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Through scaffold modification to 3,5-diaryl-4,5-dihydroisoxazoles: new potent and selective inhibitors of monoamine oxidase B

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Abstract

3,5-Diaryl-4,5-dihydroisoxazoles were synthesized and evaluated as monoamine oxidase (MAO) enzyme inhibitors and iron chelators. All compounds exhibited selective inhibitory activity towards the B isofrom of MAO in the nanomolar concentration range. The best performing compound was preliminarily evaluated for its ability to bind iron II and III cations, indicating that neither iron II nor iron III is coordinated. The best compounds racemic mixtures were separated and single enantiomers inhibitory activity evaluated. Furthermore, none of the synthesised compounds exhibited activity towards MAO A. Overall, these data support our hypothesis that 3,5-diaryl-4,5-dihydroisoxazoles are promising scaffolds for the design of neuroprotective agents.

Introduction

Amine oxidase is a widely diffused family of enzymes responsible for the oxidative deamination of both endogenous and exogenous monoamines, diamines and polyamines. These enzymes are divided into two classes, flavin adenine dinucleotide amino oxidase (FAD-AO) (EC 1.4.3.4) and copper topoquinone amino oxidase (Cu/TPQ-AO). The former, named monoamine oxidase (MAO), contains flavin adenin dinucleotide (FAD) as a cofactor while the latter contains 2,4,5-trihydroxyphenylalanine quinone as cofactor (TPQ-Cu) and are inhibited by semicarbazide. Regarding FAD-AO, two isofoms, MAO-A and MAO-B, have been isolated, differing from central nervous system (CNS) location, substrate specificity and sensitivity to inhibitor.

Methods

Materials and apparatus

Starting materials and reagents were obtained from commercial suppliers and were used without purification. All melting points were determined on a Stuart SMP11 melting points apparatus and are uncorrected (East Yorkshire, UK). Electron ionization mass spectstra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 mA, ion source temperature 200 °C) (Loughborough, UK). Samples were directly introduced into the ion source. Found mass values are in agreement with theoretical ones. Melting points, yield of reactions and the analytical and descriptive data of derivatives are reported in Table 1.

1H-NMR (Table 2) were registered on a Bruker AMX 300 MHz spectrometer (Billerica, MA). All samples were measured in CDCl3. Chemical shifts are reported referenced to the solvent in which they were measured. Coupling constants \(J\) are expressed in Hz. Elemental analyses were obtained on a Perkin-Elmer 240 B microanalyser (Waltham, MA). Analytical data of the synthesised compounds are in agreement within ±0.4% of the theoretical values.
TLC chromatography was performed using silica gel plates (Merck F 254, Kenilworth, NJ), spots were visualised by UV light.

**General procedure for the synthesis of compound derivatives**

In a typical experiment, 0.0038 mol of the appropriate \( \alpha,\beta \)-unsaturated ketone is dissolved in 30 ml of boiling absolute ethanol. On this solution, 10 ml of a freshly prepared hydroxylamine hydrochloride (0.0057 mol) and potassium hydroxide (0.013 mol) water solution are added dropwise. The solution becomes progressively orange and the reaction is monitored by thin layer chromatography (TLC) (CH\(_2\)Cl\(_2\)/n-hexane: 20/1). By cooling the reaction, the formation of a precipitate is observed which was filtered and crystallised from ethanol.

By this procedure (Scheme 1), compounds EMAC II (i–m) were synthesised.

| Compound | R          | Cryst. solvent | Calc. M.P. (°C) | Found M.P. (°C) | Yield % | E.I. 70 eV mass spectra m/z (rel. ab.) |
|----------|------------|----------------|-----------------|-----------------|--------|--------------------------------------|
| EMAC II i | 4-Cl       | Ethanol        | 70.72, 5.19, 5.15 | 70.67, 5.17, 5.12 | 134-5  | 42                                    |
| EMAC II j | 4-CH\(_3\) | Ethanol        | 81.24, 6.82, 5.57 | 81.27, 6.83, 5.54 | 103-4  | 46                                    |
| EMAC II k | 4-F        | Ethanol        | 75.28, 5.53, 5.49 | 75.31, 5.54, 5.43 | 132-4  | 41                                    |
| EMAC II l | 3,4-Cl     | Water/ethanol  | 62.76, 4.28, 4.57 | 62.72, 4.31, 4.54 | 134-5  | 65                                    |
| EMAC II m | 4-OCH\(_3\)| ethanol       | 76.38, 6.41, 5.24 | 76.33, 6.40, 5.22 | 131-3  | 44                                    |

