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Synthesis, molecular modeling studies, and selective inhibitory activity against monoamine oxidase of *N*,*N*′-bis[2-oxo-2*H*-benzopyran]-3-carboxamides

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Abstract—A novel series of *N*,*N*′-bis[2-oxo-2*H*-1-benzopyran]-3-carboxamide derivatives have been synthesized and investigated for the ability to inhibit the activity of the A and B isoforms of monoamine oxidase (MAO). Some of the synthesized compounds show good selective inhibitory activity against the MAO-A isoform. Both the MAO-A and -B isoforms, deposited in the Protein Data Bank as the 2BXR and 1GOS models, respectively, were considered in a computational study performed with docking techniques on the most active and selective inhibitors.

Recently many researchers have shown great interest in the study of MAO thanks to the key role played by the two MAO isoforms (MAO-A and MAO-B), which differ in substrate specificity, sensitivity to inhibitors, and amino acid sequence.[1] MAO-A preferentially oxidizes norepinephrine and serotonin, and is selectively inhibited by nanomolar concentrations of clorgyline,[2] whereas MAO-B preferentially deaminates β-phenylethylamine and benzylamine, and is irreversibly inhibited by nanomolar concentrations of L-deprenyl.[3] Dopamine, tyramine, and tryptamine are non-selective substrates for MAO-A and -B.[4]

Therefore, selective and reversible inhibitors of MAO-A or MAO-B may be useful therapeutic agents, devoid of undesirable side-effects, the so-called 'cheese effect.'[5] In humans MAO-B inhibitors (MAO-B-Is) are useful as coadjuvants in the treatment of Parkinson's disease and perhaps Alzheimer's disease,[6–8] while MAO-A inhibitors (MAO-A-Is) are antidepressant and antianxiety agents.[9–11]

The recent description of the crystal structure of the two isoforms of human MAO, by Binda et al., provides to elucidate the mechanism underlying the selective interactions between these proteins and their ligands, to probe the catalytic mechanism, and to gain a better understanding of the pharmacophoric requirements necessary for the rational design of potent and selective enzyme inhibitors with therapeutic potential.[12–17]

Between MAO inhibitors some (1*H*)-benzopyran derivatives, otherwise called coumarins, are known for their MAO inhibitory activity. Esuprone,[18] LU 53439,[19] 7-benzylossi derivatives (I),[20] and some 3-acyl coumarins (II)[21] have been the object of recent studies (Fig. 1).

To the best of our knowledge nothing has been reported about the MAO inhibitory activity of *N*,*N*′-bis[2-oxo-2*H*-benzopyran]-3-carboxamides (dicoumarinoyl

Keywords: Monoamine oxidase; Reversible monoamine oxidase-A inhibitors; Selective monoamine oxidase-A inhibitors; (1*H*)-Benzopyran derivatives.
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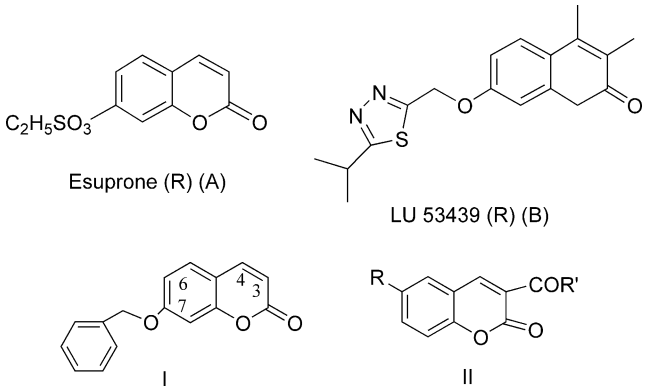


Figure 1. Chemical structures of reversible (R) selective MAO-A or MAO-B inhibitors (A or B).

diamides). In this study, we performed the synthesis and the evaluation of the MAO-A and MAO-B inhibitory activity of the dicoumarinoyl diamides **3a**–**g**. Furthermore, a molecular modeling work was performed by docking techniques to explain the selective inhibitory activity toward the MAO-A and MAO-B enzymes.

