of the side chain increases. A side chain linker length of 3 atoms (e.g. **14b**) seems to be optimal for avoiding steric conflicts and/or maximising productive interactions with the entrance cavity. The placement of the side chain in the entrance cavity thus appears to be an important determining factor for MAO-B inhibition potency, and further enhancement of activity should focus on modification of the 3-aminomethylidene side chain and binding interactions with the entrance cavity. As outlined in the introduction, MAO-B inhibitors are useful agents in the treatment of Parkinson's disease. The chromane-2,4-diones may be particularly suitable for further development since, as exemplified by **14b**, they are fully reversible MAO-B inhibitors with comparatively weak MAO-A inhibition potencies. This will greatly reduce the liability for hypertensive crisis which is associated with irreversible MAO-A inhibition.

Experimental section

Chemicals and instrumentation

All reagents and chemicals were purchased from Sigma-Aldrich and were used without further purification. All solvents used were obtained from ACE, while deuterated solvents used for NMR spectroscopy were purchased from Merck. Routine reaction monitoring was performed by thin-layer chromatography (TLC). Silica gel 60 F254 sheets (Merck) were used for TLC with mobile phases consisting of ethyl acetate: methanol (8:2) and ethyl acetate: petroleum ether (8:2). Carbon (^{13}C) and proton (^1H) NMR spectra were

recorded on a Bruker Avance III 600 spectrometer at frequencies of 150 MHz and 600 MHz, respectively. All NMR samples were dissolved in either deuterated chloroform (CDCl_3) or deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$). The chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hz. The spin multiplicities are given in abbreviated format: s (singlet), br s (broad singlet), d (doublet), br d (broad doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), br t (broad triplet), q (quartet), p (pentet/quintet), s (septet) or m (multiplet). Chemical shifts are referenced to the residual solvent signal, at 7.26 ppm for ^1H and 77.0 ppm for ^{13}C in CDCl_3 and at 2.50 ppm for ^1H and 39.5 ppm for ^{13}C in $\text{DMSO}-d_6$. High-resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionisation (APCI) mode, in positive ionisation mode. Melting points were determined with a Büchi B-545 melting point apparatus and are uncorrected. An Alpha FTIR spectrometer (Bruker) was used to record IR spectra. The OPUS/Mentor software interface was used to process the spectrometer readings. Fluorescence spectrophotometry was carried out using a Varian Cary Eclipse fluorescence spectrophotometer. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/mL) and kynuramine dihydrobromide were obtained from Sigma-Aldrich.

Purity determination by HPLC

Purity of the synthesised compounds was determined by HPLC using an Agilent 1200 HPLC system equipped with a quaternary pump and an Agilent 1200 series diode array detector. HPLC grade acetonitrile (Merck) and Milli-Q water (Millipore) were used for the chromatography. A Venusil XBP C18 column (4.60×150 mm, $5 \mu\text{m}$) was used with acetonitrile (30%) and Milli-Q water (70%) as the initial mobile phase at a flow rate of 1 mL/min. At the start of each injection, a solvent gradient programme was initiated by linearly increasing the composition of the acetonitrile in the mobile phase to 85% acetonitrile over a period of 5 min. Each run lasted 15 min and a time period of 5 min was allowed for equilibration between runs. A volume of 20 μL of solutions of the test compounds in acetonitrile (1 mM) was injected into the HPLC system, and the eluent was monitored at wavelengths of 210, 254 and 300 nm.

X-ray crystallography

X-ray crystallographic studies were carried out with a Bruker SMART X2S benchtop crystallographic system. For this purpose a single colourless crystal of compound **14j** was mounted on a MiTeGen MicroMount. Intensity measurements were taken using monochromated (doubly curved

silicon crystal) $\text{Mo}-\text{K}_\alpha$ -radiation ($0.71073 \text{ \AA}$) from a sealed microfocus tube. Generator settings were 50 kV, 1 mA. APEX2 software was used for preliminary determination of the unit cell and determination of integrated intensities, and unit cell refinement was performed using Bruker SAINT. The data collection temperature was -73°C . Data were corrected for absorption effects with SADABS using the multi-scan technique. The structure was solved with XS, and subsequent structure refinements were performed with XL. The structure was refined by anisotropic full-matrix least-squares refinement on F^2 : $\text{C}_{17}\text{H}_{14}\text{N}_2\text{O}_3$, $M = 294.30 \text{ g mol}^{-1}$, crystal size: $0.20 \times 0.20 \times 0.60 \text{ mm}^3$, monoclinic, space group $C 1 2/c 1$, $a = 33.050(5) \text{ \AA}$, $b = 4.9754(7) \text{ \AA}$, $c = 21.666(3) \text{ \AA}$, $\alpha = \gamma = 90^\circ$, $\beta = 128.152(9)^\circ$, $V = 2801.6(8) \text{ \AA}^3$, $Z = 8$, $\rho_{\text{calcd}} = 1.395 \text{ g cm}^{-3}$, $\mu = 0.096 \text{ mm}^{-1}$, $\lambda = 0.71073 \text{ \AA}$, $T = 200(2) \text{ K}$, $\theta_{\text{range}} = 1.56\text{--}25.04^\circ$, reflections collected: 13307, independent: 2474 ($R_{\text{int}} = 0.0338$), 203 parameters, final R indices [$I > 2\sigma(I)$]: $R_1 = 0.0584$; $wR_2 = 0.1593$, max/min residual electron density: $0.468/-0.368 \text{ e}^-/\text{\AA}^3$, goodness of fit on F^2 1.061, $F(000) = 1232$. CCDC 1859532 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

Procedure for synthesis of ester derivatives **12a–e**

1,1'-Carbonyldiimidazole (CDI) (5.26 mmol, 1 equiv.) was added to a stirred suspension of chromone 3-carboxylic acid (5.26 mmol, 1 equiv.) in N,N -dimethylformamide (DMF) (20 mL). This mixture was stirred for 2–3 h at 60°C under nitrogen, after which the reaction was cooled to 0°C and the alcohol (5.26 mmol, 1 equiv.) was added. The mixture was allowed to return to room temperature and stirred overnight. On completion, 40 mL of distilled water was added and the aqueous phase was extracted to ethyl acetate ($3 \times 20 \text{ mL}$). This organic fraction was washed with distilled water ($5 \times 40 \text{ mL}$) and brine, dried over magnesium sulphate and concentrated in vacuo. The resulting crude product was purified by recrystallisation from methanol.