**Table 2.** \(^1\)H NMR data and main fragments in E.I. mass spectrometry of derivatives EMAC II (i–m).

| Compound | \(^1\)H NMR δ (ppm) |
|----------|----------------------|
| EMAC II I | \(1H\text{-NMR: (300 MHz, CDCl}_3\) \(\delta 2.38\ (s, 3H, CH}_3\), 3.23 (dd, 1H, Jax 16.5, Jax 7.8, CH, isoxazole), 3.76 (dd, 1H, Jaz 16.5, Jaz 10.1, CH, isoxazole), 5.59 (dd, 1H, Jaz 10.8, Jaz 8.1, CH, isoxazole), 7.21 (d, 2H, J 7.8, CH, arom.), 7.33 (m, 4H, CH, arom.), 7.57 (d, 2H, J 8.1, CH, arom.).] |
| EMAC II j | \(1H\text{-NMR: (300 MHz, CDCl}_3\) \(\delta 2.37\ (s, 3H, CH}_3\), 3.24 (s, 3H, CH, isoxazole), 3.30 (dd, 1H, Jax 16.5, Jaz 8.3, CH, isoxazole), 3.72 (dd, 1H, Jaz 16.5, Jaz 10.1, CH, isoxazole), 5.66 (dd, 1H, Jaz 10.8, Jaz 8.3, CH, isoxazole), 7.16–7.29 (m, 6H, CH, arom.), 7.57 (d, 2H, J 8.3, CH, arom.).] |
| EMAC II k | \(1H\text{-NMR: (300 MHz, CDCl}_3\) \(\delta 2.35\ (s, 3H, CH}_3\), 3.27 (dd, 1H, Jax 16.5, Jaz 8.0, CH, isoxazole), 3.74 (dd, 1H, Jaz 16.5, Jaz 10.7, CH, isoxazole), 5.69 (dd, 1H, Jaz 10.7, Jax 8.3, CH, isoxazole), 7.04 (t, 2H, J 8.3, CH, arom.), 7.21 (d, 2H, J 8.1, CH, 4-CH\(_3\)-phenyl), 7.36 (dd, 2H, CH, J 8.3/5.3, arom.), 7.56 (d, 2H, J 8.2, CH, 4-CH\(_3\)-phenyl).] |
| EMAC II l | \(1H\text{-NMR: (300 MHz, CDCl}_3\) \(\delta 2.38\ (s, 3H, CH}_3\), 3.27 (dd, 1H, Jax 16.5, Jaz 7.8, CH, isoxazole), 3.78 (dd, 1H, Jaz 16.4, Jaz 10.8, CH, isoxazole), 5.67 (dd, 1H, Jaz 10.8, Jax 7.8, CH, isoxazole), 7.21 (m, 3H, CH, arom.), 7.44 (d, 1H, J 8.2, CH, arom.), m, 7.49 (d, 1H, J 1.2, CH, arom.), 7.56 (d, 2H, J 8.1, CH, arom.).] |
| EMAC II m | \(1H\text{-NMR: (300 MHz, CDCl}_3\) \(\delta 2.38\ (s, 3H, CH}_3\), 3.30 (dd, 1H, Jax 16.5, Jaz 7.8, CH, isoxazole), 3.71 (dd, 1H, Jaz 16.5, Jaz 10.6, CH, isoxazole), 3.80 (s, 3H, OCH\(_3\)), 5.66 (dd, 1H, Jaz 10.5, Jax 8.6, CH, isoxazole), 6.89 (d, 2H, J 8.3, CH, arom.), 7.21 (d, 2H, J 8.2, CH, 4-CH\(_3\)-phenyl), 7.31 (d, 2H, J 8.7, CH, arom.), 7.58 (d, 2H, J 8.2, CH, 4-CH\(_3\)-phenyl).] |