Very little is reported in the literature about the preparation of these compounds. Their synthesis could be performed by two main routes:

(1) Reaction of coumarin-3-acyl chloride[22] or coumarin-3-ethyl ester[23] with the suitable alkyliden diamine.
(2) Heterocyclization reaction of the suitable *N*,*N*′-bis(salicylidene)diamine with carbon suboxide.[24]

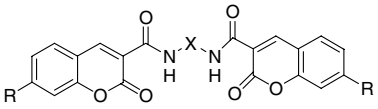
In the present work, we investigate the routes of synthesis depicted in Scheme 1 in order to find the best way to obtain **3a**–**g** in good yields.

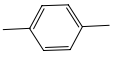
In Route 1 the reaction of esters **1a** and **1c** with the diamines always gave the dicoumarinoyl diamides **3a**–**g** in different yields ranging from 11% to 30% (Table 1), while no reaction was observed starting from coumarin-3-acyl chloride.

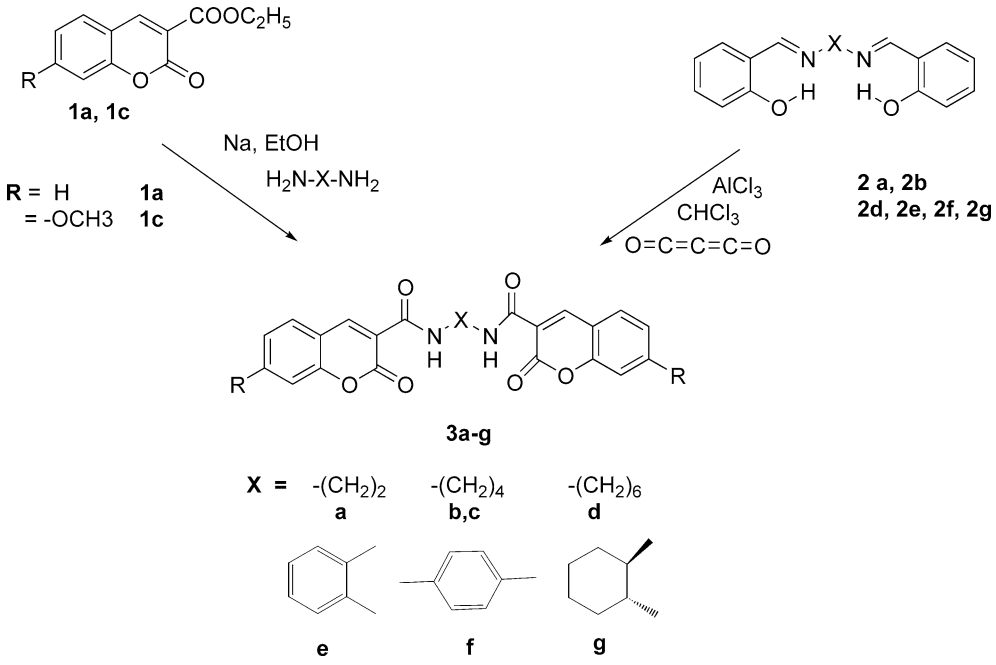
In Route 2 the reaction of *N*,*N*′-bis(salicylidene)diamines **2a**–**g** with carbon suboxide gave the dicoumarinoyl diamides **3a**–**g** with yields ranging from 0% to 72% (Table 1).

On the basis of these results for the synthesis of compounds **3b**–**d** we choose Route 2, while for the synthesis of compounds **3a**, **3e**–**g** we preferred Route 1 starting from coumarin-3-ethyl esters.

