

NMR and DFT studies for the tautomeric characterization of a novel quinoxalinone derivative; *In silico* testing against monoamine oxidases

Vasiliki Paschou¹, Filippou Panteleimon Chatzipieris¹, Demeter Tzeli², Stella Manta³, Thomas Mavromoustakos^{1,*}

¹ Laboratory of Organic Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, 15771 Athens, Greece

² Laboratory of Physical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, 15771 Athens, Greece

³ Laboratory of Organic Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

* Correspondence author: tmavrom@chem.uoa.gr

DOI: [10.62579/JAGC0020](https://doi.org/10.62579/JAGC0020)

Abstract

Quinoxalinones are extensively studied in medicinal chemistry, serving as promising pharmacophores with broad biological activity. More specifically, researchers focus on exploring their activity as central nervous system (CNS) agents. An interesting phenomenon that concerns many researchers is that various quinoxalinone analogues are characterized by imine-enamine tautomerism. However, there is limited research on how each tautomeric form acts on a certain biological target. This study investigates the tautomerism of a novel quinoxalinone derivative in conjunction with the *in silico* investigation of its inhibitory effect against monoamine oxidases (MAOs). NMR spectroscopy as well as theoretical DFT calculations were employed to examine the tautomeric equilibrium. A plausible transition state geometry based on DFT computational studies is proposed as well. Additionally, molecular docking simulations and MM/GBSA calculations were carried out to test the inhibition efficacy of the synthesized compound on the MAO enzymes.

Key words

Quinoxalinones, imine-enamine tautomerism, NMR spectroscopy, DFT calculations, transition state, Molecular Docking studies, MM/GBSA, Monoamine oxidase B, Monoamine oxidase A, MAO-B, MAO-A

1. Introduction

Quinoxalinones—a class of nitrogen-containing heteroaromatic compounds—hold great significance in medicinal chemistry [1-7]. They exhibit a broad range of biological properties (Fig. 1), notably acting as potent antiviral (Opavirline) [1], anticancer (Dovitinib), neuroprotective (Caroverine, NBQX) and antiepileptic (Becampanel) agents [2,3]. Several quinoxalinone derivatives exhibit imine-enamine tautomerism, which can be easily studied using both experimental and theoretical methods [2-5].

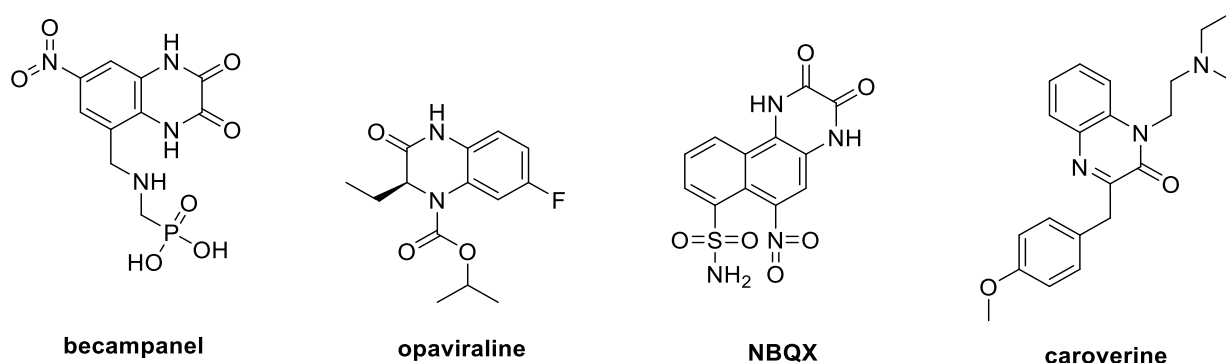


Figure 1. Chemical structures of quinoxalinone-based investigational drugs.

This research describes the tautomeric study of a newly synthesized quinoxalinone derivative, employing NMR spectroscopy and DFT calculations. A plausible transition state, resulting from theoretical DFT calculations, is also suggested. Since these molecules showcase such a diverse mode of action against different types of diseases, including neuroprotection, we decided to test their inhibitory activity against monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B). Both enzymes play an important role in the pathophysiology of Parkinson's [2] and Alzheimer's [3] diseases, as well as depressive disorders [4]. For that purpose, we conducted molecular docking and molecular mechanics/generalized Born surface area (MM/GBSA) simulations on each tautomeric form of the synthesized quinoxalinone.

2. Materials and Methods

2.1 Spectral characterization

NMR analysis

NMR spectra were recorded with an Agilent-500/54 or a Bruker DRX 500 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), using DMSO-*d*₆ as solvent. All ¹H (500 MHz) and ¹³C NMR (125 MHz) spectra were recorded with chemical shifts (ppm) and coupling constants (*J*) in hertz (Hz).

MS analysis

Mass spectra were obtained on a Finnigan, Surveyor MSQ Plus Mass Spectrometer (electrospray ionization).

2.2 Theoretical DFT calculations

DFT calculations were conducted using the Gaussian 16 software suite [5], and the Gauss View 6.0 [6] molecular visualization program. Optimal geometry and transition state calculations were carried out on the B3LYP 6-311+G(d,p) level of theory, using the density functional theory (DFT) Becke-3-Lee Yand Parr (B3LYP) technique with the 6-311+G(d,p) basis set in the ground state [7,8].

2.3 In silico studies

All computational studies (*in silico* docking and MM/GBSA studies) were performed utilizing Maestro 2021-2 software of Schrödinger.

2.3.1. Molecular Modelling

The tautomers studied underwent molecular modelling simulations to evaluate their structural characteristics. The simulations employed molecular docking and binding free energy calculations. By utilizing these techniques, we gained insight into the atomic-level interactions between the selected compounds and the target proteins. Additionally, the simulations enabled the prediction of the binding affinity and binding mode of the compounds.

2.3.2. Target Protein Preparation

Maestro 2021-2 of Schrödinger utilizes different algorithms and scoring functions to simulate molecular binding interactions and is also used for enzymes with metals in their active site. The preparation of each molecular structure was carried out separately using the “Protein Preparation Wizard”, application of the Maestro 2021-2 program from Schrödinger [7]. The molecular structures of the enzymes studied are found deposited in the online database “Protein DataBank-PDB” as monomeric or polymeric structures and can be downloaded directly into the Maestro program. For our studies the PDB: 6FVZ [8] for the crystallized MAO-B was utilized, as well as the PDB: 2Z5X [9] for the crystallized MAO-A. For the preparation of the protein, bond orders were assigned, the CCD database was used, hydrogens were added, the original hydrogens were removed, zero-order bonds to metals were created, as well as disulfide bonds, selenomethionines to methionines were converted, missing side chains and loops were filled in using Prime, the termini were capped, waters were deleted beyond 5 Å from het groups and het states were generated using Epik [10], while the pH was set to physiological range (pH = 7.4). Based on these parameters the protein was pre-processed. Then the workspace was analysed and states were regenerated. Sample water orientations using PROPKA [11] at pH 7.4 were optimized. Finally, the heavy atoms were converged to RMSD 0.30 Å and hydrogens only were allowed to move, to minimize the protein structure. The Force Field used was the Optimized Potentials for Liquid Simulations (OPLS) 4 [12].

2.3.3. Ligand Preparation

Ligands were firstly minimized utilizing minimization (macromodel). Their 3D conformations were saved as .mol2 files and loaded on Maestro’s interface. Then the minimization begun. The force field used was OPLS4 with solvent water. After the minimization of the ligands’ conformations, the ligands were prepared through LigPrep. The Force Field used was OPLS4. The possible ionization states were generated at physiological pH 7.4 using Epik [10], which uses a Hammett and Taft methodology, predicting the ionization states and penalties related to these states. Inclusion of the original state was added, in case the structure generated is not plausible in the pH range chosen. The ligands were desalted, removing counter-ions and disconnected salt fragments from the input structures, leaving only the main neutral organic molecule (the ligand), while tautomer generation was performed. Specified chiralities were retained, while it was chosen for LigPrep to generate at most 15 conformations per ligand. This search generated multiple conformers for each molecule, which were then energetically ranked. The most favourable conformation was selected as input for subsequent docking calculations.

2.3.4. Molecular Docking Studies

Following the preparation of the protein and ligands, induced fit docking was performed using the OPLS4 force field. The grid box was centred on the active site amino acid residues of MAO-A (Tyr407 and Tyr444) [13] and MAO-B (Tyr326, Tyr398, and Tyr435) [14], with the box size set to 46.0 Å. Ring conformations were sampled within a 2.5 kcal/mol energy window. During the initial Glide docking stage, constrained refinement was performed alongside side-chain trimming. The van der Waals scaling factors for both the receptor and ligand were set to 0.50, and the maximum number of poses was limited to 8.

For the subsequent Prime refinement stage, protein residues within 5.0 Å of the docked ligand poses were refined while optimizing the side chains. Following Prime protein refinement, the ligands were redocked into the newly refined receptor structures using Extra Precision (XP) mode. Redocking was constrained within a 30.0 kcal/mol energy window of the top-performing refined structure, keeping the top 8 structures overall. Through this workflow, the most stable conformations for the protein-ligand complexes were generated.