**Figure 1.** Scaffold evolution of MAO B inhibitors: from dihydro-pyrazoles to dihydro-isoxazoles.
HPLC enantioseparations and stereochemical characterisation

Direct separation of the enantiomers of EMAC II (i–m) was achieved by HPLC using pure dichloromethane as the eluent. The resolving power of the immobilized-type was sufficiently high to achieve a baseline enantioseparation of all compounds in a short-time analysis. The optimized analytical conditions were easily scaled up to semipreparative level employing a 1 cm i.d. IA column. An amount of <20 mg of racemic samples was resolved for each chromatographic run, and both enantiomers were collected with high enantiomeric purity. The stereochemical characterization of compounds EMAC II (i–m) was performed by CD correlation comparing the maximum and minimum of ellipticity of the CD spectra of the isolated enantiomers of compound EMAC II i of known stereochemistry (Figure 2), as previously described by some of us. Single enantiomers were resubmitted to biological assay.

Biological assay

The inhibitory activities of compounds EMAC II (i–m) on human recombinant MAO A and B isoforms, expressed in baculovirus infected BTI infected cells, as IC_{50}, are reported in Table 1. Tested compounds demonstrated no interference with the measurements, since they were unable to directly react with the Amplex Red reagent. The kinetic parameters of hMAO-A and hMAO-B were evaluated in the presence of different tyramine concentrations. In our experiments, hMAO-A displayed a Michaelis constant (Km) of 514 ± 46.8 mM and a maximum reaction velocity (Vmax) of 301.4 ± 27.9 nmol/min/mg protein, whereas hMAO-B showed a Km of 104.7 ± 16.3 mM and a Vmax of 28.9 ± 6.3 nmol/min/mg protein (n = 5). Active compounds showed reversible behaviour according to the method proposed by Cer et al. Hence, reported IC_{50} is a useful tool to determine the relative activity of the compounds within the series as well as to determine substituent and key positions in the scaffold. All the tested compounds exhibited inhibitory activity towards hMAO-B at nM concentration. Single enantiomers activity was evaluated indicating that stereochemistry plays a relevant role in determining inhibitory concentrations. None of the tested compounds exhibited activity towards the A isoform of the enzyme at the tested concentration of 100 μM.

Coordination studies

The interaction between EMAC II I and the Fe^{2+} or Fe^{3+} ions was evaluated by recording the UV/VIS absorbance spectra of EMAC II I (Figure 3) in absence and in presence either of FeCl3 or FeSO4. EMAC II I was solubilised in DMSO then diluted to 1 mM in
The UV/vis spectrum of the solution was recorded before and after addition of 10 mM final concentration either of FeCl₃ or FeSO₄. The spectra were recorded with an Ultrospec 2100 pro (Amersham Biosciences, Little Chalfont, UK) and analysed with SWIFT II-METHOD software (GE Healthcare, Little Chalfont, UK).

### Molecular modelling studies

#### Protein preparation

Three-dimensional coordinates of the receptor were obtained from the Protein Data Bank (PDB). The protein was processed and the internal hydrogen bonding network of the receptor was optimized using the algorithm implemented in Protein Preparation wizard.

#### Ligand preparation

The ligands were built using Maestro GUI. The lowest energy conformer was considered for the subsequent computational studies. This was obtained with MacroModel version 7.2, considering MMFF94 as force field and solvent effects by adopting the implicit solvation model Generalized Born/Surface Area (GB/SA) water. The simulation was performed allowing 1000 steps Monte Carlo analysis with Polak-Ribier Conjugate Gradient (PRCG) method and a convergence criterion of 0.05 kcal/(mol Å).

#### Docking experiments

Previous reported and validated QMPL docking protocol was applied using default settings. In order to better take into account the induced fit phenomena, the most energy favoured generated complexes were fully optimized with the AMBER united atoms force field in GB/SA implicit water, setting 5000 steps iterations analysis with PRCG method and a convergence criterion of 0.1 kcal/(mol Å). Analysis of the results of the complex minimization was carried out taking into account the state equations (free energy of complex formation) computed at 300 K, applying molecular mechanics and continuum solvation models with the molecular mechanics generalized Born. The resulting complexes were considered for the binding modes graphical analysis with Pymol and Maestro.