Phenyl 4-oxo-4H-chromene-3-carboxylate (12a) The title compound was prepared from chromone-3-carboxylic acid and phenol in a yield of 22%: mp. $154.7\text{--}155.5^\circ\text{C}$ (methanol), pale yellow crystals. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 9.26 (s, 1H, H-2), 8.13 (dd, $J = 7.9, 1.7 \text{ Hz}$, 1H, H-5), 7.88 (ddd, $J = 8.7, 7.2, 1.7 \text{ Hz}$, 1H, H-7), 7.76 (dd, $J = 8.5, 1.0 \text{ Hz}$, 1H, H-8), 7.58 (ddd, $J = 8.1, 7.2, 1.1 \text{ Hz}$, 1H, H-6), 7.50–7.43 (m, 2H, H-3'/5'), 7.34–7.27 (m, 1H, H-4'), 7.28–7.22 (m, 2H, H-2'/6'). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 172.5 (C-4), 164.0 (C-2), 161.1 (C-9), 155.2 (C-8a), 150.2 (C-1'), 135.0 (C-7), 129.6 (C-3'/5'), 126.7 (C-6), 126.1 (C-4'), 125.5 (C-5), 124.3 (C-4a), 121.9 (C-2'/6'), 118.7 (C-8),

114.8 (C-3). IR $\nu_{\max}$ (cm⁻¹) 3057, 1746, 1645, 1614, 1486, 1094, 750. APCI-HRMS m/z calcd. for C₁₆H₁₁O₄, 267.0652, $[M+H]^+$ found 267.0658. Purity (HPLC): 81%.

Benzyl 4-oxo-4H-chromene-3-carboxylate (12b) The title compound was prepared from chromone-3-carboxylic acid and benzyl alcohol in a yield of 16%: mp. 107.3–108.3 °C (methanol), pale brown crystals. ¹H NMR (600 MHz, CDCl₃) δ 8.65 (s, 1H, H-2), 8.27 (dd, $J=8.0$, 1.7 Hz, 1H, H-5), 7.67 (ddd, $J=8.7$, 7.1, 1.7 Hz, 1H, H-7), 7.48–7.40 (m, 4H, H-6, H-8, H-2'/6' or H-3'/5')*, 7.39–7.33 (m, 2H, H-2'/6' or H-3'/5')*, 7.34–7.28 (m, 1H, H-4'), 5.35 (s, 2H, H-10). ¹³C NMR (151 MHz, CDCl₃) δ 173.3 (C-4), 163.1 (C-9), 161.9 (C-2), 155.5 (C-8a), 135.5 (C-1'), 134.2 (C-7), 128.6 (C-2'/6' or C-3'/5'), 128.24 (C-4'), 128.16 (C-2'/6' or C-3'/5'), 126.5 (C-5 or C-6), 126.2 (C-5 or C-6), 125.1 (C-4a), 118.1 (C-8), 116.0 (C-3), 66.9 (C-10). IR $\nu_{\max}$ (cm⁻¹) 3087, 1727, 1650, 1613, 1462, 1079, 754. APCI-HRMS m/z calcd. for C₁₇H₁₃O₄, 281.0808, $[M+H]^+$ found 281.0809. Purity (HPLC): 97%. *In no particular order.

4-Chlorophenyl 4-oxo-4H-chromene-3-carboxylate (12c) The title compound was prepared from chromone-3-carboxylic acid and 4-chlorophenol in a yield of 21%: mp. 181.9–182.3 °C (methanol), pale yellow crystals. ¹H NMR (600 MHz, CDCl₃) δ 8.83 (s, 1H, H-2), 8.29 (dd, $J=8.0$, 1.7 Hz, 1H, H-5), 7.72 (ddd, $J=8.7$, 7.2, 1.7 Hz, 1H, H-7), 7.54–7.45 (m, 2H, H-6, H-8)*, 7.35 (d, $J=8.8$ Hz, 2H, H-3'/5'), 7.16 (d, $J=8.8$ Hz, 2H, H-2'/6'). ¹³C NMR (151 MHz, CDCl₃) δ 173.0 (C-4), 162.8 (C-2), 161.6 (C-9), 155.5 (C-8a), 148.7 (C-1'), 134.5 (C-7), 131.5 (C-4'), 129.5 (C-3'/5'), 126.61, 126.57 (C-5, C-6)*, 125.0 (C-4a), 123.1 (C-2'/6'), 118.2 (C-8), 115.3 (C-3). IR $\nu_{\max}$ (cm⁻¹) 3056, 1752, 1643, 1615, 1461, 1070, 758. APCI-HRMS m/z calcd. for C₁₆H₁₀ClO₄, 301.0262, $[M+H]^+$ found 301.0268. Purity (HPLC): 83%. *In no particular order.