Table 1. Chemical-physical properties of derivatives **3a**–**g**



Compound	X	R	Yield % Route 1	Yield % Route 2	Mp (°C)
3a	$(CH_2)_2$	H	30	10	118–120[24]
3b	$(CH_2)_4$	H	12	65	83–85[24]
3c	$(CH_2)_4$	$-OCH_3$	12	68	176–178
3d	$(CH_2)_6$	H	11	72	68–70[24]
3e		H	26	0	237–240
3f		H	19	0	212–214[23]
3g		H	30	0	88–90



Scheme 1.

The reactions for the preparation of **3a** and **3e–g**[25] derivatives were performed by addition of the ethyl ester of 2-oxo-2*H*-1-benzopyran-3-carboxylic acid to a solution of the appropriate diamine in absolute ethanol and in the presence of natrium. For the synthesis of **3b–d** derivatives the reactions were carried out by addition of carbon suboxide at −70 °C to a solution of the suitable *N*,*N*′-bis(salicylidene)diamine in dry chloroform. Their structures were confirmed by observing the formation of a singlet for the amidic proton at 13.35 ppm and the disappearance of the broad peak of the aminic protons (NH_2) in the ^1H NMR spectra.[25]

The final compounds **3a–g** were assayed for their MAO-A and MAO-B inhibitory activity.

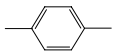
Bovine brain mitochondria were isolated according to Basford.[26] In all experiments the MAO activities of the bovine brain mitochondria were determined by a fluorimetric method according to Matsumoto et al.[27] using kinuramine as a substrate at four different final concentrations, ranging from 5 μM to 0.1 mM. Briefly, the incubation mixtures contained: 0.1 mL of 0.25 M potassium phosphate buffer (pH 7.4), mitochondria (6 mg/mL), and drug solutions with a final concentration ranging from 0 to 10^{-3} μM.

The solutions were incubated at 38 °C for 30 min. Addition of perchloric acid ended the reaction. The samples were centrifuged at 10,000*g* for 5 min and the supernatant was added to 2.7 mL of 0.1 N NaOH. The coumarine derivatives were dissolved in dimethylsulfoxide (DMSO), added to the reaction mixture from 0 to 10^{-3} μM. In order to study the inhibition of coumarine derivatives on the activities of both MAO-A and -B separately, the mitochondrial fractions were pre-incubated at 38 °C for 30 min before adding the specific inhibitors (L-deprenyl 0.5 μM to estimate MAO-A activity, and clorgyline 0.05 μM to assay the isoform B), considering that MAO-A is irreversibly inhibited by a low concentration of clorgyline, but is unaffected by a low concentration of L-deprenyl, which is used in the MAO-B form. Fluorimetric measurements were recorded with a Perkin-Elmer LS 50B Spectrofluorimeter. The protein concentration was determined according to Bradford.[28] The data are means of three experiments performed in duplicate. It is interesting to note that all compounds act through the reversible mode, as shown by dialysis performed over 24 h in a cold room against a 0.1 M potassium phosphate buffer (pH 7.2) capable of restoring 90–100% of the activity of the enzyme. The MAO-A and MAO-B inhibition data, expressed as pK_i, are reported in Table 2 together with the selectivity index.

The molecular modeling work was carried out considering the most active compound **3b** to explain by docking techniques its selectivity toward the MAO-A isoform (Table 2).

The work was performed with compound **3b**, built and energy minimized with AMBER* united atoms notation in GB/SA water as implemented in MacroModel software.[29] The crystallographic models 2BXR[14] for the MAO-A and 1GOS[12] for the MAO-B enzymes deposited into the Protein Data Bank (PDB) were pre-treated with a 48 kcal/mol constrained energy minimization of those residues out of a radius of 15 Å from the N5 of the isoalloxazine ring in order to restore the natural planarity of the isoalloxazine FAD ring and to relax the active site amino acids. The resulting energy minimum structures, after removing covalent ligands (clorgyline for 2BXR and pargyline for 1GOS), were used as receptor models.

