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ARTICLE *in* BIOORGANIC & MEDICINAL CHEMISTRY LETTERS · APRIL 2003

Impact Factor: 2.42 · DOI: 10.1002/chin.200314117 · Source: PubMed

Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 3629–3633

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of Amine Oxidases Activity by 1-Acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole Derivatives

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Received 13 April 2002; revised 28 June 2002; accepted 14 August 2002

Abstract—A novel series of 1-acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole derivatives have been synthesised and investigated for the ability to inhibit selectively monoamine oxidases, swine kidney oxidase, and bovine serum amine oxidase. The newly synthesised compounds **1**–**6** proved to be reversible and non-competitive inhibitors of all types of the assayed amine oxidases. Compounds inhibit monoamine oxidases potently, displaying low I_{50} values of particular interest. In particular 1-acetyl-3-(2,4-dihydroxyphenyl)-5-(3-methylphenyl)-4,5-dihydro-(1H)-pyrazole **6** showed to be a potent monoamine oxidase inhibitor with a K_i of about 10^{-8} M. Further insights in the theoretical evaluation of the possible interactions between the compounds and monoamine oxidase B have been developed through a computational approach.

Amine oxidases (AOs) are enzymes widely distributed among all living organisms. Without doubt their widespread occurrence accounts for important oxidatively deaminated biological functions, such as biogenic amine metabolism.

Two major classes of amine oxidases (AOs) are known: the AOs containing flavin adenin dinucleotide as cofactor (FAD-AOs) and the semicarbazide sensitive AOs (ssAOs) containing copper II and 2,4,5-trihydroxyphenilalanine quinone as cofactors (TPQ-Cu AOs). The former sub-class includes the mitochondrial monoamine oxidases (MAOs), which can be distinguished by substrate specificity and amino acid sequence in the two major isoforms MAO-A and MAO-B.[1,2] Recently a critical role of Ile-335 in MAO-A and Tyr-326 in MAO-B in determining substrate and inhibitor specificities in human MAO-A and -B has been reported.[3]

The initial interest in MAO inhibitors (MAOIs) for the treatment of depression faded because of their severe side effects and irreversible mechanism of action.[4] Only recently the development of new generation of inhibitors led to a renewed interest in the design, synthesis, and study of reversible, selective compounds.[5–7]

The finding that 1,3,5-triphenyl pyrazolines show monoamine oxidase inhibitory properties unrelated to tranquilizing, muscle relaxant, psychoanaleptic and anticonvulsant activities,[8–10] pushed us to synthesize a series of substituted 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazoles.[11] These compounds showed reversible and selective inhibitory activity on AOs, belonging to the third generation of AOIs. Modelling studies of the interaction with the isoalloxazine nucleus of FAD, however, revealed that the structure of the inhibitors did not allow the formation of a charge-transfer bonding interaction between the N2 of the pyrazoline ring and the N5 of isoalloxazine, reported to be particularly important for MAOs inhibition.[12a] The compounds showed in fact low inhibitory activity on MAOs, while inhibited BSAO.

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PII: S0960-894X(02)00699-6

The results of this work encouraged us to study the influence of the 1,3,5-phenyl substituents of the 4,5-dihydro-(1H)-pyrazole nucleus on the selective inhibitory activity towards AOs. We hypothesised that replacing the substituted phenyl ring at N1 by an acetyl group could increase the inhibitory activity towards MAOs. Such a substitution, in fact, was expected to decrease the bulkiness of the molecules and to increase the positive charge on N1 of the heterocycle, so as to strengthen the charge-transfer bonding interaction with isoalloxazine, then postulated as one of the major interactions with MAOs of reversible inhibitors.[12b] A preliminary computational analysis, performed on 1-acetyl-3-(2-hydroxyphenyl)-5-(3-methylphenyl)-4,5-dihydro-(1H)-pyrazole (reported as **4** in Table 1) to verify this hypothesis, demonstrated a more favourable interaction with isoalloxazine nucleus of this compound[13] with respect to our old-generation compounds.[11]

On the basis of this data a new series of 1-acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole derivatives was synthesised (Scheme 1 and Table 1) and biological results were examined by theoretical methods.