2.3.5. Binding Free Energies Calculated by Molecular Mechanics/Generalized Born Surface Area (MM/GBSA)

The MM/GBSA method offers a rapid and effective approach for estimating the binding free energy between multiple ligands and a protein target [15]. This method combines molecular mechanics calculations, which account for the interactions between the compound and protein, with a Generalized Born solvent model that describes the solvation free energy of the complex. The following equation is used to calculate binding free energy (ΔG_{bind}):

$$\Delta G_{\text{bind}} = \Delta G_{\text{solv}} + \Delta E_{\text{MM}} + \Delta G_{\text{SA}}$$

where ΔG_{solv} is the difference between the complex's GBSA solvation energy and the sum of the unliganded protein and inhibitor solvation energies; ΔE_{MM} is the difference between the sum of the unliganded protein and inhibitor energy and the minimized energies of the complex; and ΔG_{SA} is the difference between the complex's surface area energies and the sum of the unliganded protein and inhibitor's surface area energies. Energies for the optimized complex, free receptor, and free ligand were computed using the Prime MM-GBSA module within Schrödinger. The solvation energy was determined utilizing the Generalized Born model, which estimates the solvation energy based on the molecular surface area and electrostatic potential of the complex. The binding free energy of each studied compound was then estimated employing Schrodinger's Prime module. The VSGB solvation model was used during the simulations. During conformational sampling, residues located within a flexible distance of 5.0 Å from the ligand were allowed to move [16].

3. Results and Discussion

3.1 Characterization of compound SM041

Compound SM041 was fully assigned using a combination of 1D-NMR (^1H , ^{13}C) and 2D-NMR (^1H - ^1H COSY, ^1H - ^1H NOESY, ^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC) experiments. Both tautomeric forms of the synthesized derivative were observed in the NMR spectra (Fig. 2), and the structure of each tautomer was separately elucidated.

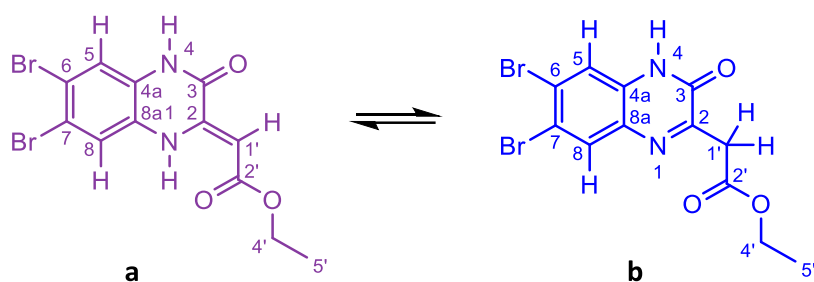
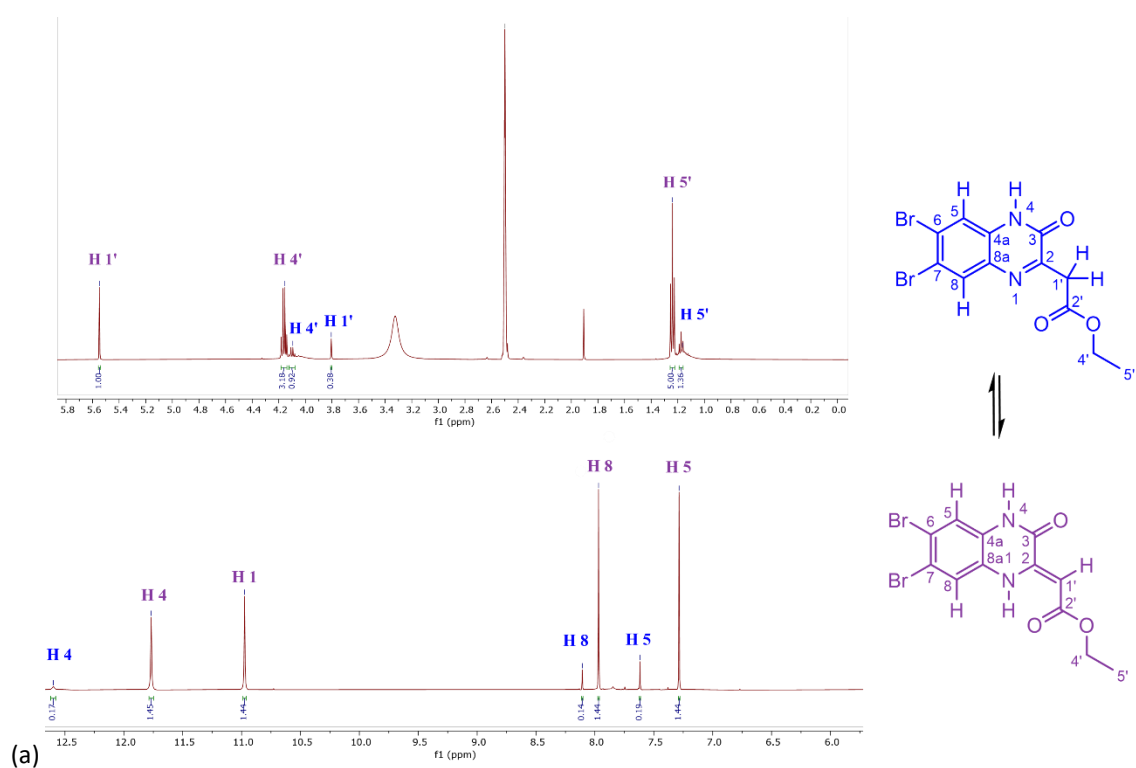


Figure 2. Tautomeric forms of the synthesized quinoxalinone SM041; enaminooester **SM041a** in pink, iminoester **SM041b** in blue.

Assigned ^1H - and ^{13}C -NMR spectra are depicted in Fig. 3, whereas the strategy used for the structure elucidation of each tautomer is outlined in Scheme 1.



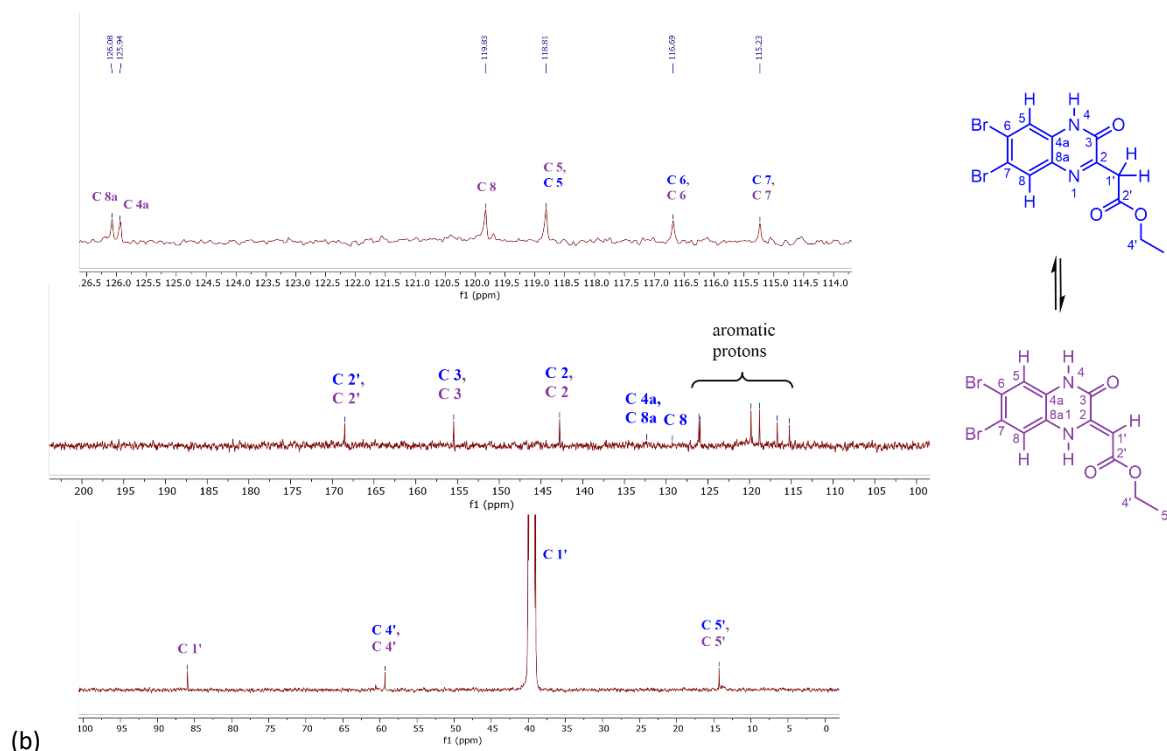
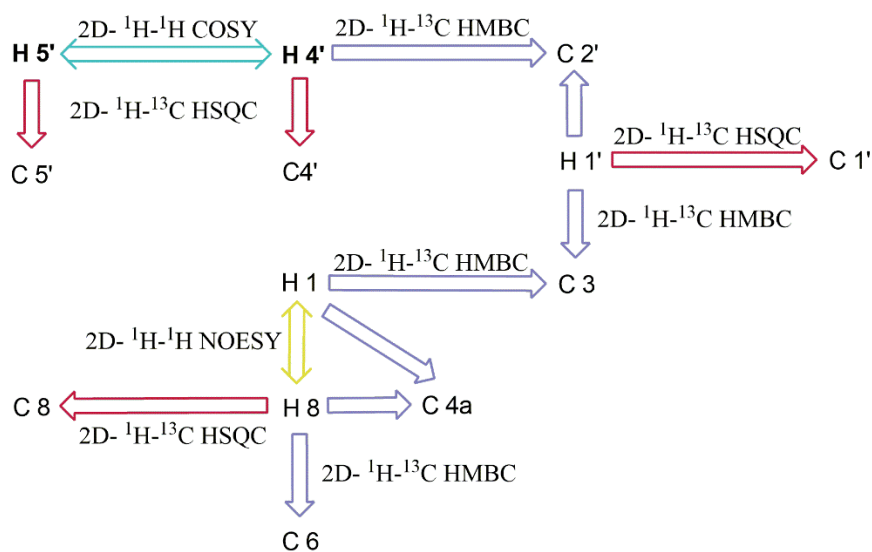


Figure 3. (a) Assigned ^1H -NMR spectrum (500 MHz) for compound SM041 in $\text{DMSO}-d_6$. Enaminoester **SM041a** in pink and iminoester **SM041b** in blue, (b) Assigned ^{13}C -NMR spectrum (125 MHz) for compound SM041 in $\text{DMSO}-d_6$. Enaminoester **SM041a** in pink and iminoester **SM041b** in blue.