3-Chlorophenyl 4-oxo-4H-chromene-3-carboxylate (12d) The title compound was prepared from chromone-3-carboxylic acid and 3-chlorophenol in a yield of 19%: mp. 151.2–155 °C (methanol), yellow crystals. ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.27 (s, 1H, H-2), 8.13 (dd, $J=7.9$, 1.7 Hz, 1H, H-5), 7.88 (ddd, $J=8.7$, 7.2, 1.7 Hz, 1H, H-7), 7.76 (dd, $J=8.5$, 1.0 Hz, 1H, H-8), 7.58 (ddd, $J=8.1$, 7.2, 1.1 Hz, 1H, H-6), 7.50 (t, $J=8.1$ Hz, 1H, H-5'), 7.44 (t, $J=2.1$ Hz, 1H, H-2'), 7.39 (ddd, $J=8.1$, 2.1, 0.9 Hz, 1H, H-4'), 7.26 (ddd, $J=8.2$, 2.3, 1.0 Hz, 1H, H-6'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 172.5 (C-4), 164.4 (C-2), 160.7 (C-9), 155.2 (C-8a), 150.9 (C-1'), 135.1 (C-7), 133.4 (C-3'), 131.1 (C-5'), 126.8 (C-6), 126.3 (C-4'), 125.6 (C-5), 124.3 (C-4a), 122.4 (C-2'), 121.0 (C-6'), 118.8 (C-8), 114.4 (C-3). IR $\nu_{\max}$ (cm⁻¹) 3072, 1738, 1642, 1615, 1462, 1088, 755. APCI-HRMS m/z calcd. for C₁₆H₁₀ClO₄, 301.0262, $[M+H]^+$ found 301.0272. Purity:

sample decomposed upon standing and purity was thus deemed acceptable based on NMR.

(4-Chlorophenyl)methyl 4-oxo-4H-chromene-3-carboxylate (12e) The title compound was prepared from chromone-3-carboxylic acid and 4-chlorobenzyl-alcohol in a yield of 59%: mp. 116.6–117.7 °C (methanol), yellow crystals. ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.00 (s, 1H, H-2), 8.09 (dd, $J=8.0$, 1.7 Hz, 1H, H-5), 7.83 (ddd, $J=8.6$, 7.1, 1.7 Hz, 1H, H-7), 7.70 (br d, $J=8.4$, 1H, H-8), 7.57–7.49 (m, 3H, H-6, H-2'/6' or H-3'/5'), 7.45 (d, $J=8.4$ Hz, 2H, H-2'/6' or H-3'/5'), 5.29 (s, 2H, H-10). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 172.5 (C-4), 162.9 (C-2), 162.6 (C-9), 155.2 (C-8a), 135.0 (C-1'), 134.8 (C-7), 132.6 (C-4'), 129.6 (C-2'/6' or C-3'/5'), 128.4 (C-2'/6' or C-3'/5'), 126.5 (C-6), 125.5 (C-5), 124.4 (C-4a), 118.6 (C-8), 115.5 (C-3), 65.2 (C-10). IR $\nu_{\max}$ (cm⁻¹): 3080, 1732, 1647, 1614, 1461, 1083, 766. APCI-HRMS m/z calcd. for C₁₇H₁₂ClO₄, 315.0419 $[M+H]^+$ found 315.0388. Purity (HPLC): 97%.

Procedure for synthesis of 3-aminomethylidene-2,4-chromandiones 14a–j

CDI (5.26 mmol, 1 equiv.) was added to a stirred suspension of chromone 3-carboxylic acid (5.26 mmol, 1 equiv.) in 20 mL DMF. This mixture was stirred for 2–3 h at 60 °C under nitrogen, after which the reaction was cooled to 0 °C and the amine (5.26 mmol, 1 equiv.) was added. The mixture was allowed to return to room temperature and stirred overnight. On completion of the reaction, 40 mL of distilled water was added and the aqueous phase was extracted to ethyl acetate (3 × 20 mL). This organic fraction was washed with distilled water (5 × 40 mL) and brine, dried over magnesium sulphate and concentrated *in vacuo*. The resulting crude product was purified by recrystallisation from methanol.

(3E/Z)-3-[(Phenylamino)methylidene]-3,4-dihydro-2H-1-benzopyran-2,4-dione (14a) The title compound was prepared from chromone-3-carboxylic acid and aniline in a yield of 62%: mp. 206.7–209.5 °C (methanol), (lit. 208–210 °C, [41]; 204–208 °C; [55], 201–202 °C (DMSO), [42]; 194–236 °C (CHCl₃—petroleum ether), [45]), yellow fluffy crystals. Ratio of major isomer: minor isomer 1:0.9. ¹H NMR (600 MHz, CDCl₃) δ Major isomer: 13.68 (d, $J=13.5$ Hz, 1H, NH), 8.88 (d, $J=13.6$ Hz, 1H, H-9), 8.05 (dd, $J=7.8$, 1.7 Hz, 1H, H-5), 7.62–7.55 (m, 1H, H-7), 7.49–7.42 (m, 2H, H-2'/6' or H-3'/5'), 7.39–7.22 (m, 5H, H-2'/6' or H-3'/5', H-4', H-6, H-8). Minor isomer: 11.92 (d, $J=14.4$ Hz, 1H, NH), 9.02 (d, $J=14.5$ Hz, 1H, H-9), 8.12 (dd, $J=7.8$, 1.7 Hz, 1H, H-5), 7.62–7.55 (m, 1H, H-7), 7.49–7.42 (m, 2H, H-2'/6' or H-3'/5'), 7.39–7.22 (m, 5H, H-2'/6' or H-3'/5', H-4', H-6, H-8). ¹³C NMR (151 MHz, CDCl₃) δ

Major isomer: 181.8 (C-4), 163.6 (C-2), 154.95 (C-8a), 154.92 (C-9), 137.7 (C-1'), 134.8 (C-7), 130.18 (C-3'/5'), 127.5 (C-4'), 126.6 (C-5), 124.2 (C-6), 120.3 (C-4a), 118.45 (C-2'/6'), 117.43 (C-8), 98.7 (C-3). Minor isomer: 178.6 (C-4), 165.2 (C-2), 154.7 (C-8a), 153.4 (C-9), 137.7 (C-1'), 134.7 (C-7), 130.16 (C-3'/5'), 127.4 (C-4'), 125.8 (C-5), 124.4 (C-6), 120.6 (C-4a), 118.51 (C-2'/6'), 117.37 (C-8), 98.8 (C-3). IR $\nu_{\max}$ (cm⁻¹) 3180, 1684, 1594, 1567, 755. APCI-HRMS m/z calcd. for C₁₆H₁₂NO₃, 266.0812, [M+H]⁺ found 266.0807. Purity (HPLC): 90%.