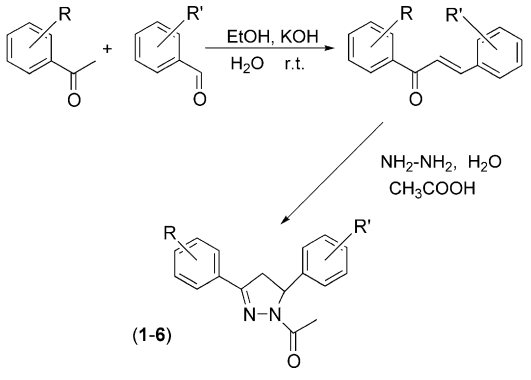
Compounds **1–6** were obtained starting from chalcone, obtained from condensation of substituted acetophenone with the required benzaldehyde by adding hydrazine hydrate dropwise (2.5:1 mol) in acetic acid (30 mL) during 8 h under stirring.[11]

All the compounds were tested on MAOs, on swine kidney diamine oxidase (SKDAO) and on bovine serum amine oxidase (BSAO) (Table 2).[14] SKDAO and BSAO are TPQ-Cu AOs and are both tested because of their specificity on different substrates: histamine and putrescine for DAO and spermine and spermidine for BSAO. The most active compounds **5** and **6** were also tested on MAO-A and MAO-B in the presence of specific substrates serotonine and benzylamine, respectively. This test did not show any significant different inhibiting activity between MAO-A and B of **5** and **6** (Table 3), suggesting residues Ile335 and Tyr326 not to be strongly involved in the binding of these inhibitors.[3]

Table 1. Chemical and physical data (**1–6**)

Compd	R	R′	Yield (%)	Mp (°C)	Formula[a]
1	H	3-CH_3	82	78–81	$C_{18}H_{18}N_2O$
2	H	4-Cl	74	110–112	$C_{17}H_{15}N_2OCl$
3	2-OH	2-CH_3	72	120–121	$C_{18}H_{18}N_2O_2$
4	2-OH	3-CH_3	67	128–130	$C_{18}H_{18}N_2O_2$
5	2,4-OH	2-CH_3	63	132–136	$C_{18}H_{18}N_2O_3$
6	2,4-OH	3-CH_3	62	141–143	$C_{18}H_{18}N_2O_3$

[a]Results of elemental analysis were ±0.4% of theoretical values; NMR and IR spectra confirmed the assigned structures.



Scheme 1.

Table 2. Inhibiting activity [K_i (M) ±SD] on MAOs, SKDAO and BSAO (**1–6**)

Compd	MAOs	SKDAO	BSAO
1	$2.0\times10^{-4}\pm0.08$	N.I.[a]	N.I.[a]
2	$6.0\times10^{-6}\pm0.4$	N.I.[a]	N.I.[a]
3	$3.0\times10^{-4}\pm0.5$	$5.5\times10^{-6}\pm0.4$	$5.7\times10^{-5}\pm0.2$
4	$5.5\times10^{-6}\pm0.6$	$8.0\times10^{-6}\pm0.8$	$5.5\times10^{-6}\pm0.3$
5	$8.0\times10^{-8}\pm0.09$	$1.0\times10^{-4}\pm0.2$	$1.0\times10^{-4}\pm0.2$
6	$5.0\times10^{-8}\pm0.04$	$2.5\times10^{-5}\pm0.7$	$1.0\times10^{-5}\pm0.1$

[a]N.I., no inhibition, corresponding to values $>10^{-4}$.

It is interesting to point out that all the compounds were found to belong to the third generation of inhibitors and to act through the non-competitive and reversible mode as demonstrated by the fact that dialysis for 24 h in a cold room against 0.1 M potassium phosphate buffer pH 7.2 was able to restore 90–100% of the enzyme activity. The most active compound (**6**) showed I_{50} values of 40 nM accompanied by a selectivity factor of 4000 for MAOs.

The experimental crystal structure of human MAO-B, deposited in late 2001 in RCSB Protein Data Bank (1GOS),[20] was exploited to perform a molecular modelling study. While synthesized compounds were, in fact, tested on bovine MAOs, the very high sequence identity (91%) between bovine and human MAO-B[21] stands 1GOS as a candidate eligible for such an approach. The recognition process between *R*- and *S*- enantiomers of **6** (chosen as the reference compound) was theoretically investigated by flexible docking experiments, assuming it was accommodated into the catalytic site of the enzyme, in accordance with a protocol already defined by some of us and well validated.[22] Using the isoalloxazine nucleus as a counterpart to study π–π stacking interactions, in fact,[11,12a,b] is now to be considered an oversimplification leading to unuseful, if not erroneous, models. Our approach was expected to give an insight into major binding interactions inside the catalytic site of the enzyme, helpful to explain the pharmacological behaviour of our compounds and to assist the design of new reversible inhibitors. Moreover, the calculation of enzyme-inhibitor interaction energies might allow a theoretical evaluation of which enantiomer of every inhibitor could be better accommodated into the catalytic site of MAO-B.

