



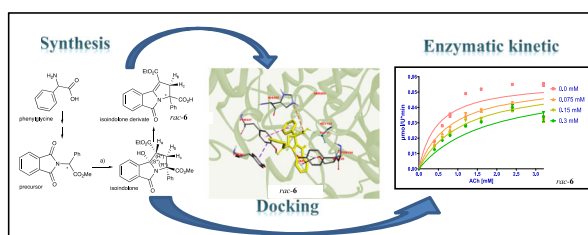
# Synthesis, in silico, and evaluation of AChE inhibitory activity of *N*-phthaloylphenylglycine derivatives as potential anti-Alzheimer's agents

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## Abstract

Mental illnesses are one of the most relevant health problems today, among which Alzheimer's disease (AD) stands out. This is a severe disease that entails different alterations such as chronic cognitive impairment. Commercial therapy drugs have not had the expected success due to their notable and rapid pharmacological efficacy reduction, therefore, we aimed to find new compounds capable of stopping the progression of this disease by cholinesterase inhibition. We synthesized and evaluated nine new racemic compounds (two precursors and their corresponding pyrrolo[2,1-*a*]isoindol-5-ones with different substituents) derived from phenylglycine as potential acetylcholinesterase inhibitors. Three of them (*rac*-4, *rac*-5, and *rac*-6) showed good enzyme inhibition ( $K_i$  117.5, 90.62, and 77.30  $\mu$ M, respectively), with a pattern of competitive inhibition type supported by in silico and in vitro experiments, being the *rac*-6 derivative the best inhibitor. The structural analysis showed that the presence of the ethyl ester group in the structure favors inhibition, likewise, the presence of double bonds increases the affinity of the inhibitor for the enzyme, so these new pyrrolo[2,1-*a*]isoindol-5-ones derivatives might be helpful for the treatment of Alzheimer's disease.



**Keywords** Isoindolone · Phenylglycine derivatives · Alzheimer's disease · Acetylcholinesterase inhibitor · Neurodegenerative disorders · Pyrrolo[2,1-*a*]isoindol-5-ones

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## Introduction

Dementia is a disorder characterized by a decline in cognition that interferes with daily functioning. One of the most common forms of this kind of disorder is Alzheimer's disease (AD), accounting for 60–80% of all cases [1, 2]. Worldwide, around 50 million people suffer a type of dementia, of which nearly 60% belong to developing countries. The estimated proportion of the general population aged 60 and over with dementia at a given time is between 5 and 8%. The total number of people with dementia is projected to reach 82 million in 2030 and 152 million in 2050 [2, 3]. The mainstay of AD treatment includes acetylcholinesterase (AChE) inhibitors and *N*-methyl-D-aspartate (NMDA) receptor antagonists, due to several clinical trials have demonstrated small improvements in cognition and activities of daily living after administration of these two classes of drugs. In this sense, currently, the Food and Drug Administration (FDA) has only approved 4 AChE inhibitors (AChEi) i.e., donepezil, rivastigmine, galantamine, and tacrine [4–6]: this last is no longer in use because of hepatotoxicity and poor tolerability [7, 8]. AD patients often have reduced amounts of choline acetyltransferase cerebral, leading to decreased acetylcholine synthesis and thus impaired cortical cholinergic function. AChEi inhibits acetylcholinesterase in the synaptic cleft, thus increasing cholinergic transmission [9]. Other potential drugs that suggest being useful for the treatment of Alzheimer's are isoindolones since it is known that a series of [1,3]dioxolo[4,5-*f*]isoindolone derivatives are potent inhibitors of AChE [10]. Furthermore, their effects on memory impairment in mice induced by scopolamine were investigated with a step-through test [10]. Other reported effects by isoindolone derivatives include antitumor activity by inhibition of PI3K/AKT/GSK3 $\beta$  signaling pathway [11] or by strong glycogen synthase kinase 3/cyclin dependent kinase 5 dual inhibition [12]. In addition, some isoindolone derivatives isolated from endophytic fungus *Emericella* sp. (HK-ZJ) isolated from the mangrove plant *Aegiceras corniculatum* have anti-influenza A (H1N1) viral activity [13], and synthetic derivatives demonstrated being potent and selective 5-HT<sub>2C</sub> antagonists [14]. The present contribution aimed to evaluate the novel pyrrolo[2,1-*a*]isoindol-5-ones derivatives of phenylglycine as acetylcholinesterase inhibitors, employing in silico and in vitro approaches.