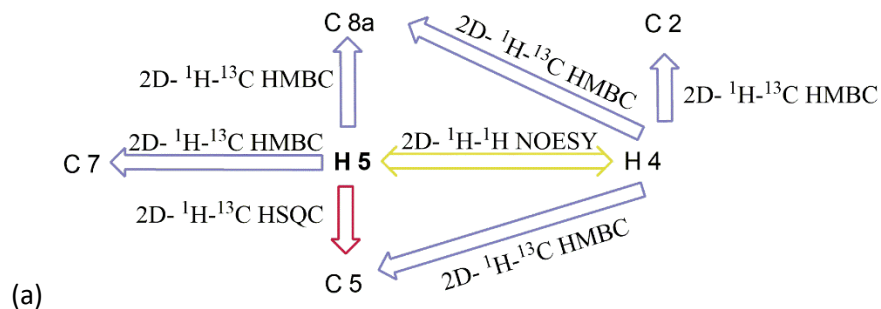
Initial characterization focused on the enaminoester structure. In the ^1H NMR spectrum (Fig. 3a), a triplet at δ 1.24 (methyl protons H-5') and a quartet at δ 4.15 (methylene protons H-4') showed vicinal coupling, which was confirmed by COSY cross-peaks. The corresponding carbons, C-4' (δ 59.33) and C-5' (δ 14.29), were assigned *via* HSQC (Fig. 3b), while an HMBC correlation from H-4' identified the C-2' position (δ 168.52). The enamine tautomer was identified by a characteristic singlet at δ 5.55 (^1H NMR), assigned to the vinylic proton H-1' based on its HMBC correlation with C-2'. Further HSQC and HMBC correlations of H-1' revealed C-1' (δ 85.95) and C-3 (δ 155.46) (Fig. 3b).

Singlets at δ 10.98 and δ 7.97 were assigned to the enamine proton H-1 and the aromatic proton H-8, respectively. This assignment was supported by an HMBC correlation between H-1 and C-3, alongside NOESY cross-peaks between H-1 and H-8. The carbon signal at δ 125.94 was assigned to C-4a due to HMBC correlations with both H-1 and H-8. In the ^{13}C NMR spectrum (Fig. 3b), C-8 (δ 119.83) and C-6 (δ 116.69) were resolved *via* HSQC and HMBC correlations with H-8. In the ^1H NMR spectrum, the amide proton H-4 and aromatic proton H-5 appeared as singlets at δ 11.77 and δ 7.29, respectively, with their proximity validated by NOESY cross-peaks. The lack of an HSQC signal for H-4 confirmed the absence of a carbon atom to its environment, whereas H-5 exhibited an HSQC correlation to C-5 (δ 118.81), which was further corroborated by an HMBC signal from H-4. Moreover, HMBC correlations from both H-4 and H-5 successfully identified C-8a (δ 126.08). Finally, C-2 (δ 142.77) was assigned based on an HMBC correlation with H-4, while a separate HMBC correlation from H-5 successfully revealed the C-7 signal (δ 115.23) (Fig. 3b).

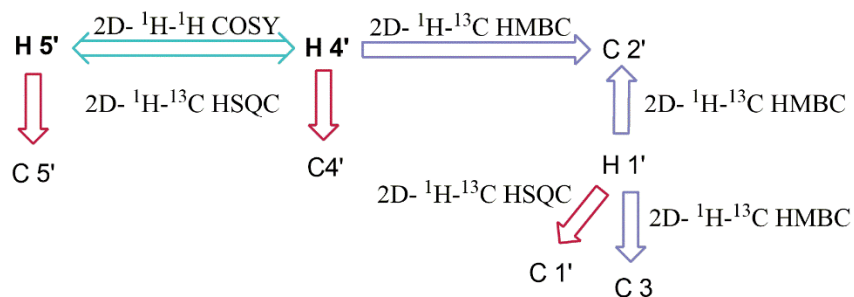
1st series of steps



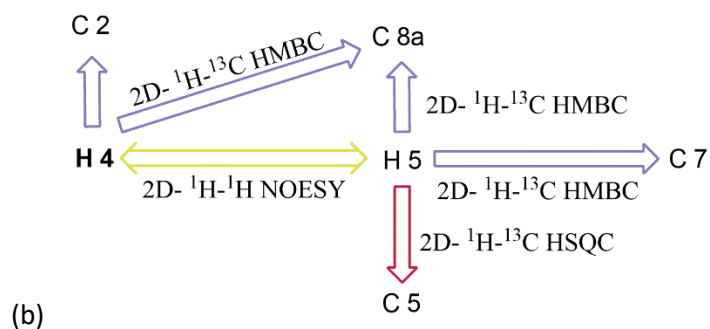
2nd series of steps



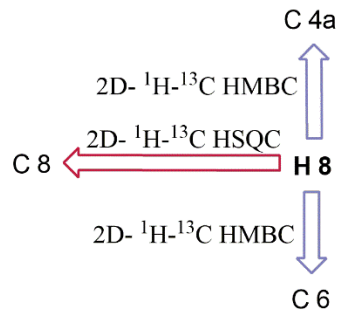
1st series of steps



2nd series of steps



3rd series of steps



Scheme 1. Rationale of the structural elucidation of SM041; (a) enaminoester **SM041a**, (b) iminoester **SM041b**

Subsequent characterization of the iminoester tautomer began with the identification of methyl protons H-5' and methylene protons H-4', which appeared as a triplet at δ 1.17 and a quartet at δ 4.09, respectively (Fig. 3a). Their mutual coupling was verified by COSY cross-peaks, while the corresponding C-4' (δ 59.33) and C-5' (δ 14.29) signals (Fig. 3b) were assigned *via* HSQC correlations with H-4' and H-5', respectively. The carbonyl carbon C-2' (δ 168.52) was identified through an HMBC correlation with H-4'. A key characteristic of the imine tautomer was a singlet peak integrating to two protons at δ 3.81 (^1H NMR), which was assigned to the methylene group H-1' based on its HMBC interaction with C-2'. Consequently, the C-1' (δ 40.00) and C-3 (δ 155.46) carbons were mapped using HSQC and HMBC cross-peaks from H-1', respectively.

The amide proton H-4 (singlet at δ 12.60), aromatic proton H-5 (δ 7.62), and carbons C-2 (δ 142.77), C-5 (δ 118.81), C-7 (δ 115.23), and C-8a (δ 132.33) were elucidated following the same logic as the enaminoester. The final proton signal was assigned to the aromatic proton H-8 (δ 8.11), which was coupled to C-8 (δ 129.25) as confirmed by HSQC. Furthermore, HMBC correlations from H-8 successfully resolved the C-6 (δ 116.69) and C-4a (δ 132.33) positions. Notably, carbons C-6 and C-7 exhibited identical chemical shifts across both tautomeric forms. This consistency is structurally expected, given their distance from the dynamic tautomeric center.

Chemical shifts of all protons and carbon atoms are summarized in Table 1.

Table 1. Chemical Shifts of protons and carbons of compound SM041 expressed in ppm.

<i>Enaminoester</i>			<i>Iminoester</i>		
<i>Position</i>	^1H (ppm)	^{13}C (ppm)	<i>Position</i>	^1H (ppm)	^{13}C (ppm)
1	10.98	-	1	-	-
2	-	142.77	2	-	142.77
3	-	155.46	3	-	155.46
4	11.78	-	4	12.62	-
4a	-	125.94	4a	-	132.33
5	7.28	118.81	5	7.62	118.81
6	-	116.69	6	-	116.69
7	-	115.23	7	-	115.23
8	7.97	119.83	8	8.11	129.25
8a	-	126.08	8a	-	132.33
1'	5.55	85.95	1'	3.80	40.00
2'	-	168.52	2'	-	168.52
4'	4.15	59.33	4'	4.09	59.33
5'	1.24	14.29	5'	1.17	14.29

Additional information was obtained *via* mass spectrometry. The mass spectrum of compound SM041, as depicted in Fig. 4, confirms the molecular weight and important moieties of the molecular structure of the quinoxalinone derivative. More specifically, the basic peak (m/z = 388) corresponds to the molecular ion $[\text{M}-\text{H}]^+$. Furthermore, the fragments $[\text{M}-73]^+$ and $[\text{M}-45]^+$ correspond to the loss of an ethoxycarbonyl ($[\text{M}-\text{COOCH}_2\text{CH}_3]^+$) and an ethoxy group ($[\text{M}-\text{OCH}_2\text{CH}_3]^+$), respectively, which are characteristic for the compound's structure.