Table 3. Inhibiting activity [K_i (M) ±SD] on MAO A/B of **5** and **6**

Compd	MAO A	MAO B
5	$5.2\times10^{-8}\pm1.40$	$3.0\times10^{-8}\pm1.20$
6	$1.3\times10^{-8}\pm0.30$	$3.8\times10^{-8}\pm0.35$

MAO-B active site has been accurately described by C. Binda et al.[23] who crystallized the pargyline inhibitor covalently bound to the N5 atom on the *re* side of the flavin moiety of the enzyme and identified, in this way, the residues bounding the catalytic cavity. In our studies, the lowest energy conformation of both enantiomers of **6** in the gas phase, manually predocked in place of the inhibitor,[22] were used as the starting geometries for several consecutive Monte Carlo runs into the active site of the enzyme. During each run, only the side chains of twelve residues (Tyr60, Tyr435, Gln206, Tyr188, Ile198, Phe168, Ile199, Tyr326, Leu171, Cys172, Tyr398 and Phe343), reported to line the pargyline binding site,[23] were allowed to relax together with the ligand and isoalloxazine (fully oxidized planar form), while the remainder of the enzyme was fixed in 3-D-space.[24]

A sole binding mode per enantiomer was found through the docking experiments within a 10 kcal/mol energy window over the global minimum. In the case of (*R*)-**6**, (see Fig. 1), the 2,4-substituted phenyl and the dihydro-(1H)-pyrazole rings were found to face the isoalloxazine nucleus (the nearest atoms—$N1_{(azole)}$ and $C6_{(phenyl)}$—were located at about 3.1 Å from the isoalloxazine plane). Moreover, the disubstituted phenyl ring was sandwiched between the side chains of Tyr435 and Tyr398 and involved in a network of two hydrogen bonding interactions with the backbone CO of residues Gly434 (at a distance of 2.1 Å) and Cys172 (at a distance of 2.0 Å).[27] The CO of the acetyl group was favourably directed towards the protonated nitrogen of the side chain of Lys296 (distance between the carbonyl oxygen and the nitrogen equal to 3.2 Å), in proximity of the flavin-N5 (distance between the carbonyl carbon and the nitrogen equal to 3.8 Å), while the phenyl ring at C5 was accommodated in a pocket of the catalytic site formed by residues Phe343, Leu171, Leu328 and Tyr326.

Compound (*S*)-**6** showed a binding geometry very similar to the one displayed by (*R*)-**6** as for the disubstituted phenyl and pyrazoline rings, which displayed the set of intermolecular interactions already described in the case of the *R*-enantiomer. The main difference with respect to the recognition of (*R*)-**6** was in the spatial orientation of the phenyl ring at C5, accommodated this time in proximity of residues Tyr60, Tyr326 and Gln206 (see Fig. 1). Such a different orientation was mainly responsible of the lower stability theoretically calculated for the complex between MAO-B and (*S*)-**6** with respect to the one between MAO-B and (*R*)-**6** ($\Delta E_{(S\text{-}R)} \cong 3$ kcal/mol).

The docking studies rationalized the relevant inhibitory activity of **6** towards MAO-B, as due to the formation of several favourable interactions with the catalytic site of the enzyme. Notably, residues Tyr398 and Tyr435, both interacting with the disubstituted phenyl ring of **6**, have been already underlined in the paper by Binda et al. to play a role in the substrate specificity of MAO-B.[23] The importance of the 4-OH group in properly positioning the disubstituted phenyl ring of **6** by H-bond formation was pointed out. It is worth noting that compounds lacking this OH, like in the case of **1**–**4**, showed their inhibitory activity lowered at least by two orders of magnitude. As shown in Table 2, the introduction of one Cl in position 4 of the phenyl ring at C5 increased the activity of **2** by two orders of magnitude with respect to **1**. Notably, the in-silico introduction of such a substituent in the docking geometry of **6** revealed that this atom might be properly accommodated between the side chains of residues Leu171, Leu345, Tyr326 and Phe343, in a favourable hydrophobic environment. Unfortunately, the modelling studies could not rationalise the selectivity towards MAO-A and MAO-B of our derivatives due to the lack, up-to-date, of a reliable 3-D structure of MAO-A.