(3E/Z)-3-[(Benzylamino)methylidene]-3,4-dihydro-2H-1-benzopyran-2,4-dione (14b) The title compound was prepared from chromone-3-carboxylic acid and benzylamine in a yield of 58%: mp. 163.8–164.6 °C (methanol), (lit. 165–167 °C, [41]), pink crystals. Ratio of major isomer: minor isomer 1:0.3. ¹H NMR (600 MHz, DMSO-*d*₆) δ Major isomer: 11.96–11.88 (m, 1H, NH), 8.62 (d, *J* = 14.6 Hz, 1H, H-9), 7.89 (dd, *J* = 7.8, 1.4 Hz, 1H, H-5), 7.67–7.58 (m, 1H, H-7), 7.44–7.23 (m, 7H, H-6, H-8, H-2'–6')*, 4.82 (d, *J* = 6.2 Hz, 2H, H-10). Minor isomer: 10.71–10.65 (m, 1H, NH), 8.72 (d, *J* = 15.4 Hz, 1H, H-9), 7.93 (br d, *J* = 7.7 Hz, 1H, H-5), 7.67–7.58 (m, 1H, H-7), 7.44–7.23 (m, 7H, H-6, H-8, H-2'–6')*, 4.82 (d, *J* = 6.2 Hz, 2H, H-10). ¹³C NMR (151 MHz, DMSO-*d*₆) δ Major isomer: 179.4 (C-4), 162.7 (C-2), 162.2 (C-9), 154.2 (C-8a), 136.7 (C-1'), 134.3 (C-7), 128.8 (C-2'/C-6' or C-3'/C-5'), 128.0 (C-2'/C-6' or C-3'/C-5'), 127.98 (C-4'), 125.3 (C-5), 123.9 (C-6), 120.2 (C-4a), 116.9 (C-8), 96.0 (C-3), 53.2 (C-10). Minor isomer: 177.1 (C-4), 163.2 (C-2), 160.6 (C-9), 154.1 (C-8a), 137.0 (C-1'), 134.4 (C-7), 128.8 (C-2'/C-6' or C-3'/C-5'), 128.0 (C-2'/C-6' or C-3'/C-5'), 127.9 (C-4'), 125.8 (C-5), 124.0 (C-6), 120.4 (C-4a), 117.0 (C-8), 95.9 (C-3), 53.1 (C-10). IR $\nu_{\max}$ (cm⁻¹) 3033, 1708, 1592, 1463, 746. APCI-HRMS m/z calcd. for C₁₇H₁₄NO₃, 280.0968, [M+H]⁺ found 280.0972. Purity (HPLC): 100%. *In no particular order.

(3E/Z)-3-[(2-Phenylethyl)amino]methylidene-3,4-dihydro-2H-1-benzopyran-2,4-dione (14c) The title compound was prepared from chromone-3-carboxylic acid and phenethylamine in a yield of 59%: mp. 178.2–178.7 °C (methanol), white crystals. Ratio of major isomer: minor isomer 1:0.5. ¹H NMR (600 MHz, DMSO-*d*₆) δ Major isomer: 11.68–11.62 (m, 1H, NH), 8.34 (d, *J* = 14.3 Hz, 1H, H-9), 7.90 (dd, *J* = 7.7, 1.7 Hz, 1H, H-5), 7.66–7.59 (m, 1H, H-7), 7.33–7.17 (m, 7H, H-2'–6', H-8, H-6)*, 3.83 (q, *J* = 6.7 Hz, 2H, H-10), 2.96 (t, *J* = 7.2 Hz, 2H, H-11). Minor isomer: 10.40 (br s, 1H, NH), 8.45 (d, *J* = 14.8 Hz, 1H, H-9), 7.90 (dd, *J* = 7.7, 1.7 Hz, 1H, H-5), 7.66–7.59 (m, 1H, H-7), 7.33–7.17 (m, 7H, H-2'–6', H-8, H-6)*, 3.83 (q, *J* = 6.7 Hz, 2H, H-10), 2.96 (t, *J* = 7.2 Hz, 2H, H-11). ¹³C NMR (151 MHz, DMSO-*d*₆) δ Major isomer: 179.3 (C-4), 162.7 (C-2), 162.2 (C-9), 154.2 (C-8a), 137.9 (C-1'), 134.3 (C-7), 128.9, 128.5 (C-2'/6',

C-3'/5')*, 126.6 (C-4'), 125.3 (C-5), 123.95 (C-6), 120.3 (C-4a), 116.9 (C-8), 95.6 (C-3), 51.6 (C-10), 35.8 (C-11). Minor isomer: 177.0 (C-4), 163.2 (C-2), 160.6 (C-9), 154.1 (C-8a), 137.9 (C-1'), 134.4 (C-7), 128.9, 128.5 (C-2'/6', C-3'/5')*, 126.6 (C-4'), 125.8 (C-5), 124.04 (C-6), 120.4 (C-4a), 117.0 (C-8), 95.3 (C-3), 51.7 (C-10), 35.9 (C-11). IR $\nu_{\max}$ (cm⁻¹) 3063, 1708, 1590, 1463, 741. APCI-HRMS m/z calcd. for C₁₈H₁₆NO₃, 294.1125, [M+H]⁺ found 294.1129. Purity (HPLC): 100%. *In no particular order.