SM041_ESI-50_t500 #1-22 RT: 0.00-0.71 AV: 22 NL: 1.99E4
T: {0,0} - p ESI !corona sid=50.00 det=1306.00 Full ms [150.00-850.00]

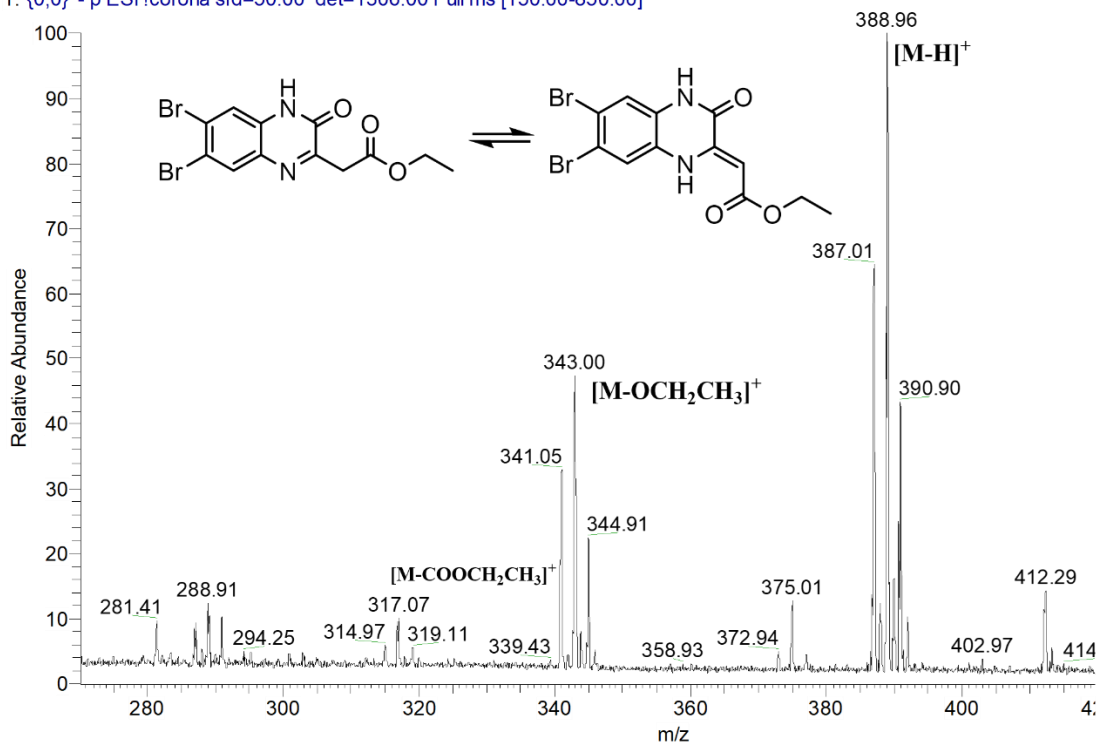


Figure 4. MS spectrum (ESI, -50 eV, 500 K) for compound SM041 in DMSO- d_6 .

NMR tautomeric analysis of compound SM041

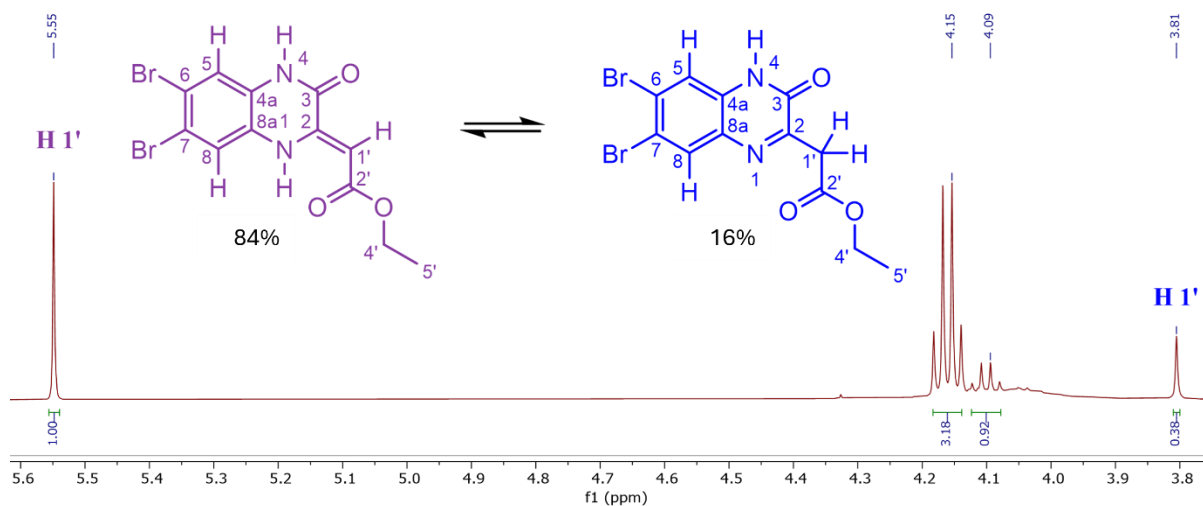


Figure 5. (a) Assigned ^1H -NMR spectrum (500 MHz) for compound SM041 in DMSO- d_6 , zoomed in tautomeric protons region. Enaminoester **SM041a** in pink and iminoester **SM041b** in blue.

It is evident that both tautomers can be observed in DMSO- d_6 , which indicates a slow tautomeric exchange between the enaminoester **SM041a** and iminoester **SM041b**. The presence of both tautomers can be attributed to the high stability of their aromatic bicyclic quinoxalinone skeleton.

As can be observed in Fig. 5, the enaminoester: iminoester tautomeric ratio for compound SM041 is 84: 16.

The fact that enaminoester **SM041a** is the major tautomer can be explained by the formation of an intramolecular hydrogen bond between the enamine proton and the ester carbonyl group, as illustrated in Fig. 6. As a result, the energy of tautomer **a** is not only further decreased, but the enamine proton (H-1) is also highly deshielded due to the hydrogen bond. The latter explains its high chemical shift (δ 10.98).

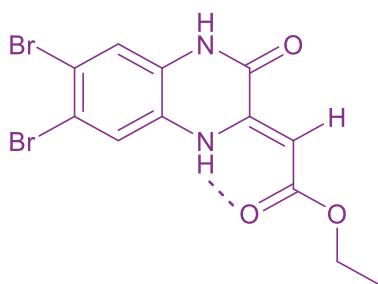


Figure 6. Intramolecular hydrogen bond formation of enaminoester **a**.

3.2 Tautomeric study *via* DFT

Two different series of theoretical DFT calculations were carried out at the B3LYP/6-311+G(d,p) level of theory; geometry optimization and transition state prediction. DMSO was modeled as the solvent across all calculations using an implicit solvation method.

Optimal geometries

Firstly, the geometry of each tautomer was optimized, as shown in Fig. 7. Both tautomers are characterized by a bicyclic aromatic system that stabilizes each structure. Additionally, DFT results of the enaminoester evidently support the experimental findings; the formation of an intramolecular hydrogen bond is favored in the tautomer's optimal conformation.