### Results and discussion

Pursuing in our research on the design and synthesis of new agents for the selective inhibition of MAO B, we have synthesised a series of 3,5-diaryl-4,5-dihydro-isoxazoles EMAC II (i–m). All the

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**Table 3. Inhibitory activities towards hMAO-A and hMAO-B of EMAC II (i–m) derivatives.**

| Compound | Structure | MAO-A (IC₅₀) | MAO-B (IC₅₀) | Ratio<sup>a</sup> |
|----------|-----------|--------------|--------------|-----------------|
| EMAC II i | ![](image) | 104.04 ± 3.69 nM | >961<sup>d</sup> |
| EMAC II j | ![](image) | 41.05 ± 1.52 nM | >2436<sup>d</sup> |
| EMAC II k | ![](image) | 320.22 ± 13.61 nM | >312<sup>d</sup> |
| EMAC II l | ![](image) | 11.97 ± 0.37 nM | >8354<sup>d</sup> |
| EMAC II m | ![](image) | 449.57 ± 18.02 nM | >222<sup>d</sup> |

Clobrine 4.46 ± 0.32 nM<sup>c</sup> 61.35 ± 1.13 μM 0.000073
L-Deprenyl 67.25 ± 1.02 μM 19.60 ± 0.86 nM 3431.12
Iproniazide 6.56 ± 0.76 μM 7.54 ± 0.36 μM 0.87
Moclobemide 361.38 ± 19.37 μM a <0.36<sup*e</sup>

<sup>a</sup>inactive at 1 mM (highest concentration tested).
<sup>b</sup>inactive at 100 μM (highest concentration tested). At higher concentration, the compounds precipitate.
<sup>c</sup>Results are mean ± SEM from five experiments. Level of statistical significance: P < 0.01 versus the corresponding IC₅₀ values obtained against MAO-B, as determined by ANOVA/Dunnett’s.
<sup>d</sup>Values obtained under the assumption that the corresponding IC₅₀ against MAO-B is the highest concentration tested (100 μM).
<sup>e</sup>Selectivity ratios [IC₅₀(MAO-A)]/[IC₅₀(MAO-B)].
compounds were submitted to biological evaluation to investigate their ability to inhibit both MAO isoforms. The structure and the MAO A and MAO B IC\textsubscript{50} values and selectivity ratios of the new derivatives are reported in Table 3. All the compounds inhibit MAO B isozyme with IC\textsubscript{50} values ranging from higher to lower nanomolar range. Accordingly to what is observed in our previous report on similar derivatives\textsuperscript{21}, compound EMAC II I, bearing a 3,4-dichlorophenyl moiety in the position 5 of the dihydroisoxazole ring resulted, the most active within all the tested compounds. Based on this observation we assumed that, in the formation of the enzyme–ligand complex, this portion of the inhibitor is, in all probability, oriented similarly with respect to our previously reported compounds. Interestingly, none of the new compounds exhibited activity towards the MAO A isoform up to 100 \textmu M concentration. Considering the presence of an asymmetric carbon at the position 5 of the dihydro-isoxazole nucleus we performed, semipreparative chromatographic enantioseparation of all the new derivatives EMAC II (i–m). The separated enantiomers were then submitted to biological evaluation (Table 4). The obtained data pointed out the importance of enantiomeric separation for both potency and selectivity. In particular, in the case of compound R-(-)-EMAC II I, the IC\textsubscript{50} value towards MAO B, drops from 11.97 nM to 1.89 nM, while the selectivity towards MAO B is increased by more than six folds. Moreover, considering the growing interest in the development of iron chelators for the treatment of neurodegenerative disorders\textsuperscript{35–37}, we have preliminarily investigated our best compound able to bind iron II and iron III cations following a previously reported procedure\textsuperscript{38}. According to our preliminary results (Figure 3), compound EMAC II I do not have the ability to bind neither iron II nor iron III cations.