(3E/Z)-3-[(3-Phenylpropyl)amino]methylidene-3,4-dihydro-2H-1-benzopyran-2,4-dione (14d) The title compound was prepared from chromone-3-carboxylic acid and 3-phenyl-1-propylamine in a yield of 58%: mp. 171.3–171.6 °C (methanol), white crystals. Ratio of major isomer: minor isomer 1:0.7. ¹H NMR (600 MHz, DMSO-*d*₆) δ Major isomer: 11.76–11.57 (m, 1H, NH), 8.44 (d, *J* = 14.7 Hz, 1H, H-9), 7.96–7.89 (m, 1H, H-5), 7.68–7.61 (m, 1H, H-7), 7.40–7.08 (m, 7H, H-6, H-8, H-2'–6')*, 3.60 (q, *J* = 6.8 Hz, 2H, H-10), 2.64–2.57 (m, 2H, H-12), 1.95 (p, *J* = 7.3 Hz, 2H, H-11). Minor isomer: 10.52–10.21 (m, 1H, NH), 8.55 (d, *J* = 15.5 Hz, 1H, H-9), 7.96–7.89 (m, 1H, H-5), 7.68–7.61 (m, 1H, H-7), 7.40–7.08 (m, 7H, H-6, H-8, H-2'–6')*, 3.60 (q, *J* = 6.8 Hz, 2H, H-10), 2.64–2.57 (m, 2H, H-12), 1.95 (p, *J* = 7.3 Hz, 2H, H-11). ¹³C NMR (151 MHz, DMSO-*d*₆) δ Major isomer: 179.3 (C-4), 162.7 (C-2), 162.2 (C-9), 154.2 (C-8a), 140.9 (C-1'), 134.28 (C-7), 128.4, 128.3 (C-2'/6', C-3'/5')*, 125.9 (C-4'), 125.3 (C-5), 123.9 (C-6), 120.3 (C-4a), 116.9 (C-8), 95.7 (C-3), 50.0 (C-10), 31.9 (C-12), 31.15 (C-11). Minor isomer: 177.1 (C-4)**, 163.5 (C-2), 160.6 (C-9), 154.2 (C-8a), 140.9 (C-1'), 134.33 (C-7), 128.4, 128.3 (C-2'/6', C-3'/5')*, 125.7 (C-4'), 125.3 (C-5), 124.0 (C-6), 120.5 (C-4a), 117.0 (C-8), 95.7 (C-3), 50.2 (C-10), 31.9 (C-12), 31.2 (C-11). IR $\nu_{\max}$ (cm⁻¹) 3023, 1711, 1603, 1462, 752. APCI-HRMS m/z calcd. for C₁₉H₁₈NO₃, 308.1281, [M+H]⁺ found 308.1280. Purity (HPLC): 100%. *In no particular order. **Shift based on HMBC correlation, signal not visible on ¹³C NMR.

(3E/Z)-3-[(4-Phenylbutyl)amino]methylidene-3,4-dihydro-2H-1-benzopyran-2,4-dione (14e) The title compound was prepared from chromone-3-carboxylic acid and 4-phenylbutylamine in a yield of 13%: mp. 142.4–143.3 °C (methanol), white crystals. Ratio of major isomer: minor isomer 1:0.4. ¹H NMR (600 MHz, CDCl₃) δ Major isomer: 11.86 (br s, 1H, NH), 8.34 (d, *J* = 14.1 Hz, 1H, H-9), 7.99 (dd, *J* = 7.8, 1.7 Hz, 1H, H-5), 7.54 (ddd, *J* = 8.4, 7.4, 1.7 Hz, 1H, H-7), 7.30–7.12 (m, 7H, H-6, H-8, H-2'–H-6')*, 3.50 (q, *J* = 6.4 Hz, 2H, H-10), 2.65 (t, *J* = 6.8 Hz, 2H, H-13), 1.79–1.66 (m, 4H, H-11, H-12)*. Minor isomer: 10.21 (br s, 1H, NH), 8.50 (d, *J* = 14.9 Hz, 1H, H-9), 8.07 (dd, *J* = 7.8, 1.6 Hz, 1H, H-5), 7.57–7.53 (m, 1H, H-7), 7.30–7.12 (m, 7H, H-6, H-8, H-2'–H-6')*, 3.52 (q, *J* = 6.4 Hz, 2H, H-10),

2.65 (t, $J=6.8$ Hz, 2H, H-13), 1.79–1.66 (m, 4H, H-11, H-12)*. ^{13}C NMR (151 MHz, CDCl_3) δ Major isomer: 181.3 (C-4), 163.9 (C-2), 162.2 (C-9), 154.8 (C-8a), 141.08 (C-1'), 134.2 (C-7), 128.5, 128.3 (C-2'/6', C-3'/5')*, 126.1 (C-5), 125.6 (C-4'), 123.9 (C-6), 120.5 (C-4a), 117.3 (C-8), 96.83 (C-3), 50.9 (C-10), 35.2 (C-13), 29.57, 28.0 (C-11, C-12)*. Minor isomer: 178.4 (C-4), 165.2 (C-2), 160.6 (C-9), 154.6 (C-8a), 141.10 (C-1'), 134.3 (C-7), 128.5, 128.3 (C-2'/6', C-3'/5')*, 126.3 (C-5), 125.6 (C-4'), 124.1 (C-6), 120.7 (C-4a), 117.2 (C-8), 96.76 (C-3), 50.8 (C-10), 35.2 (C-13), 29.64, 28.0 (C-11, C-12)*. IR ν_{max} (cm^{-1}) 3195, 1687, 1606, 1463, 754. APCI-HRMS m/z calcd. for $\text{C}_{20}\text{H}_{20}\text{NO}_3$, 322.1438, $[M+H]^+$ found 322.1445. Purity (HPLC): 100%. *In no particular order.

