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Supporting Information for

Exploring coumarin potentialities: development of new enzymatic inhibitors based on the 6-methyl-3-carboxamidocoumarin scaffold

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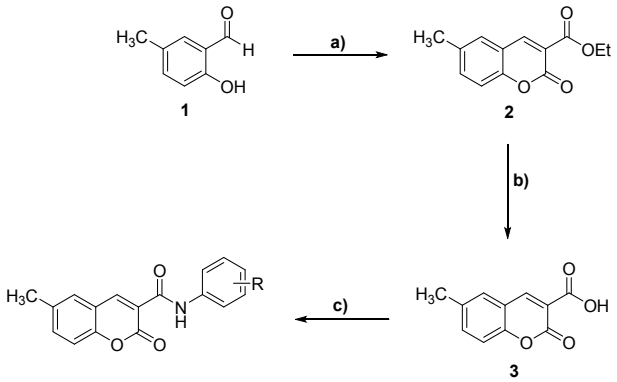

Scheme 1 – Synthesis of coumarins **4**-**15.** Reagents and conditions: a) diethyl malonate, EtOH, piperidine, reflux, overnight. b) NaOH (0.5% aq./EtOH), reflux, 4h. c) EDC, DMAP, DCM, corresponding amine, 0 ºC to r.t., 4h.

Reagents and materials.

All starting materials and reagents were obtained from commercial suppliers and were used without further purification (Sigma–Aldrich). Melting points (mp) were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and were not corrected. ^{1}H (250 MHz) and ^{13}C

(63 MHz) NMR spectra were recorded on a Bruker AMX spectrometer, using DMSO-d6 or $CDCl_3$ as solvents. Chemical shifts (δ) and coupling constants (*J)* were expressed in ppm and in Hz, respectively, using TMS as internal standard. The notations used for spin multiplicities were: s (singlet), d (doublet), dd (double doublet), t (triplet), dt (double triplet) and m (multiplet). Mass spectrometry data was acquired with a Hewlett- Packard-5972-MSD spectrometer. Elemental analyses were performed with a PerkinElmer 240B microanalyzer and are within 0.4% of calculated values in all cases. Silica gel (Merck 60, 230–400 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). Organic solutions were dried over anhydrous Na_2SO_4. The solvents were evaporated on a rotary evaporator (Büchi Rotavapor). The purity of all the compounds was higher than 95%.

***Synthesis of 6-methylcoumarin-3-carboxylic acid* (3).**

Firstly, 5-methylsalicylaldehyde (**1**) (1 mmol), diethyl malonate (1 mmol) and catalytic amounts of piperidine were refluxed in ethanol (10 mL) overnight. After cooling to room temperature, the suspension was filtered off and ethyl 6-methylcoumarin-3-carboxylate (**2**) was attained. Afterwards, compound **2** was hydrolyzed in 20 mL of an ethanolic solution with 0.5% NaOH (aq.) at reflux for 1h. After reaction 10% HCl (aq.) was added and the desired carboxylic acid (**3**) was then filtered off and washed with water to yield 89%.[1]

***General procedure for the synthesis of compounds* 4-15.**

To a solution of 6-methylcoumarin-3-carboxylic acid (**3**) (1 mmol) in dichloromethane (DCM) (5 mL) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (1.10 mmol) and 4-dimethylaminopyridine (DMAP) (1.10 mmol) were added. The mixture was kept in a round bottom flask with a flux of argon at 0 ºC for five minutes. Shortly after, the aromatic amine (1 mmol) with the pretended substitution pattern was added in small portions. The reaction mixture was stirred for 4 h at room temperature. The obtained precipitate was filtered and purified by column chromatography (hexane/ethyl acetate 9:1) or by recrystallization with ethanol to give the desired product.[2]

Determination of h*MAO-A *and h*MAO-B *inhibitory activity.

The effect of synthesized coumarins (compounds **4-15**) on *h*MAO isoforms was evaluated by a fluorimetric assay, following a previously described method, by measuring their outcome on the production of hydrogen peroxide (H_2O_2) from p-tyramine. In the assays the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, OR, U.S.) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for *h*MAO-A or *h*MAO-B (Sigma-Aldrich Química S.A) have been used. The production of H_2O_2 catalyzed by MAO isoforms was detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a nonfluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce resorufin, a