Table 2. Structures and monoamine oxidase inhibitory activity of derivatives **3a–g**[a]

Compound	X	R	pK_i MAO-A	pK_i MAO-B	pSI[b] A selectivity
3a	$(CH_2)_2$	H	7.48	5.20	2.28
3b	$(CH_2)_4$	H	9.00	6.00	3.00
3c	$(CH_2)_4$	$-OCH_3$	6.77	5.85	0.92
3d	$(CH_2)_6$	H	8.40	5.74	2.66
3e		H	7.79	5.52	2.27
3f		H	5.62	5.76	−0.14
3g		H	6.74	5.85	0.89

[a] Data represent mean values of at least three separate experiments.
[b] pSI, log selectivity index = $pK_{i(MAO\text{-}A)} - pK_{i(MAO\text{-}B)}$.

The starting position of compound **3b** into the enzymatic clefts of both isoforms was built according to previously reported conformational results,[21] that is, with one coumarine ring stacked between Tyr398 and Tyr435 into the MAO-B. Similarly, this compound was built into the MAO-A in stacking configuration with Tyr407 and Tyr444.

The docking study was performed by a 1000 iterations of MC in situ search, allowing all rotatable bonds of the inhibitor to freely rotate and to translate into the enzymatic clefts within 10 Å from the original position, using the MOLS directive in the command file. In this search all residues within 10 Å from N5 of the isoalloxazine ring were allowed to move, while all other atoms were kept fixed by a 48 kcal/mol constant force. All configurations within 11.95 kcal/mol above the global minimum were fully energy minimized with the same force field and implicit solvation conditions.

The MOLINE approach[30] was used in the estimation of the state functions related to the recognition process of MAO-A and MAO-B complexes with **3b**. In order to analyze more relevant intermolecular contacts between ligands and the enzyme isoform, the most stable complexes were submitted to a Ligplot[31] analysis and visualized with the Pymol software.[32]

All the assayed compounds showed a selective inhibitory activity toward MAO-A. Among the compounds containing a phenyl or cyclohexyl as separator between the two coumarin-3-carboxamide moieties, the best activity was found for derivative **3e** ($pK_{iMAO\text{-}A} = 7.79$).

The lowest activity was detected for compound **3f** in which the phenyl ring is linked to the carboxamide groups in the 1,4 positions. Among all compounds, **3b** and **3d** showed the best MAO-A inhibitory activity ($pK_{iMAO\text{-}A}$ = 9.00 and 8.40, respectively) together with the best selectivity ($pSI = pK_{iMAO\text{-}A} - pK_{iMAO\text{-}B}$ = 3.00 and 2.66, respectively).

The results of the docking into the MAO-A and MAO-B clefts performed on the most potent and selective inhibitor **3b** consisted, respectively, of 34 and 72 configurations. In agreement with the experimental selectivity, the thermodynamic analysis of both configurational ensembles revealed a consistent difference in the free energy of complexation ($\Delta\Delta G = -14.91$ kcal/mol) favoring MAO-A molecular ($\Delta G = -58.20$ kcal/mol) versus MAO-B recognition ($\Delta G = -43.29$ kcal/mol).

The analysis of the most stable complex configurations was carried out by the LigPlot software considering the most stable complex of **3b** in both enzyme isoforms. A summary of the intermolecular contacts is reported in Figure 2.

In the MAO-A model the compound assumed a partially folded conformation interacting with 15 residues including the FAD. The recognition with the Tyr407 and Tyr444 was remarkably strong and extended,