(3E/Z)-3-[(4-Chlorophenyl)amino]methylidene)-3,4-dihydro-2H-1-benzopyran-2,4-dione (14f) The title compound was prepared from chromone-3-carboxylic acid and 4-chloroaniline in a yield of 57%: mp. 251.7–252.7 °C (methanol), (lit. 250 °C, [56]), yellow solid. Ratio of major isomer: minor isomer 1:0.4. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ Major isomer: 13.37 (d, $J=13.8$ Hz, 1H, NH), 8.84 (d, $J=13.8$ Hz, 1H, H-9), 8.00–7.95 (m, 1H, H-5), 7.74–7.67 (m, 3H, H-7, H-2'/6'), 7.56–7.49 (m, 2H, H-3'/5'), 7.39–7.30 (m, 2H, H-6, H-8)*. Minor isomer: 11.83 (d, $J=14.5$ Hz, 1H, NH), 8.86 (d, $J=14.8$ Hz, 1H, H-9), 8.00–7.95 (m, 1H, H-5), 7.74–7.67 (m, 3H, H-7, H-2'/6'), 7.56–7.49 (m, 2H, H-3'/5'), 7.39–7.30 (m, 2H, H-6, H-8)*. ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ Major isomer: 180.3 (C-4), 162.3 (C-2), 155.8 (C-9), 154.4 (C-8a), 137.1 (C-1'), 135.1 (C-7), 131.2 (C-4'), 129.6 (C-3'/5'), 125.5 (C-5), 124.4 (C-6), 121.3 (C-2'/6'), 119.9 (C-4a), 117.2 (C-8), 98.3 (C-3). Minor isomer: 177.5 (C-4), 163.1 (C-2), 154.5 (C-9), 154.2 (C-8a), 137.6 (C-1'), 135.0 (C-7), 131.1 (C-4'), 129.5 (C-3'/5'), 125.9 (C-5), 124.5 (C-6), 121.7 (C-2'/6'), 120.2 (C-4a), 117.3 (C-8), 98.2 (C-3). IR ν_{max} (cm^{-1}) 3076, 1711, 1614, 1463, 750. APCI-HRMS m/z calcd. for $\text{C}_{16}\text{H}_{11}\text{ClNO}_3$, 300.0422, $[M+H]^+$ found 300.0439. Purity (HPLC): 79%. *In no particular order.

(3E/Z)-3-[(4-Chlorophenyl)methyl]amino} methylidene)-3,4-dihydro-2H-1-benzopyran-2,4-dione (14g) The title compound was prepared from chromone-3-carboxylic acid and 4-chlorobenzylamine in a yield of 37%: mp. 182.7–183.1 °C (methanol), white crystals. Ratio of major isomer: minor isomer 1:0.5. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ Major isomer: 11.87 (br s, 1H, NH), 8.61 (br s, 1H, H-9), 7.95–7.86 (m, 1H, H-5), 7.67–7.60 (m, 1H, H-7), 7.48–7.38 (m, 4H, H-2'/6', H-3'/5')*, 7.32–7.23 (m, 2H, H-6, H-8), 4.80 (s, 2H, H-10). Minor isomer: 10.66 (br s, 1H, NH), 8.71 (br s, 1H, H-9), 7.95–7.86 (m, 1H, H-5), 7.67–7.60 (m, 1H, H-7), 7.48–7.38 (m, 4H, H-2'/6', H-3'/5')*, 7.32–7.23 (m, 2H, H-6, H-8), 4.80 (s, 2H, H-10). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ Major isomer: 179.5 (C-4), 162.8

(C-2), 162.3 (C-9), 154.3 (C-8a), 135.9 (C-1'), 134.5 (C-7), 132.7 (C-4'), 130.0, 128.8 (C-2'/6', C-3'/5')*, 125.3 (C-5), 124.0 (C-6), 120.3 (C-4a), 117.0 (C-8), 96.2 (C-3), 52.5 (C-10). Minor isomer: 177.3 (C-4), 163.2 (C-2), 160.8 (C-9), 154.3 (C-8a), 136.1 (C-1'), 134.5 (C-7), 132.7 (C-4'), 130.0, 128.8 (C-2'/6', C-3'/5')*, 125.8 (C-5), 124.1 (C-6), 120.4 (C-4a), 117.1 (C-8), 96.0 (C-3), 52.5 (C-10). IR ν_{max} (cm^{-1}) 3183, 1698, 1617, 1464, 753. APCI-HRMS m/z calcd. for $\text{C}_{17}\text{H}_{13}\text{ClNO}_3$, 314.0578, $[M+H]^+$ found 314.0557. Purity (HPLC): 100%. *In no particular order.