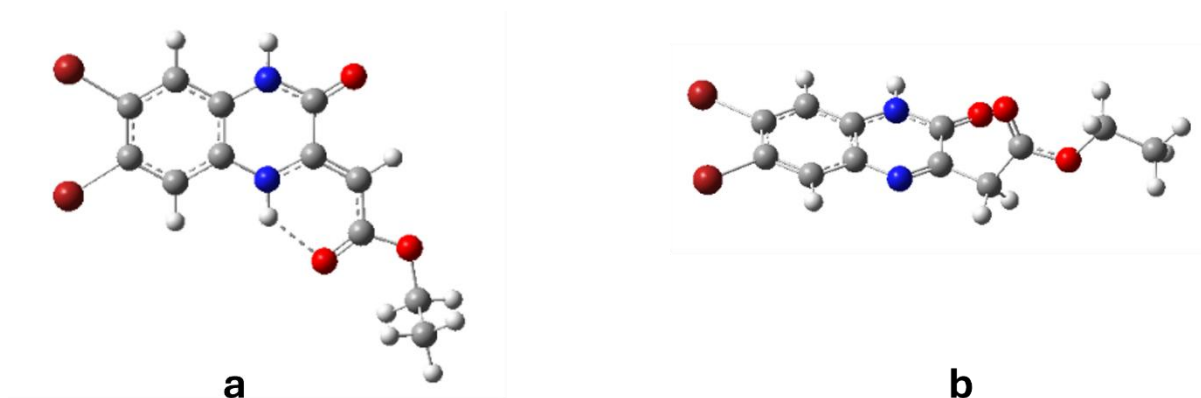
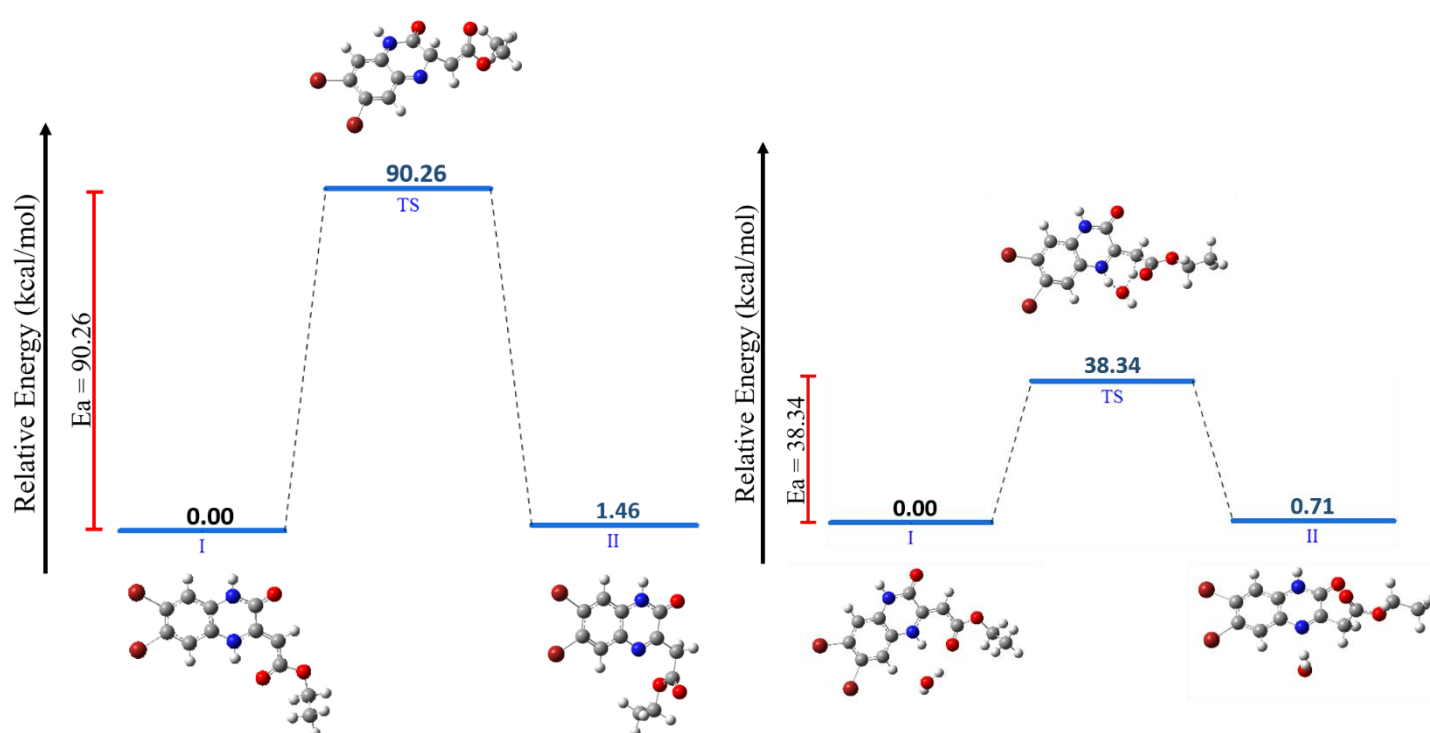


Figure 7. Optimal geometries of the two tautomeric forms for compound SM041; enaminoester **SM041a** (left) iminoester **SM041b** (right).

Transition State

Two different tautomeric mechanisms were investigated, and two transition states were, therefore, calculated. On the one hand a non-catalysed intramolecular proton exchange was examined. On the other hand, a water-mediated tautomeric mechanism was explored. In each case, the proton exchange took place between the enamine proton (H-1) of the enamino-tautomer and the methylene proton (H-1') of the imino-tautomer. The energy diagrams of both cases are depicted in Scheme 2.

Results indicated that the energy barrier for the intramolecular tautomeric mechanism is very high (90.26 kcal/mol), indicating that this transition state is energetically unfavourable in the quinoxalinone ground state. However, the water molecule in the catalysed mechanism significantly lowers the activation energy (38.34 kcal/mol). Nonetheless, the energy barrier remains notably high and this explains why tautomerism is slow on the NMR timescale. Thus, it can be concluded that the residual water of DMSO- d_6 solvent catalyses the proton exchange between the tautomers.



Scheme 2. Energy diagrams of the non-catalysed (left) and catalysed (right) transition state of compound SM041. TS; transition state I; enaminoester (a) II; iminoester (b)

3.3 Molecular Docking analysis

Given the fact that quinoxalinones play an intrinsic part in a variety of pathological conditions, offering anticancer and neuroprotective effects, we decided to study their ability to bind to two important enzymes implicated in Alzheimer's and Parkinson's diseases, as well as depressive disorders, monoamine oxidases (MAOs). Monoamine oxidases exist in two isoforms MAO-A and MAO-B [3], and both play a critical role in the oxidative deamination of monoamines and neurotransmitters leading to neuronal oxidative damage and death [17]. Due to the important role of MAO-B's deregulation in Parkinson's [18] and Alzheimer's diseases [3], as well as the implications of MAO-A in depression and anxiety disorders [4], the affinities of the synthesized compound for these drug targets were tested *in silico*. Computational docking studies were conducted utilizing Maestro 2021-2 software from Schrödinger, following an induced fit protocol, allowing flexibility in the amino acid side chains, to properly accommodate the ligand within the studied cavities. In particular, tautomers **SM041a** (enamine) and **SM041b** (imine) were anchored in the active sites of both enzymes. The highest affinity conformations of every tautomer for each target are analysed below.

Close examination of the docking results showcases the tautomers' affinity for each target. The catalytic site of MAO-B is described by amino acid residues Tyr326, Tyr398 and Tyr435 [14]. When anchored to MAO-B's active site, **SM041a** forms a hydrogen bond with Cys172, while establishing hydrophobic interactions with Tyr326, Leu328, Tyr398, Tyr60, Tyr435, Leu171, Ile198, Ile199, Phe168, Leu167, Pro102, Pro104 and Ile316 (Fig. 8). The exact same interactions are observed for **SM041b** within the catalytic site (Fig. 8). These results are compared to known MAO-B inhibitor and drug against Parkinson's disease, rasagiline [19]. Rasagiline is an irreversible inhibitor of MAO-B, covalently bound to the flavin adenine dinucleotide (FAD) (Fig. 9). FAD acts as a co-factor within the active site [20]. To bind irreversibly to FAD, rasagiline must first be stabilized within the active site cavity (Fig. 9).

Therefore, the stabilization of the non-covalent conformation is compared to those of **SM041a** and **SM041b**. As can be observed in Figure 9, rasagiline positions its terminal propargylamine group in close proximity to the FAD ring, allowing covalent binding to occur. The compound is stabilized through hydrogen bonding with Ile199 and non-polar interactions with Ile198, Leu171, Phe168, Leu167, Leu164, Pro104, Phe103, Pro102, Tyr326 and Phe99. Consequently, the newly synthesized **SM041a** and **SM041b** interact with the same amino acid residues as rasagiline. The minor differences in their binding modes, however, exert a profound impact on their binding free energy, as discussed below. Notably the interaction with Cys172 provides greater stabilization for both the enamine and imine compounds when compared to rasagiline's interaction with Ile199. Specifically, residue Cys172 forms a hydrogen bond with the keto group of the pyrazine moiety in both tautomeric structures.

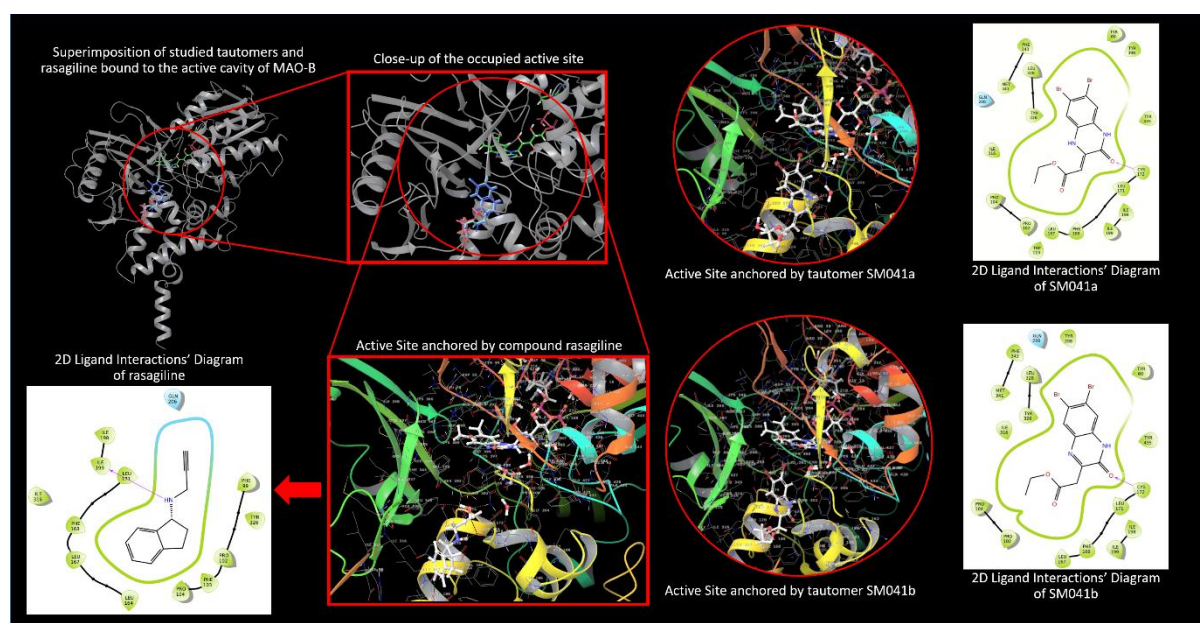


Figure 8. Top left: Superimposition of tautomers **SM041a** (enamine) and **SM041b** (imine) and compound rasagiline in the active site of monoamine oxidase B (MAO-B) (PDB: 6FVZ). The enamine tautomer is shown in blue, while the imine tautomer as well as the reference compound rasagiline are shown in light blue and red colour, respectively. Top and bottom right: Representation of the enamine and imine tautomers docked at the active site of MAO-B. The interactions between the ligands and the amino acid residues of the active site are presented in the 3D complex structures and 2D ligand interactions' diagrams. Bottom left: 3D complex structures and the 2D ligand interactions' diagrams for known drug rasagiline, irreversible inhibitor of MAO-B, before its covalent binding to flavin adenine dinucleotide (FAD) co-factor. Purple arrows symbolize hydrogen bonds, while light blue and green residues symbolize polar and hydrophobic residues, respectively.