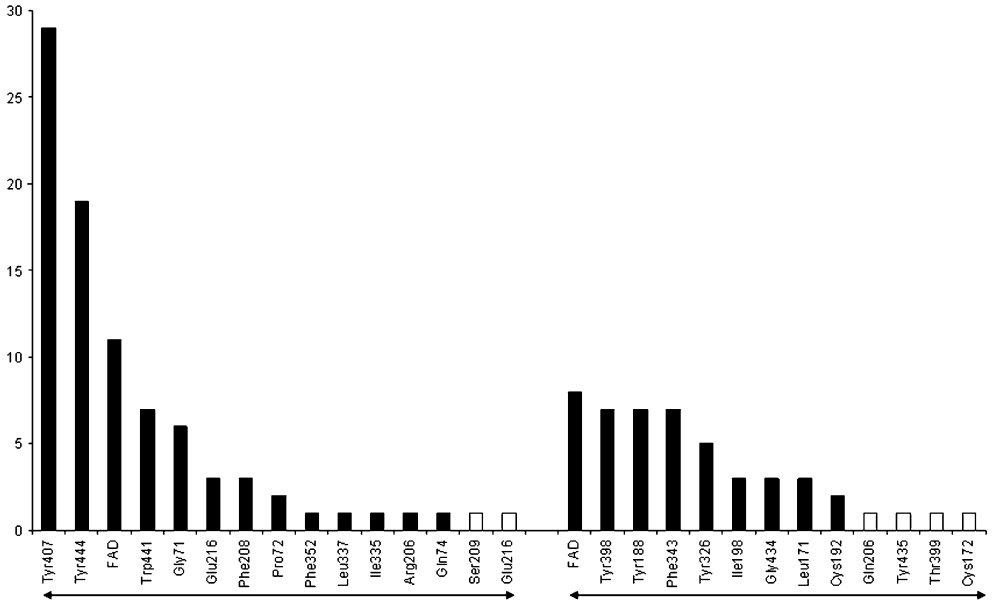


Figure 2. Number of non-bonded **3b** contacts within MAO-A and MAO-B clefts obtained by the LigPlot analysis of most stable complexes. Empty bars are related to intermolecular hydrogen bonds.

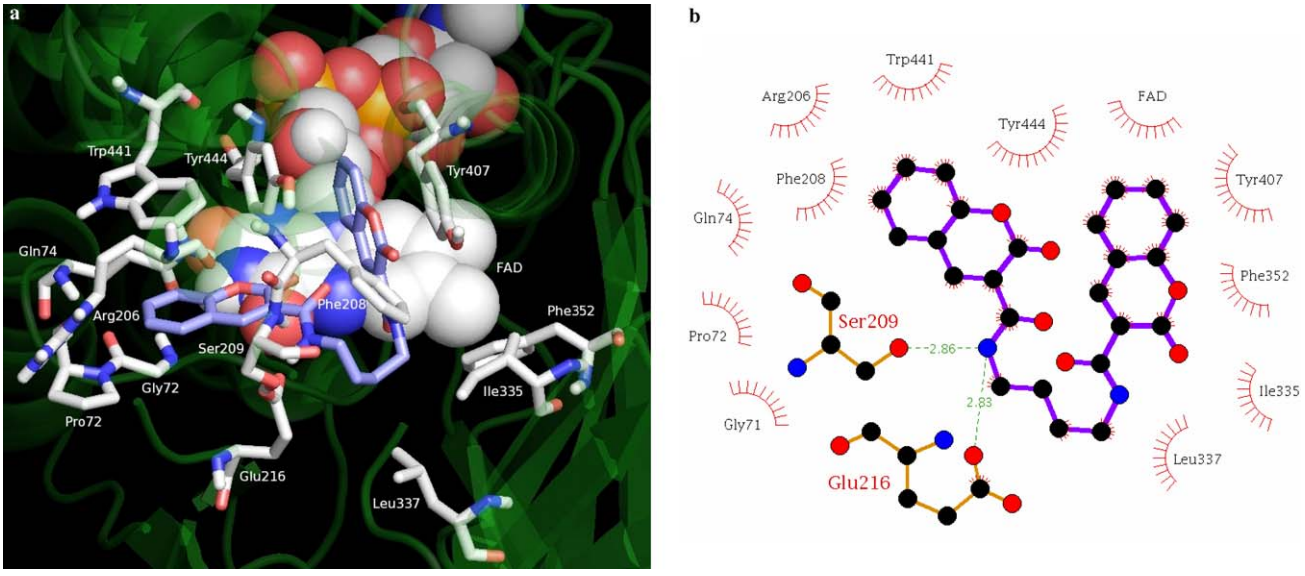


Figure 3. (a) Most stable complex between **3b** (blue carbon polytube) and MAO-A. Interacting residues of the active site are depicted in labelled polytubes, FAD in CPK rendering, other amino acids in ribbon. (b) LigPlot representation without hydrogen atoms: hydrogen bonds are reported as dashed lines with distances between heavy atoms.