(3E/Z)-3-[(2-(3-Chlorophenyl)ethyl)amino] methylidene)-3,4-dihydro-2H-1-benzopyran-2,4-dione (14h) The title compound was prepared from chromone-3-carboxylic acid and 2-(3-chlorophenyl)ethan-1-amine in a yield of 70%: mp. 216.3–217.6 °C (methanol), white fluffy crystals. The ratio of major isomer: minor isomer 1:0.5. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ Major isomer: 10.45–10.35 (m, 1H, NH), 8.37 (d, $J=14.6$ Hz, 1H, H-9), 7.94–7.88 (m, 1H, H-5), 7.68–7.60 (m, 1H, H-7), 7.39–7.18 (m, 6H, H-2', H-4', H-5', H-6', H-6, H-8), 3.83 (q, $J=7.0$ Hz, 2H, H-10), 3.01–2.94 (m, 2H, H-11). Minor isomer: 11.66–11.61 (m, 1H, NH), 8.48 (d, $J=15.5$ Hz, 1H, H-9), 7.94–7.88 (m, 1H, H-5), 7.68–7.60 (m, 1H, H-7), 7.39–7.18 (m, 6H, H-2', H-4', H-5', H-6', H-6, H-8), 3.83 (q, $J=7.0$ Hz, 2H, H-10), 3.01–2.94 (m, 2H, H-11). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ Major isomer: 179.3 (C-4), 162.7 (C-2), 162.3 (C-9), 154.2 (C-8a), 140.5 (C-1'), 134.3 (C-7), 133.1 (C-3'), 130.3, 128.9, 127.7, 126.6 (C-2', C-4', C-5', C-6')*, 125.3 (C-5), 124.0 (C-6), 120.3 (C-4a), 116.9 (C-8), 95.6 (C-3), 51.2 (C-10), 35.3 (C-11). Minor isomer: 177.0 (C-4), 163.2 (C-2), 160.7 (C-9), 154.1 (C-8a), 140.5 (C-1'), 134.4 (C-7), 133.1 (C-3'), 130.3, 128.9, 127.7, 126.5 (C-2', C-4', C-5', C-6')*, 125.8 (C-5), 124.1 (C-6), 120.3 (C-4a), 117.0 (C-8), 95.4 (C-3), 51.3 (C-10), 35.3 (C-11). IR ν_{max} (cm^{-1}) 3140, 1705, 1623, 1464, 752. APCI-HRMS m/z calcd. for $\text{C}_{18}\text{H}_{15}\text{ClNO}_3$, 328.0735, $[M+H]^+$ found 328.0734. Purity (HPLC): 98%. *In no particular order.

(3E/Z)-3-[(Pyridin-2-yl)amino]methylidene)-3,4-dihydro-2H-1-benzopyran-2,4-dione (14i) The title compound was prepared from chromone-3-carboxylic acid and 2-aminopyridine in a yield of 65%: mp. 212.2–214.9 °C (methanol), (lit. 237–240 °C (DMF), [47]), yellow solid. Ratio of major isomer: minor isomer 1:0.4. ^1H NMR (600 MHz, CDCl_3) δ Major isomer: 13.57 (d, $J=12.5$ Hz, 1H, NH), 9.61 (d, $J=12.8$ Hz, 1H, H-9), 8.49–8.43 (m, 1H, H-6'), 8.05 (dd, $J=7.8, 1.7$ Hz, 1H, H-5), 7.77 (ddd, $J=8.1, 7.4, 1.9$ Hz, 1H, H-4'), 7.63–7.56 (m, 1H, H-7), 7.32–7.23 (m, 2H, H-6, H-8), 7.20 (ddd, $J=7.4, 4.8, 0.9$ Hz, 1H, H-5'), 7.10 (br d, $J=8.1$ Hz, 1H, H-3'). Minor isomer: 11.91 (d, $J=13.7$ Hz, 1H, NH), 9.71 (d, $J=13.7$ Hz, 1H, H-9), 8.49–8.43 (m, 1H, H-6'), 8.13 (dd, $J=7.8, 1.7$ Hz, 1H, H-5), 7.77 (ddd, $J=8.1,$

7.4, 1.9 Hz, 1H, H-4'), 7.63–7.56 (m, 1H, H-7), 7.32–7.23 (m, 2H, H-6, H-8), 7.20 (ddd, $J=7.4, 4.8, 0.9$ Hz, 1H, H-5'), 7.12 (br d, $J=8.1$ Hz, 1H, H-3'). ^{13}C NMR (151 MHz, CDCl_3) δ Major isomer: 182.4 (C-4), 163.3 (C-2), 155.1 (C-9), 154.4 (C-8a), 149.4 (C-6'), 149.1 (C-2'), 139.0 (C-4'), 135.1 (C-7), 126.0 (C-5), 124.2 (C-6), 122.1 (C-5'), 120.3 (C-4a), 117.5 (C-8), 113.4 (C-3'), 99.7 (C-3). Minor isomer: 178.8 (C-4), 165.3 (C-2), 154.7 (C-9), 154.5 (C-8a), 149.3 (C-6'), 149.25 (C-2'), 139.0 (C-4'), 134.9 (C-7), 126.7 (C-5), 124.5 (C-6), 122.0 (C-5'), 120.7 (C-4a), 117.4 (C-8), 113.2 (C-3'), 100.0 (C-3). IR ν_{max} (cm^{-1}) 3218, 1685, 1640, 1459, 760. APCI-HRMS m/z calcd. for $\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_3$, 267.0764, $[M+H]^+$ found 267.0748. Purity (HPLC): 99%.

(3E/Z)-3-([2-(Pyridin-2-yl)ethyl]amino)methylidene)-3,4-dihydro-2H-1-benzopyran-2,4-dione (14j) The title compound was prepared from chromone-3-carboxylic acid and 2-(2-pyridyl)ethylamine in a yield of 53%: mp. 144.7–145.8 °C (methanol), colourless crystals. Ratio of major isomer: minor isomer 1:0.4. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ Major isomer: 11.81 (s, 1H, NH), 8.56–8.49 (m, 1H, H-6'), 8.40 (br s, 1H, H-9), 7.93–7.86 (m, 1H, H-5), 7.76–7.69 (m, 1H, H-4'), 7.66–7.58 (m, 1H, H-7), 7.33–7.21 (m, 4H, H-3', H-5', H-6, H-8), 4.01 (t, $J=6.7$ Hz, 2H, H-10), 3.16 (t, $J=6.6$ Hz, 2H, H-11). Minor isomer: 10.61 (s, 1H, NH), 8.56–8.49 (m, 2H, H-9, H-6'), 7.93–7.86 (m, 1H, H-5), 7.76–7.69 (m, 1H, H-4'), 7.66–7.58 (m, 1H, H-7), 7.33–7.21 (m, 4H, H-3', H-5', H-6, H-8), 4.01 (t, $J=6.7$ Hz, 2H, H-10), 3.16 (t, $J=6.6$ Hz, 2H, H-11). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ Major isomer: 179.2 (C-4), 162.7 (C-2), 162.2 (C-9), 157.8 (C-2'), 154.1 (C-8a), 149.1 (C-6'), 136.7 (C-4'), 134.2 (C-7), 125.3 (C-5), 123.9 (C-6), 123.6, 121.9 (C-3', C-5)*, 120.3 (C-4a), 116.9 (C-8), 95.6 (C-3), 49.3 (C-10), 36.97 (C-11). Minor isomer: 176.9 (C-4), 163.2 (C-2), 160.6 (C-9), 157.9 (C-2'), 154.2 (C-8a), 149.0 (C-6'), 136.7 (C-4'), 134.3 (C-7), 125.7 (C-5), 124.0 (C-6), 123.6, 121.9, (C-3', C-5)*, 120.4 (C-4a), 117.0 (C-8), 95.4 (C-3), 49.5 (C-10), 37.1 (C-11). IR ν_{max} (cm^{-1}): 3197, 1708, 1605, 1463, 762. APCI-HRMS m/z calcd. for $\text{C}_{17}\text{H}_{15}\text{N}_2\text{O}_3$, 295.1077, $[M+H]^+$ found 295.1071. Purity (HPLC): 100%. *In no particular order.