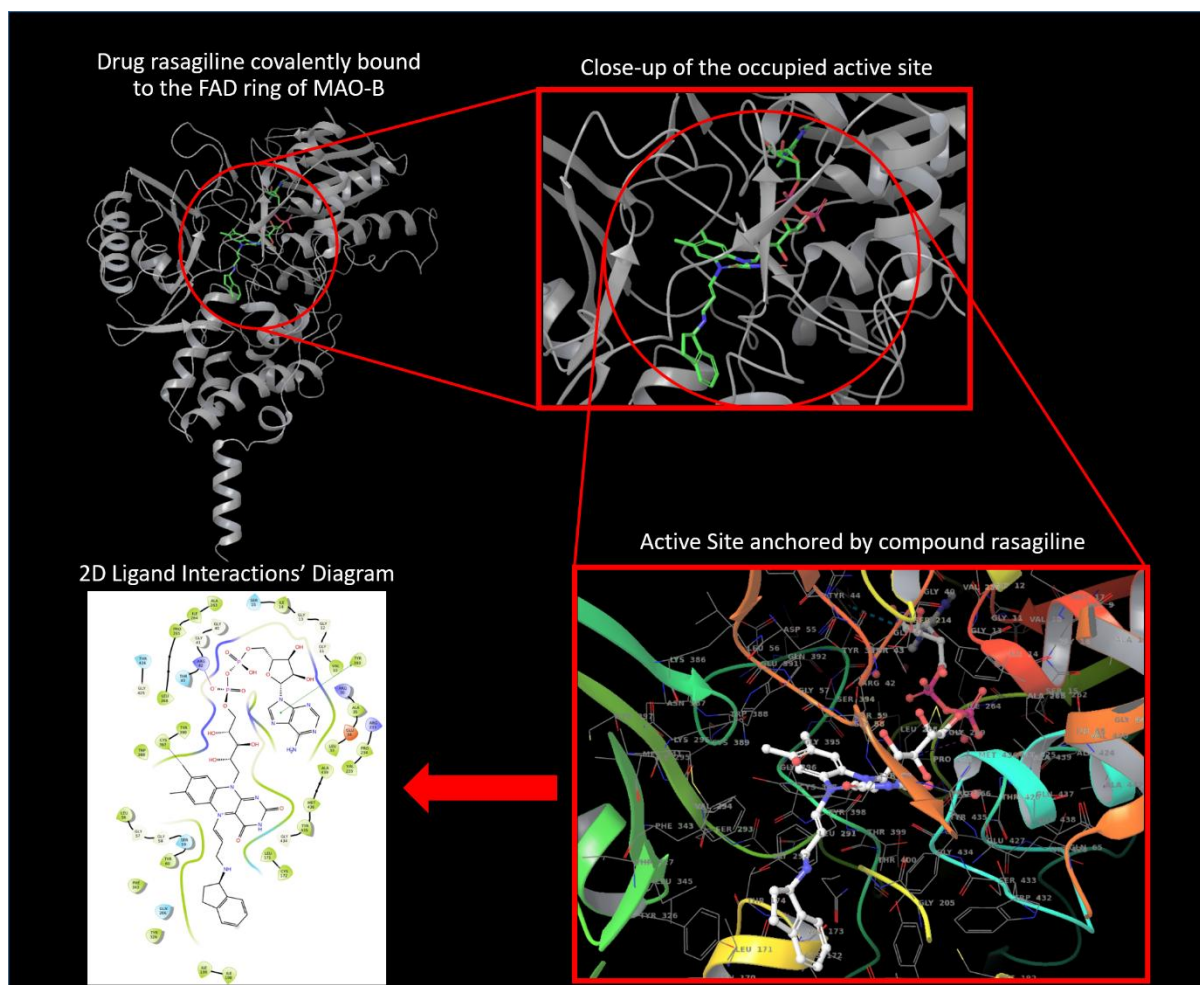


Figure 9. Representation of rasagiline covalently bound to flavin adenine dinucleotide (FAD) co-factor of the MAO-B enzyme (PDB: 2XFQ). The interactions between the ligand and the amino acid residues of the active site are presented in the 3D complex structure (bottom right) and the 2D ligand interactions' diagram (bottom left). As it is presented, its inhibitory potency derives mainly through the covalent bonding to the FAD ring, with a limited number of amino acids stabilizing it through hydrophobic interactions (Ile199, Ile198, Tyr326 and Tyr60). Green arrows symbolize π - π stacking interactions, purple arrows symbolize hydrogen bonds and deep blue-red arrows symbolize salt bridges, while light blue, green, deep blue and red amino acid residues symbolize polar, hydrophobic, positively and negatively charged residues, respectively.

Next, the molecular interactions between the **SM041a** and **SM041b** tautomers, and the catalytic cavity of MAO-A were examined (Fig. 10). The active site of MAO-A is described by the amino acid residues Tyr407, and Tyr444 [13]. Regarding MAO-A, both compounds were capable of developing a vast network of interactions when compared to the **SM041** – MAO-B complexes. Tautomer **SM041a** developed a hydrogen bond with amino acid residue Asn181 *via* a water molecule. Moreover, it could form two halogen bonds with amino acid residues Met350 and Lys305. **SM041a** was further stabilized *via* hydrophobic interactions with amino acid residues Phe352, Leu337, Ile335, Ile180, Val210, Phe208, Ile207 and Tyr69 and polar interactions with Thr336 and Gln215. On the other hand, **SM041b** was capable of forming hydrogen bonds with Gln215 *via* its carbonyl group of the ester moiety and Tyr407 *via* its amide group of the pyrazine moiety, a π - π interaction between Tyr59 and the compound's benzyl ring, as well as a halogen bond with Met350. These results are compared to the reference drug moclobemide. Moclobemide is a reversible inhibitor of MAO-A and has been extensively evaluated in

the treatment of a wide spectrum of depressive disorders and less extensively studied in anxiety disorders [21]. As shown in Figure 10, Phe208 interacts *via* hydrogen bonding with the nitrogen atom of the morpholine ring *via* a water molecule, while Asn181 forms a hydrogen bond as well with the amide group of moclobemide again through the mediation of a water molecule. Moreover, the chlorobenzene ring of the compound interacts *via* π - π stacking with amino acid residue Phe352. The molecule is further stabilized by hydrophobic interactions with residues Met350, Tyr407, Ile180, Pro113, Phe112, Ala111, Phe173, Leu97, Phe108, Ile335, Leu337 and Tyr69. Based on these results, it seems that tautomers **SM041a** and **SM041b** showcase a different mode of binding when compared to moclobemide, which interacts with different amino acid residues. These differences are directly translated to significant differences in binding free energies, as it will be discussed later.

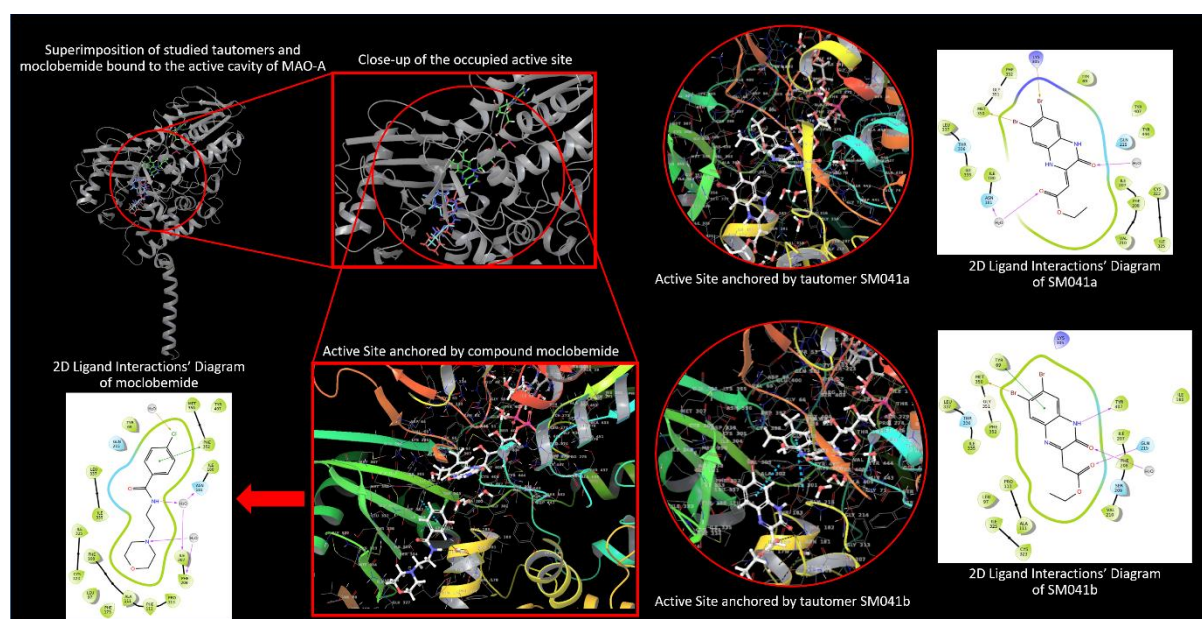


Figure 10. Top left: Superimposition of tautomers **SM041a** (enamine) and **SM041b** (imine) in the active site of monoamine oxidase A (MAO-A) (PDB: 2Z5X). The enamine tautomer is shown in blue, while the imine tautomer as well as the reference compound moclobemide is shown in light blue and red colour, respectively. Top and bottom right: Representation of the enamine and imine tautomers docked at the active site of MAO-A. The interactions between the ligands and the amino acid residues of the active site are presented in the 3D complex structures and 2D ligand interactions' diagrams. Bottom left: 3D complex structures and the 2D ligand interactions' diagrams for known drug moclobemide, reversible inhibitor of MAO-A. Green arrows symbolize π - π stacking interactions, purple arrows symbolize hydrogen bonds, while light blue, green and deep blue amino acid residues symbolize polar, hydrophobic and positively charged residues, respectively.

Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) calculations

In order to assess the binding free energy (ΔG_{bind}) of the tautomers and reference compounds for the respective target, molecular mechanics/Generalized Born surface area (MM/GBSA) calculations were conducted. Both tautomers showcase the same or even greater binding affinity for each target, when compared with reference compound rasagiline for MAO-B and moclobemide for MAO-A (Scheme 3). Specifically, both **SM041a** ($\Delta G_{\text{bind}} = -52.01$ kcal/mol) and **SM041b** ($\Delta G_{\text{bind}} = -53.28$ kcal/mol) exhibited much higher affinity for MAO-B enzyme when compared to rasagiline ($\Delta G_{\text{bind}} = -37.76$ kcal/mol). Regarding the affinity for MAO-A, **SM041a** displayed the lowest free energy value ($\Delta G_{\text{bind}} = -69.38$

kcal/mol), while **SM041b** ($\Delta G_{\text{bind}} = -52.10$ kcal/mol) and moclobemide ($\Delta G_{\text{bind}} = -51.42$ kcal/mol), were of the same order of magnitude. This indicates the possible use of these tautomers as MAO-A and MAO-B inhibitors. Further *in vitro* studies are of interest for these molecules' inhibitory potency.



Scheme 3. Bar graph of the docking scores (blue) as well as free binding energies (orange) of tautomers **SM041a** and **SM041b** compared with rasagiline and moclobemide for the active site of MAO-B and MAO-A, expressed in kcal/mol.

In Table 2, component analysis for the ligand-protein complexes following MM/GBSA binding free energy calculations, are depicted. SM041a showcases the most favourable predicted binding energy ($\Delta G_{\text{bind}} = -69.38$ kcal/mol), indicating the strongest binding to MAO-A among the three compounds. SM041b and moclobemide have very similar total MM-GBSA binding energies (-52.10 and -51.42 kcal/mol, respectively). SM041a benefits from the strongest van der Waals, packing, and overall hydrophobic interactions, while also incurring the smallest polar solvation penalty, making these the primary contributors to its superior binding. Moclobemide exhibits the strongest electrostatic

(Coulomb) and lipophilic contributions; however, these favourable interactions are largely offset by a large unfavourable polar solvation energy, resulting in an overall binding energy comparable to SM041b rather than SM041a. Although SM041a induces the largest receptor strain, the substantial gains in favourable intermolecular interactions more than compensate for this energetic penalty.

Regarding MAO-B, both SM041a and SM041b showcase similar binding affinities ($\Delta G_{\text{bind}} = -52.01$ and -53.28 kcal/mol, respectively). Both molecules benefit from strong van der Waals and Coulomb interactions, when compared to rasagiline. Moreover, rasagiline exhibits the strongest lipophilic contributions. Even though SM041a and SM041b suffer from a high polar solvation penalty, compared to rasagiline, the remaining energy components highly favour and stabilize these complexes.

Table 2. Component analysis for the ligand-protein complexes following MM/GBSA binding free energy calculations

Monoamine Oxidase B (MAO-B)									
<i>Name/ΔG</i>	<i>bind</i>	<i>vdW</i>	<i>Coulomb</i>	<i>Solvation</i>	<i>Lipophilic</i>	<i>Hbond</i>	<i>Packing</i>	<i>Ligand strain</i>	<i>Receptor strain</i>
SM041a	-52.01	-51.82	-10.91	+26.28	-16.73	-0.26	-2.52	+1.85	+4.27
SM041b	-53.28	-51.44	-11.37	+24.38	-18.46	-1.05	-0.78	+4.07	+2.12
rasagiline	-37.76	-33.34	-3.79	+17.45	-22.48	-0.68	-0.68	+3.30	+1.70
Monoamine Oxidase A (MAO-A)									
<i>Name/ΔG</i>	<i>bind</i>	<i>vdW</i>	<i>Coulomb</i>	<i>Solvation</i>	<i>Lipophilic</i>	<i>Hbond</i>	<i>Packing</i>	<i>Ligand strain</i>	<i>Receptor strain</i>
SM041a	-69.38	-44.35	-16.24	+16.44	-23.62	-1.60	-6.02	+3.20	+7.47
SM041b	-52.10	-38.49	-21.24	+27.52	-19.77	-1.61	-3.31	+3.97	+5.87
moclobemide	-51.42	-25.24	-23.11	+28.22	-27.11	-0.99	-1.95	+4.16	+2.94

4. Conclusions

The primary aim of this study was the tautomeric investigation of the synthesized quinoxalinone derivative. Spectroscopic results indicate the presence of both imino- and enamino-tautomer in dms- d_6 solution, alongside the predominance of enaminoester. The latter can be justified by the intramolecular hydrogen bonding that is prominent in this structure. Moreover, computational data are in accordance with the experimental ones, and they were further proved as an efficient tool to investigate plausible transition state mechanisms. Finally, molecular docking simulations provided insights into the distinct binding modes of each tautomer and revealed how their interactions with the enzyme may differ. Specifically, it was found that while both tautomers exhibit almost identical binding to MAO-B, the binding mode of each tautomeric form differs in the case of MAO-A. In both cases, however, they show greater binding affinity than the reference drugs.

5. Author Contributions

Visualization, conceptualization, investigation, project administration, review and editing, supervision, T.M.; Synthetic methodology, S.M. and V.P.; general computational and DFT methodology, writing — review and editing, V.P., F.P.C., D.T.

6. Declaration of Competing Interest

None declared.

7. Data availability

Data will be made available on request.

8. References

- [1] X. Jiang, K. Wu, R. Bai, P. Zhang, and Y. Zhang, “Functionalized quinoxalinones as privileged structures with broad-ranging pharmacological activities,” Feb. 05, 2022, *Elsevier Masson s.r.l.* doi: 10.1016/j.ejmech.2021.114085.
- [2] A. Markham, “Erdafitinib: First Global Approval,” *Drugs*, vol. 79, no. 9, pp. 1017–1021, Jun. 2019, doi: 10.1007/s40265-019-01142-9.
- [3] P. Lampertico *et al.*, “Real-world effectiveness and safety of glecaprevir/pibrentasvir for the treatment of patients with chronic HCV infection: A meta-analysis,” *J. Hepatol.*, vol. 72, no. 6, pp. 1112–1121, Jun. 2020, doi: 10.1016/j.jhep.2020.01.025.
- [4] J. G. Taylor *et al.*, “Discovery of the pan-genotypic hepatitis C virus NS3/4A protease inhibitor voxilaprevir (GS-9857): A component of Vosevi®,” *Bioorg. Med. Chem. Lett.*, vol. 29, no. 16, pp. 2428–2436, Aug. 2019, doi: 10.1016/j.bmcl.2019.03.037.
- [5] H. Gao, E. F. Yamasaki, K. K. Chan, L. L. Shen, and R. M. Snapka, “DNA Sequence Specificity for Topoisomerase II Poisoning by the Quinoxaline Anticancer Drugs XK469 and CQS,” *Mol. Pharmacol.*, vol. 63, no. 6, pp. 1382–1388, Jun. 2003, doi: 10.1124/mol.63.6.1382.
- [6] P. Sukanya and C. V. R. Reddy, “Structural investigation, DNA interactions and in vitro anticancer studies of transition metal complexes of 3-(2-(2, 4-dihydroxy benzylidene) hydrazinyl) quinoxalin-2(1H) -one,” *J. Biomol. Struct. Dyn.*, vol. 40, no. 13, pp. 6151–6162, 2022, doi: 10.1080/07391102.2021.1877819.
- [7] N. Kaloğlu, M. Boğa, S. Ercan, E. Çınar, Y. Bektaşlı, and M. Kaloğlu, “Synthesis, Characterization, Anticholinesterase, and Antioxidant Properties of Quinoxalin-2(1H)-One Functionalized (Benz)imidazol-3-ium Salts as Hybrid Molecules in Drug Discovery,” *ChemistrySelect*, vol. 11, no. 6, Feb. 2026, doi: 10.1002/slct.202506425.
- [8] K. H. P. Moore *et al.*, “Pharmacokinetics and Tolerability of GW420867X, a Nonnucleoside Reverse Transcriptase Inhibitor, following Single Escalating Doses in Healthy Male Volunteers,” *The Journal of Clinical Pharmacology*, vol. 41, no. 10, pp. 1098–1105, Oct. 2001, doi: 10.1177/00912700122012706.