In the light of the discussion developed so far it appears reasonable to assert that the recognition of the most active compounds among **1**–**6** by MAO-B can prevent

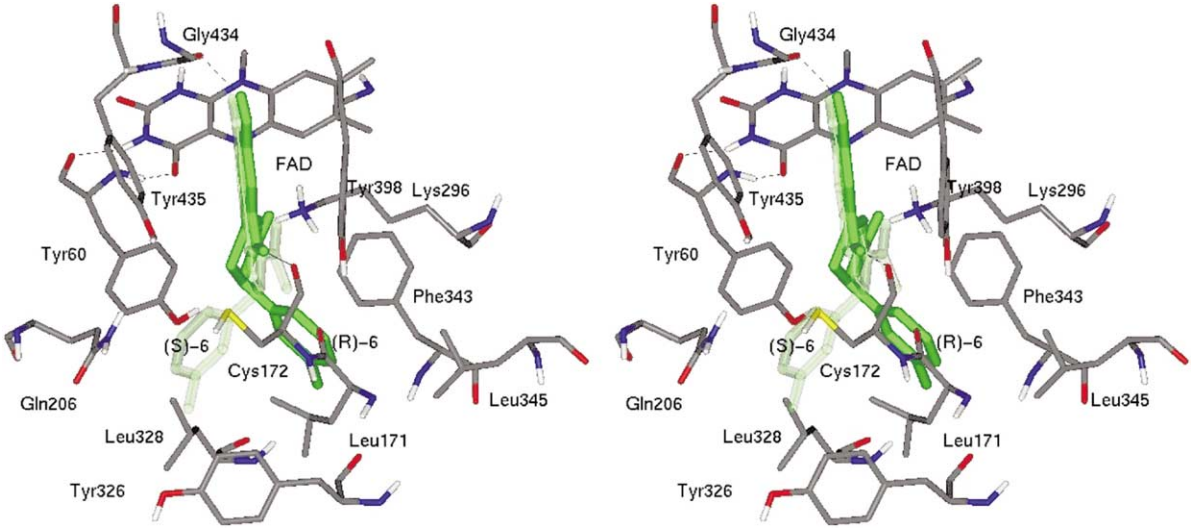


Figure 1. Superimposition (relaxed stereoview) of the calculated recognition geometries (thick) of (*R*)-**6** (green) and (*S*)-**6** (light green) located at their proper respective position inside the catalytic site of MAO-B (thin, coloured by atom types). For the sake of simplicity only the MAO residues useful for the discussion are shown. Hydrogen atoms bound to heteroatoms are displayed. Hydrogen-bonding interactions are depicted as dashed lines.

the access of substrates to the isoalloxazine nucleus hampering their oxidation by the enzyme. Moreover, the molecular modelling studies have highlighted that computational approaches like the one we followed in the past,[11] are too simplistic to have importance in assisting the drug design of new reversible AO inhibitors.

In conclusion and in partial, even though fortuitous, agreement with our preliminary hypotheses, the acetyl group proved to contribute to the inhibitory activity and selectivity towards MAOs of **1–6**, likely taking part in the interaction with the isoalloxazine nucleus. The OH groups on the phenyl ring at C3, in a similar manner, could participate in the formation of important intermolecular hydrogen bonds, increasing in this way the inhibitory activity towards MAOs of the most active compounds.

The information conveyed by the structure–activity studies reported here are considered important for ongoing projects of rational design and synthesis of new potent reversible derivatives, structurally related to the already synthesized compounds **1–6**. A new method for the synthesis of 1-acetyl-3-(2,4- and 2,6-dihydroxyphenyl) - 5 - (disubstituted - phenyl) - 4,5 - dihydro - (1H) - pyrazole derivatives is under study with the dual-purpose to prepare new derivatives, in homochiral form and with better yields than those reported here. A full computational approach will be applied as well, that will take into consideration several reversible inhibitors, with the aim to develop a SAR model of the inhibition of amine oxidases activity by 1-acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole derivatives, able to rationalize the influence of the stereoisomers on the activity/selectivity.

Acknowledgements

The authors wish to thank the IBAF (Istituto di Biotecnologie Applicate alla Farmacologia) of the CNR (Consiglio Nazionale delle Ricerche) for computational resources. This work was partly financed by MURST, by a CNR project on Biotechnology e Molecular Biology and by MIUR (cofin. 2000, ex 40%, prot. MM03237197_002).