fluorescent product. Briefly, 100 µL of sodium phosphate buffer (0.05 M, pH 7.4) containing various concentrations of the coumarins, or reference inhibitors with DMSO as co-solvent, and adequate amounts of recombinant *h*MAO-A or *h*MAO-B were incubated for 15 min at 37˚C in a flat-black-bottom 96-well microplate (BRANDplates®, pureGradeTM, BRAND GMBH, Wertheim, Germany) placed in a dark multimode microplate reader chamber. After the incubation period, the reaction was started by adding (final concentrations) 200 µM Amplex Red reagent, 1 U/mL horseradish peroxidase, and 1 mM tyramine. The production of H_2O_2 and consequently of resorufin was quantified at 37˚C in a multimode microplate reader (Biotek Synergy HT), based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, along which the fluorescence increased linearly. Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions. Additionally, assays have been performed to check the interference of the compounds with the fluorescence generated in the reaction due to a non-enzymatic inhibition (*e.g.,* for a direct reaction with Amplex Red reagent) by adding them to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. To determine the kinetic parameters of *h*MAO-A and *h*MAO-B (Km and Vmax), their enzymatic activity was evaluated (under the experimental conditions above described) in the presence of different p-tyramine concentrations. The specific fluorescence emission, used to obtain the final results, was calculated after subtraction of the background activity, which was determined from wells containing all components except the MAO isoforms (replaced by a sodium phosphate buffer solution). The final results of the *h*MAO inhibitory activity were then expressed as IC_{50}, the concentration of each drug required to yield a 50% decrease on control value concentrations. Each assay was run in triplicate and each reaction was repeated at least three independent times being the highest concentration tested 10 µM. For the reference compound selegiline was used.[3]

Determination of AChE and BuChE inhibitory activity.

The AChE and BuChE inhibitory activities of the synthesized coumarins were determined in the following assays conditions. Briefly, the samples were dissolved in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 150 mM NaCl, and 0.05% Tween 20 at pH 7.6) and AChE or BuChE solutions (50 µL, 0.25 unit/mL) from Electroporus electricus and equine serum, respectively, in the same phosphate buffer, were added. The assay solutions were pre-incubated with the enzyme for 30 min at room temperature in a 96-well microplate. After pre-incubation, a solution containing the enzyme subtract consisting of acetylthiocholine or butyrylthiocholine (0.24 mM) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (0.2 mM, DTNB, Ellman's reagent) in Na_2HPO_4 (40 mM) was added. Then, the final absorbance was measured on a microtiter plate reader (Multiskan EX, Thermo,Vantaa, Finland) at 405 nm for 5 min. The background activity was given by a well with only sodium phosphate buffer solution. The final results were calculated by means of regression analysis and then expressed as IC_{50}. The alkaloid galantamine was used as the reference compound. Each assay was run in triplicate and each reaction was repeated at least three independent times.[4]

Data analysis and statistics.

The biological results were expressed as mean ± standard deviation of at least three different experiments. Statistical comparisons between control and test groups were carried by one-way analysis of variance (ANOVA-1) followed by Dunnett comparison post-test (α = 0.05, 95% confidence intervals). Differences were considered to be significant for *p* values lower than 0.05. Plots and statistical analysis were performed using GraphPad Software, Inc. La Jolla, CA 92937, USA.

Theoretical evaluation of drug-like properties.

The ADME properties of the compounds under study were calculated using the Molinspiration property program. LogP and topological polar surface area (TPSA) were calculated as a sum of fragment-based contributions and correction factors. The method for the calculation of molecule volume developed at Molinspiration have been obtained by fitting the sum of fragment contributions to 'real' three dimensional (3D) volume for a training set of about 12 000, mostly drug-like molecules. 3D molecular geometries for a training set were fully optimized by the semi-empirical AM1 method.[5, 6]

Evaluation of hMAO-B-inhibitor kinetics.

To evaluate the mechanism of *h*MAO-B inhibition of compound **11**, substrate-dependent kinetic experiments were performed. The catalytic rates of *h*MAO-B were measured at five different concentrations of p-tyramine substrate (0.031 – 3 mM) in the absence or presence of the selected inhibitor (compound **11**), at concentrations between 1.0 and 5.0 nM. The results are presented as double reciprocal Lineweaver-Burk plots (1/V vs. 1/[S]) and the kinetic data, namely Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}), was acquired employing Michaelis-Menten equation. The Ki value was estimated using Dixon plots, by replotting the slope of each Lineweaver-Burk plot versus the inhibitor concentration. In the Dixon plots, the Ki value was obtained from the x-axis intercept ($-K_i$). The enzymatic reactions and measurements were performed using the same *h*MAO-B assay conditions as described above (n=3). Linear regression analysis was performed using Prism 5.

Evaluation of hMAO-B-inhibitior type of binding affinity.

The analysis of the type of binding of compound **11** and the standard inhibitors with *h*MAO-B was performed by a time-dependent inhibition assay. The enzyme was incubated for a 60 minute period with the coumarin based inhibitor as well as the standard inhibitors at their IC_{80} values. The final well concentrations were: compound **11** (15 nM), (R)-(−)-deprenyl (50 nM), safinamide (40 nM) and MAO-B (6.4 μg/mL). Control experiments without inhibitors were run simultaneously. The enzymatic activity was determined as described above (see determination of *h*MAO isoform activity). The percentage of enzyme activity was plotted against the incubation time to determine time-dependent enzyme-inhibition. Data are the mean ± SD of three independent experiments.

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