- [9] F. Ye *et al.*, "Design, synthesis, and biological evaluation of 3-vinyl-quinoxalin-2(1H)-one derivatives as novel antitumor inhibitors of FGFR1," *Drug Des. Devel. Ther.*, p. 1489, May 2016, doi: 10.2147/DDDT.S88587.
- [10] C. Porta, P. Giglione, W. Liguigli, and C. Paglino, "Dovitinib (CHIR258, TKI258): Structure, Development and Preclinical and Clinical Activity," *Future Oncology*, vol. 11, no. 1, pp. 39–50, Jan. 2015, doi: 10.2217/fon.14.208.
- [11] V. A. Mamedov, "Quinoxaline—As a Parent Heterocycle," in *Quinoxalines*, Springer International Publishing, 2016, pp. 1–4. doi: 10.1007/978-3-319-29773-6_1.
- [12] P. L. Follett, P. A. Rosenberg, J. J. Volpe, and F. E. Jensen, "NBQX Attenuates Excitotoxic Injury in Developing White Matter," *The Journal of Neuroscience*, vol. 20, no. 24, pp. 9235–9241, Dec. 2000, doi: 10.1523/JNEUROSCI.20-24-09235.2000.
- [13] W. Jahnke *et al.*, "A General Strategy for Targeting Drugs to Bone," *Angew. Chem. Int. Ed.*, vol. 54, no. 48, pp. 14575–14579, Nov. 2015, doi: 10.1002/anie.201507064.
- [14] W. Suter, A. Hartmann, F. Poetter, P. Sagelsdorff, P. Hoffmann, and H.-J. Martus, "Genotoxicity assessment of the antiepileptic drug AMP397, an Ames-positive aromatic nitro compound," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 518, no. 2, pp. 181–194, Jul. 2002, doi: 10.1016/S1383-5718(02)00105-5.
- [15] X. Jie, Y. Shang, Z. N. Chen, X. Zhang, W. Zhuang, and W. Su, "Differentiation between enamines and tautomerizable imines in the oxidation reaction with TEMPO," *Nat. Commun.*, vol. 9, no. 1, Dec. 2018, doi: 10.1038/s41467-018-07534-x.
- [16] Z. Rappoport, *The chemistry of enamines Part 2*. 9780471933397, 1994.
- [17] E. Kolehmainen, B. Ośmiałowski, T. M. Krygowski, R. Kauppinen, M. Nissinen, and R. Gawinecki, "Substituent and temperature controlled tautomerism: Multinuclear magnetic resonance, X-ray, and theoretical studies on 2-phenacylquinolines," *Journal of the Chemical Society. Perkin Transactions 2*, no. 6, pp. 1259–1266, 2000, doi: 10.1039/a908874a.
- [18] E. Kleinpeter, "NMR Spectroscopic Study of Tautomerism in Solution and in the Solid State," in *Tautomerism*, Wiley, 2013, pp. 103–143. doi: 10.1002/9783527658824.ch5.
- [19] L. Dezsai and L. Vecsei, "Monoamine Oxidase B Inhibitors in Parkinson's Disease," *CNS Neurol. Disord. Drug Targets*, vol. 16, no. 4, Jul. 2017, doi: 10.2174/1871527316666170124165222.
- [20] F. P. Chatzipieris *et al.*, "New Prospects in the Inhibition of Monoamine Oxidase-B (MAO-B) Utilizing Propargylamine Derivatives for the Treatment of Alzheimer's Disease: A Review," *ACS Omega*, vol. 10, no. 25, pp. 26208–26232, Jul. 2025, doi: 10.1021/acsomega.5c00134.
- [21] M. Naoi, W. Maruyama, and M. Shamoto-Nagai, "Type A monoamine oxidase and serotonin are coordinately involved in depressive disorders: from neurotransmitter imbalance to impaired neurogenesis," *J. Neural Transm.*, vol. 125, no. 1, pp. 53–66, Jan. 2018, doi: 10.1007/s00702-017-1709-8.
- [22] M. J. , Frisch *et al.*, "Gaussian 16 Revision A. 03," 2016, *Gaussian Inc. Wallingford CT*, 1(2).
- [23] T. A. K. and J. M. M. Roy Dennington, "GaussView, Version 6.0.16," 2016, *Semichem Inc., Shawnee Mission, KS*.

- [24] K. Raghavachari, "Perspective on 'Density functional thermochemistry. III. The role of exact exchange,'" *Theoretical Chemistry Accounts: Theory, Computation, and Modeling (Theoretica Chimica Acta)*, vol. 103, no. 3–4, pp. 361–363, Feb. 2000, doi: 10.1007/s002149900065.
- [25] C. Lee, W. Yang, and R. G. Parr, "Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density," *Phys. Rev. B*, vol. 37, no. 2, pp. 785–789, Jan. 1988, doi: 10.1103/PhysRevB.37.785.
- [26] L. Schrödinger, "MacroModel," 2013, *Schrödinger, New York, NY*: 10.0.
- [27] J. Reis *et al.*, "Tight-Binding Inhibition of Human Monoamine Oxidase B by Chromone Analogs: A Kinetic, Crystallographic, and Biological Analysis," *J. Med. Chem.*, vol. 61, no. 9, pp. 4203–4212, May 2018, doi: 10.1021/acs.jmedchem.8b00357.
- [28] S.-Y. Son, J. Ma, Y. Kondou, M. Yoshimura, E. Yamashita, and T. Tsukihara, "Structure of human monoamine oxidase A at 2.2-Å resolution: The control of opening the entry for substrates/inhibitors," *Proceedings of the National Academy of Sciences*, vol. 105, no. 15, pp. 5739–5744, Apr. 2008, doi: 10.1073/pnas.0710626105.
- [29] J. C. Shelley, A. Cholleti, L. L. Frye, J. R. Greenwood, M. R. Timlin, and M. Uchimaya, "Epik: a software program for pK_a prediction and protonation state generation for drug-like molecules," *J. Comput. Aided. Mol. Des.*, vol. 21, no. 12, pp. 681–691, Dec. 2007, doi: 10.1007/s10822-007-9133-z.
- [30] M. Rostkowski, M. H. Olsson, C. R. Søndergaard, and J. H. Jensen, "Graphical analysis of pH-dependent properties of proteins predicted using PROPKA," *BMC Struct. Biol.*, vol. 11, no. 1, p. 6, Dec. 2011, doi: 10.1186/1472-6807-11-6.
- [31] W. L. Jorgensen and J. Tirado-Rives, "The OPLS [optimized potentials for liquid simulations] potential functions for proteins, energy minimizations for crystals of cyclic peptides and crambin," *J. Am. Chem. Soc.*, vol. 110, no. 6, pp. 1657–1666, Mar. 1988, doi: 10.1021/ja00214a001.
- [32] R. M. Geha, K. Chen, J. Wouters, F. Ooms, and J. C. Shih, "Analysis of Conserved Active Site Residues in Monoamine Oxidase A and B and Their Three-dimensional Molecular Modeling," *Journal of Biological Chemistry*, vol. 277, no. 19, pp. 17209–17216, May 2002, doi: 10.1074/jbc.M110920200.
- [33] C. Binda, M. Li, F. Hubálek, N. Restelli, D. E. Edmondson, and A. Mattevi, "Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures," *Proceedings of the National Academy of Sciences*, vol. 100, no. 17, pp. 9750–9755, Aug. 2003, doi: 10.1073/pnas.1633804100.
- [34] T. Tuccinardi, "What is the current value of MM/PBSA and MM/GBSA methods in drug discovery?," *Expert Opin. Drug Discov.*, vol. 16, no. 11, pp. 1233–1237, Nov. 2021, doi: 10.1080/17460441.2021.1942836.
- [35] D. Bashford and D. A. Case, "Generalized Born Models of Macromolecular Solvation Effects," *Annu. Rev. Phys. Chem.*, vol. 51, no. 1, pp. 129–152, Oct. 2000, doi: 10.1146/annurev.physchem.51.1.129.
- [36] J. P. M. Finberg and J. M. Rabey, "Inhibitors of MAO-A and MAO-B in Psychiatry and Neurology," *Front. Pharmacol.*, vol. 7, Oct. 2016, doi: 10.3389/fphar.2016.00340.

- [37] D. Rinaldi, M. Alborghetti, E. Bianchini, M. Sforza, S. Galli, and F. E. Pontieri, "Monoamine-oxidase Type B Inhibitors and Cognitive Functions in Parkinson's Disease: Beyond the Primary Mechanism of Action," *Curr. Neuropharmacol.*, vol. 21, no. 5, pp. 1214–1223, May 2023, doi: 10.2174/1570159X20666220905102144.
- [38] V. Oldfield, G. M. Keating, and C. M. Perry, "Rasagiline," *Drugs*, vol. 67, no. 12, pp. 1725–1747, 2007, doi: 10.2165/00003495-200767120-00006.
- [39] D. Edmondson, "The FAD Binding Sites of Human Monoamine Oxidases A and B," *Neurotoxicology*, vol. 25, no. 1–2, pp. 63–72, Jan. 2004, doi: 10.1016/S0161-813X(03)00114-1.
- [40] U. Bonnet, "Moclobemide: Therapeutic Use and Clinical Studies," *CNS Drug Rev.*, vol. 9, no. 1, pp. 97–140, Mar. 2003, doi: 10.1111/j.1527-3458.2003.tb00245.x.