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13. Unpublished results. The pyrazoline ring and the phenyl ring at C3, especially, were found to interact with the polar pyridazine and pyrimidine portions of the isoalloxazine nucleus, respectively, while the acetyl group at N1 was directed outwards, in direction of the catalytic site.[12b]
14. All chemicals were commercial reagents of analytical grade and were used without purification. Bovine serum amine oxidase (BSAO) was purified according to Turini et al.;[15] its specific activity was 0.3 U/mg. Swine kidney diamine oxidase (SKDAO) was purified according to Mondovì et al.;[16] its specific activity was 0.4 U/mg. Bovine brain mitochondria (MAO) were isolated according to Basford.[17] In all experiments the AO activity of the beef brain mitochondria, SKDAO, and BSAO were measured fluorimetrically according to Matsumoto et al.[18] Briefly, the incubation mixtures contained the following: 0.1 mL of 0.25 M potassium phosphate buffer (pH 7.5); 0.1 mL of peroxidase (25000 UI) solution (0.5 mg/mL); 0.1 mL of homovanillic acid solution (1 mg/mL); 0.1 mL of sample (beef brain mitochondria, 6 mg/mL; SKDAO or BSAO 0.14 mg/mL), or water for the hydrogen peroxide assay; 0.1 mL of the appropriate substrate (tyramine for MAO, benzylamine for BSAO and putrescine for SKDAO) under evaluation at four different final concentration ranging from 0.01 to 1 mM or hydrogen peroxide as standard, 22 nmol/mL, and 0.1 mL of water; 0.05 mL of each pyrazole derivative solution to achieve the final concentration ranging from 0 to 10^{-8} M, respectively. The solutions were incubated for 30 min at 38 °C, the reaction was then ended by addition of 2 mL of NaOH 0.1 M, and the fluorimetric assay was performed. Pyrazole derivatives were dissolved in dimethyl-sulfoxide (DMSO), added to the reaction mixture, pre-incubated 30 min before adding the appropriate substrate, and then incubated for an additional 30 min to determine enzyme activity. To study the inhibition of pyrazole derivatives on both MAO A and B activities separately, the mitochondrial fractions were pre-incubated for 30 min at 38 °C with the specific inhibitors (L-deprenyl 1 μM to estimate the MAO A activity, and clorgyline 1 μM to assay the isoform B); the samples were then processed as described above in the presence of their specific substrates (serotonin for MAO A and benzylamine for the B). The protein concentration was determined according to Goa.[19] Fluorimetric measurements were recorded with a Perkin–Elmer LS 50B Spectrofluorimeter. The results are reported in Table 2. Dixon plots were used to estimate the inhibition constant (K_i) of the inhibitors. Data are the means of three or more experiments each of them performed in duplicate.
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24. Calculations and graphic manipulations were performed on a Silicon Graphics workstation O2. The software package MacroModel/Batchmin (version 6.0),[25] equipped with the Amber* united atoms force field, was used in this study to perform all the calculations. Solvent effects were not taken into account. The Cartesian atomic co-ordinates of the enzyme utilized for the docking experiments were obtained from the pdb file and used without further refinement by energy minimization. Hydrogen atoms were added only to the heteroatoms of the model. The BatchMin Monte Carlo Multiple Minimum methodology (MCMM)[26] was chosen to carry out flexible docking simulations of (*R*)-**6** and (*S*)-**6**, each one consisting in random rototranslations coupled with a statistical conformational search, inside the active site. Because of the high number of translational, rotational and conformational degrees of freedom, our computational power did not allow a single exhaustive run. Therefore, few consecutive runs (each one made of 1000 Monte Carlo steps) with different starting geometries of the inhibitor and different sequences of random numbers were performed. For each Monte Carlo step the rototranslations were limited by the maximum values of 180° for the rotational angle and of 10 Å for the translational movement. At the same time the five rotatable bonds of **6** were subjected to random step variations of the torsion angles in the range 60–180°. The crystallographic structure of the enzyme was fixed in 3-D-space during the runs. To speed up the calculations an internal region of MAO-B (subset), centered on the 12 residues lining the catalytic site (see the text) and comprising all the aminoacids with at least one atom within a distance of 8.0 Å was considered for the minimizations.[22] The **6**-MAO complexes found in the MCMM procedure were subjected to energy minimization until a derivative convergence of 0.01 kJ/Å-mol was reached. A set of 16 atoms of the inhibitor was selected in order to compare each new minimized output structure with all the previous minima. All the complexes, whose minimum-energy was more than 100.0 kJ/mol over those previously found, were rejected. Two further constraints were imposed in performing energy minimization of the complexes: (a) in addition to the inhibitor, the side chains of 12 residues inside the subset and located on the walls of the active site and isoalloxazine (see the text) were fully minimized to guarantee the complementarity between the surfaces of the two partners; (b) all the other atoms of the internal subset were fixed in 3-D space, even though their non-bonding interactions with all the relaxing atoms were calculated.
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27. In this binding mode, incidentally, an intramolecular H-bond between the pyrazole nitrogen and the OH group in position 2 of the disubstituted phenyl ring of **6** was not retained, which was detected in the lowest energy conformation in the gas phase (see the Graphical Abstract).