Measurement of IC_{50} values for the inhibition of MAO

Recombinant human MAO-A and MAO-B enzymes served as enzyme sources [49]. The enzyme reactions were carried out in white 96-well microtiter plates (Eppendorf) in potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl). The final volume of the reactions was 200 μL and contained kynuramine (50 μM), the test inhibitors (0.003–100 μM) and MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL). Stock solutions

of the test inhibitors were prepared in DMSO and added to the reactions to yield a final concentration of 4%. Reactions serving as negative controls were carried out in the absence of inhibitor. The enzyme reactions were initiated with the addition of the MAO enzymes and were subsequently incubated for 20 min at 37 °C in a convection oven. At endpoint, the reactions were terminated with the addition of 80 μL sodium hydroxide (2 N) and the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was measured by fluorescence spectrophotometry ($\lambda_{\text{ex}}=310$ nm; $\lambda_{\text{em}}=400$ nm). For this purpose, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.56 μM). The rates of MAO-catalysed 4-hydroxyquinoline formation thus measured were fitted to the one-site competition model of the Prism 5 software package (GraphPad). This gave sigmoidal plots of rate versus logarithm of inhibitor concentration from which the IC_{50} values were estimated. IC_{50} values were measured in triplicate and are reported as the mean $\pm$ standard deviation (SD).

Dialysis studies

Dialysis was carried out with Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with a molecular weight cut-off of 10 000 and a sample volume capacity of 0.5–3 mL [49]. The test inhibitor (at a concentration equal to $4 \times \text{IC}_{50}$) and MAO-B (0.03 mg protein/mL) were prepared to a final volume of 0.8 mL in the dialysis buffer (potassium phosphate buffer, 100 mM, pH 7.4, 5% sucrose). Stock solutions of the inhibitor were prepared in DMSO and added to the buffer to yield 4% DMSO. These samples were preincubated for 15 min at 37 °C and were subsequently dialysed at 4 °C in 80 mL of dialysis buffer. The dialysis buffer was replaced with fresh buffer at 3 h and 7 h after the start of dialysis. As positive control, MAO-B was similarly preincubated and dialysed in the presence of the irreversible inhibitor, (R)-deprenyl ($\text{IC}_{50}=0.079$ μM) [57]. As negative control dialysis of the enzyme was carried out in the absence of the inhibitor. After 24 h of dialysis, 250 μL of the dialysed samples was diluted twofold with the addition of 250 μL kynuramine (dissolved in potassium phosphate buffer, 100 mM, pH 7.4, made isotonic with KCl) to yield reactions with a final volume of 500 μL and containing kynuramine (50 μM), MAO-B (0.015 mg protein/mL) and test inhibitor ($2 \times \text{IC}_{50}$). The reactions (contained in 1.5 mL microcentrifuge tubes) were incubated at 37 °C in a waterbath and after 20 min were terminated with the addition of 400 μL sodium hydroxide (2 N). After addition of 1000 μL water, the concentrations of 4-hydroxyquinoline were measured by fluorescence spectrophotometry ($\lambda_{\text{ex}}=310$ nm; $\lambda_{\text{em}}=400$ nm) employing a 3.5-mL quartz cuvette (pathlength 10×10 mm). To quantify 4-hydroxyquinoline, a linear calibration curve was constructed with authentic 4-hydroxyquinoline

(0.047–1.56 μM). For comparison, undialysed mixtures of MAO-B and the test inhibitor were maintained at 4 °C for 24 h and subsequently diluted twofold and assayed as above. All reactions were carried out in triplicate, and the residual enzyme catalytic rates were expressed as mean $\pm$ SD.

Lineweaver–Burk plots and K_i value calculations

A set consisting of four Lineweaver–Burk plots were constructed. The first plot was constructed in the absence of inhibitor, while the remaining three plots were constructed in the presence of the test inhibitor at concentrations of $\frac{1}{2} \times \text{IC}_{50}$, $1 \times \text{IC}_{50}$ and $1\frac{1}{4} \times \text{IC}_{50}$. The enzyme–substrate, kynuramine, was used at concentrations ranging from 15 to 250 μM , while the final enzyme concentration was 0.015 mg protein/mL. All incubations were carried out in 1.5 mL microcentrifuge tubes to a volume of 500 μL . The MAO-B catalytic activity was measured by fluorescence spectrophotometry as described for the dialysis study. K_i values were estimated from plots of the slopes of the Lineweaver–Burk plots versus inhibitor concentration, where the x-axis intercept equals $-K_i$.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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