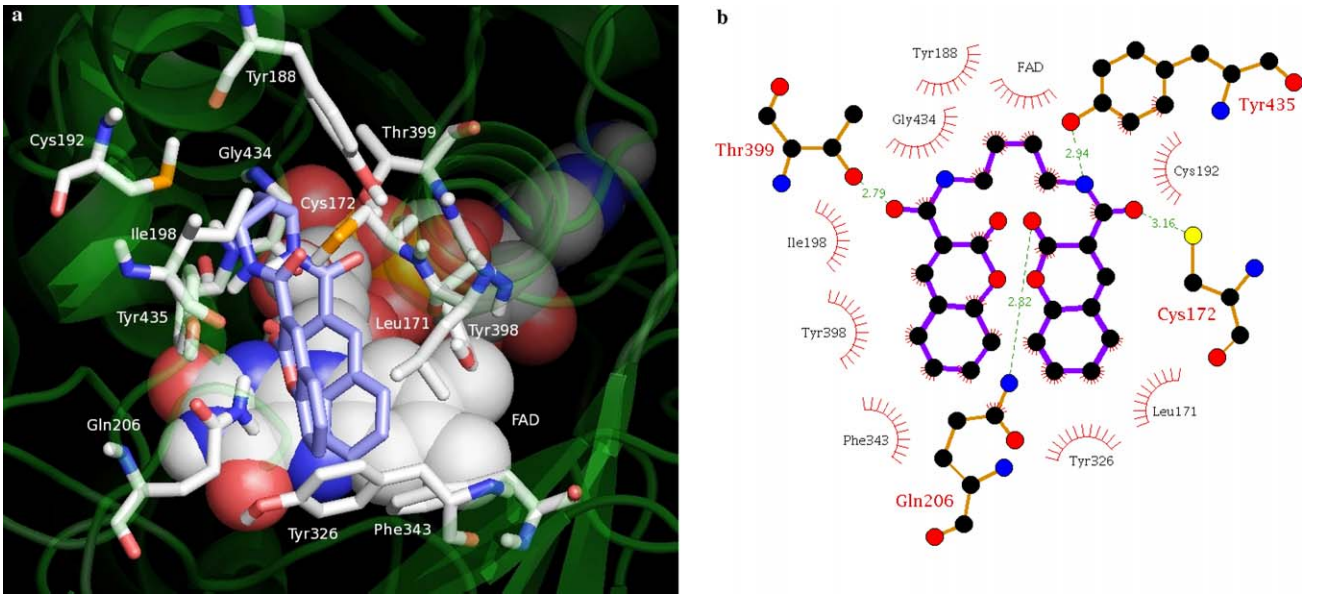


Figure 4. (a) Most stable complex between **3b** (blue carbon polytube) and MAO-B. Interacting residues of the active site are depicted in labelled polytubes, FAD in CPK rendering, other amino acids in ribbon. (b) LigPlot representation without hydrogen atoms: hydrogen bonds are reported as dashed lines with distances between heavy atoms.

configuring a double stacking with the aromatic side chains by one coumarine ring (Fig. 3). The partially folded conformation of **3b** allowed a concomitant interaction of the cofactor with both coumarine rings. Other residues were involved in the recognition of **3b** with multiple contacts. Two strong hydrogen bonds were established with the side chain of Ser209 and Glu216 (Fig. 3).