In order to explore the nature of the ligand–receptor interactions, we have carried out docking experiments considering the MAO B active site by means of QMPL docking protocol\textsuperscript{31,39,40}. We have focused our attention on the most active compound R-(-)-EMAC II I, which shows the lowest IC\textsubscript{50} value and the highest selectivity towards MAO-B. It should be pointed out that MAO-B cavity is rather narrow, therefore, small changes are reflected in the biological activity. In this respect, the best compound shows remarkable difference of activity between the R and the S enantiomers. An analogous behaviour was observed in the previous series\textsuperscript{11,20}. According to docking and post docking best scored pose, the most stable complex configurations of both R and S enantiomers are depicted in Figure 4(a,b).

### Table 4. Inhibitory activities towards hMAO-A and hMAO-B of EMAC II (i–m) single enantiomers.

| Compound | MAO-A (IC\textsubscript{50}) | MAO-B (IC\textsubscript{50}) | Ratio |
|----------|-------------------------------|-------------------------------|-------|
| R-(-) EMAC II i | 15.27 ± 0.95 nM | 6549 b |       |
| S-(-) EMAC II i | 2.55 ± 0.11 nM | 39 216 b |       |
| R-(-) EMAC II j | 31.37 ± 1.67 \textmu M | 3188 b |       |
| S-(-) EMAC II j | 299.86 ± 12.04 nM | 333.3 b |       |
| R-(-) EMAC II k | 33.82 ± 1.12 \textmu M | 2956 b |       |
| S-(-) EMAC II k | 10.96 ± 0.83 \textmu M | 9124 b |       |
| R-(-) EMAC II l | 126.16 ± 8.44 nM | 792.6 b |       |
| S-(-) EMAC II l | 291.82 ± 13.45 nM | 342.6 |       |

IC\textsubscript{50} values are the mean ± SEM from five experiments. \textsuperscript{a}Inactive at 100 \textmu M (highest concentration tested). \textsuperscript{b}Values obtained under the assumption that the corresponding IC\textsubscript{50} against MAO-B is the highest concentration tested (100 \textmu M).

Figure 4. Putative binding mode of compound EMAC II I. (a) R-(-)-EMAC II I/MAO-B complex; (c) S-(-)-EMAC II I/MAO-B complex; (b, d) compound 2D representation and binding pocket interacting residues: green, hydrophobic; cyan, polar residues. Green arrows indicate \pi – \pi interactions.
The Renantiomer is stabilized by \( \pi-\pi \) interactions with Trp119, Phe103, Phe343, Tyr326 and some hydrophobic interactions with several residues in the binding pocket: Leu 164, Leu 167, Phe 168, Ile 316, Ile 199, Ile198, Leu 171 and the Tyr of the aromatic cage 435, 298, 188.

Conversely, the S enantiomer is oriented in the opposite way: with the dichlorophenyl substituent in the entrance cavity and the 4-methylphenyl substituent in the catalytic cavity toward the FAD. The complex results in less stability and gives rise only to a single \( \pi-\pi \) interaction with Tyr 435 and several hydrophobic interactions (Figure 4(c,d)).

Compared with previous series\(^1\), the absence of acetyl group allows a better accommodation in the cavity (Figures 4(a) and 5(a)). Once again, the importance in crossing both entrance and catalytic cavity, in order to achieve the MAO-B isoform selective activity is confirmed. This essential requisite has been reported by several studies\(^2\) and can be observed in co-crystallized selective MAO B inhibitors. A typical example is the inhibitor safinamide (Figure 5(c,d))\(^3\).

**Conclusions**

In summary, a small library of 3,5-diaryl-4,5-dihydroisoxazoles have been designed and synthesized on the basis of previously reported MAO inhibitors. Our finding highlighted the capability of 3,5-diaryl-4,5-dihydroisoxazoles to exert specific recognition and inhibition against the isoform B of MAO. The stereochemical characterization of R/S enantiomers confirms an enantioselective effect against MAO B isoform. This scaffold is definitively interesting for the development of novel selective neuroprotective agents.

**Disclosure statement**

The authors have no declarations of interest to report.

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