Conversely, in the MAO-B model the conformation of the inhibitor was more highly folded than in the MAO-A complex (Fig. 4).

As consequence of the different conformation assumed by **3b** into this isoform, few residues and a minor number of contacts into the cleft were observed in this case (Fig. 4). In particular the recognition with the cofactor and the tyrosine residues 398 and 435, corresponding to Tyr407 and Tyr444 in MAO-A, was less consistent. In fact **3b** interacted with residues relatively far to the inhibitor best location close to the FAD. With some of these residues, such as Thr399, Gln206, and Cys172, the inhibitor accepted hydrogen bonds mainly with its sp^2 oxygen atoms. Another one was established by one amidic hydrogen and Tyr435 phenolic oxygen. The result was a completely different recognition energetically less stable than that of the MAO-A isoform. The interaction with Tyr326 seemed to play an important role in the selectivity of **3b** toward the MAO isoforms. This residue hindered the active site and the access to the cofactor establishing multiple steric contacts with the inhibitor (Fig. 4). Conversely, Ile335, the corresponding residue in the MAO-A, gave only one contact with **3b** suggesting that the Ile335–Tyr326 mutation can play an important role in the selectivity of the inhibitors based on coumarine scaffold.

In summary, the recognition of **3b** within the MAO clefts were driven more by the van der Waals and electrostatic contacts than by intermolecular hydrogen bonds. The advantage of two hydrogen bonds favoring the MAO-B recognition was energetically inverted by the large number of contacts especially with Tyr and FAD residues, as indicated in Figure 2.

A series of bis-coumarine derivatives has been synthesized using two different routes: (a) by reaction of coumarin-3-ethyl ester with the suitable alkyliden diamine and (b) by heterocyclization reaction of the suitable *N*,*N*′-bis(salicylidene)diamine with carbon suboxide.

The biological behavior of the bis-coumarine derivatives was investigated against both MAO-A and -B isoforms. Most of them showed potent inhibition activities in the nanomolar range with selectivity against the MAO-A isoform. The docking study carried out on the most active and selective compound **3b** provided new insights into the inhibition mechanism and for the rational drug design of more potent/selective MAO inhibitors based on the coumarin scaffold.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.04.026.

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25. *Data for compound* ***3c***. ^{1}H NMR (DMSO-d_6): δ 14.01 (s, 2H, 2NH, D_2O exch.), 8.38 (s, 2H, 2CH), 7.23–7.21 (m, 2H, 2ArH), 6.33–6.26 (m, 4H, 4ArH), 3.72 (s, 6H, $2OCH_3$), 3.55 (s, 4H, $2CH_2$), 1.65 (s, 4H, $2CH_2$). Anal. Calcd for $C_{26}H_{24}N_2O_8$: C, 63.43; H, 4.90; N, 5.69. Found: C, 63.41; H, 4.91; N, 5.69.
Compound ***3e***. ^{1}H NMR (DMSO-d_6): δ 13.15 (s, 2H, 2NH, D_2O exch.), 8.03 (s, 2H, 2CH), 7.69–7.59 (dd, 4H, 4ArH—J = 40 Hz), 7.36–7.01 (m, 8H, 8ArH). Anal. Calcd for $C_{26}H_{16}N_2O_6$: C, 69.05; H, 3.57; N, 6.17. Found: C, 69.02; H, 3.56; N, 6.19.
Compound ***3g***. ^{1}H NMR (DMSO-d_6): δ 13.29 (s, 2H, 2NH, D_2O exch.), 8.46 (s, 2H, 2CH), 7.32–7.24 (m, 4H, 4ArH), 6.81–6.76 (m, 4H, 4ArH), 3.40 (s, 2H, 2CH), 2.06–1.81 (m, 8H, $4CH_2$). Anal. Calcd for $C_{26}H_{22}N_2O_6$: C, 68.10; H, 4.83; N, 6.10. Found: C, 68.11; H, 4.84; N, 6.11.
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