## Results and discussion

### Chemistry

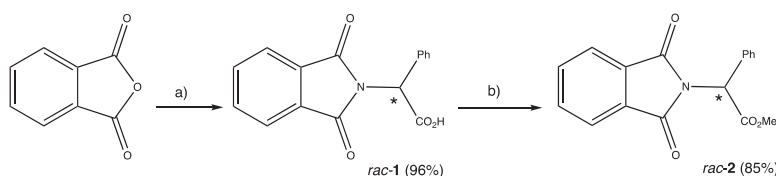
The synthesis protocol used to obtain the first compounds, *rac-1* and *rac-2*, as precursors of pyrrolo[2,1-*a*]isoindol-5-ones, is described in Scheme 1. Initially, *N*-phthaloylphenylglycine *rac-1* was prepared according to the solvent-free procedure described in the literature [15]. D-(-)- $\alpha$ -phenylglycine was condensed with phthalic anhydride at a temperature of 165–180 °C to obtain the *rac-1* compound in 96% yield, the observed racemization was confirmed by chiral HPLC of the crude product (Supplementary, Fig. 1) and agrees with what was previously reported [15, 16]. Fisher esterification reaction of *rac-1* with MeOH and H<sub>2</sub>SO<sub>4</sub> as catalyst, at reflux temperature, gave the *rac-2* compound in 85% yield.

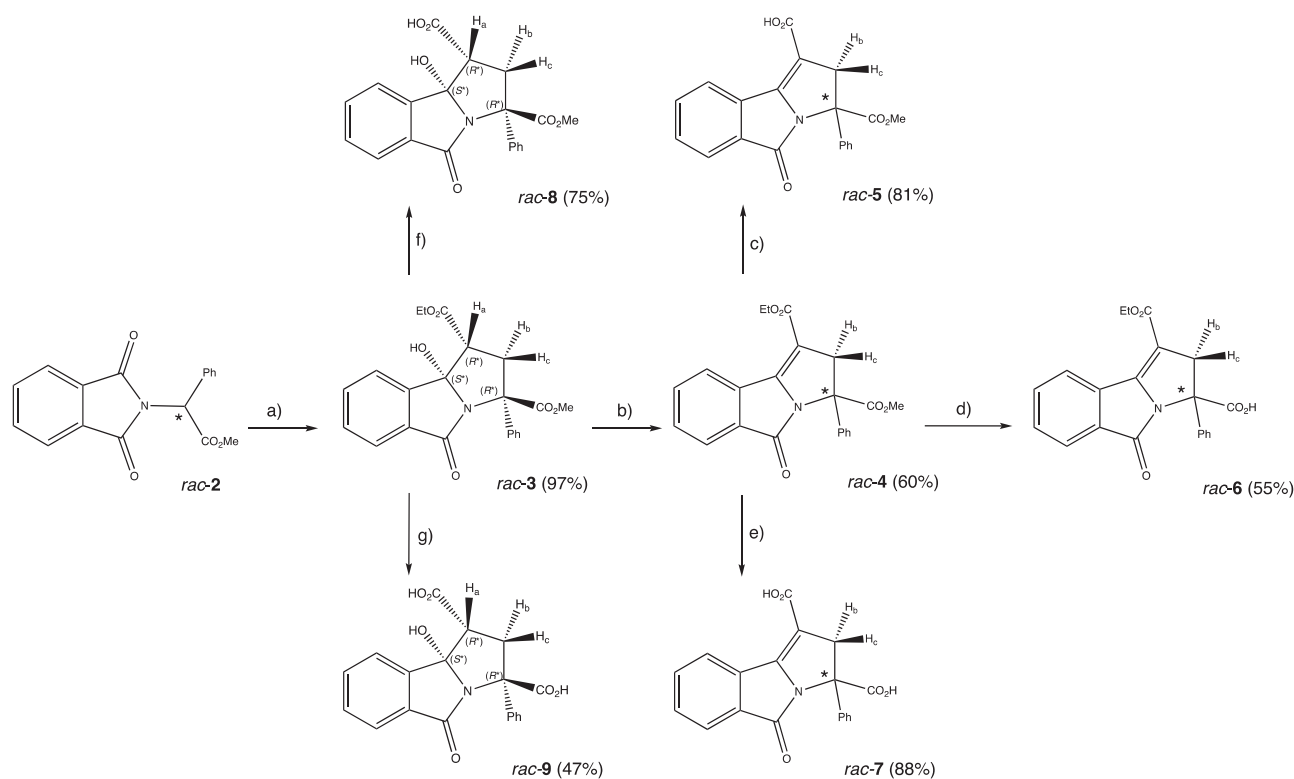
The synthesis strategy of *rac-3* and its derivatives incorporating the required pyrrolo[2,1-*a*]isoindol-5-ones scaffold has been depicted in Scheme 2. Our first pyrrolo[2,1-*a*]isoindol-5-one, *rac-3*, was prepared as previously reported with slight modifications [15, 17, 18]. The phthalimide *rac-2* and ethyl acrylate in the presence of lithium bis(trimethylsilyl)amide (LHMDS) in THF at low temperature were reacted to produce the *rac-3* compound as a single diastereoisomer in 97% yield. This relative stereochemistry in *rac-3* was explained by a type of cascade reaction involving a Michael addition when a carbanion generated from *rac-2* undergoes the 1,4-addition to the  $\alpha,\beta$ -unsaturated compound forming a new enolate, continuing with a stereospecific intramolecular nucleophilic addition between the enolate and one of the carbonyl group of the phthalimidyl moiety, this latter by a Zimmerman-Traxler type transition state, which explains the selectivity of this reaction [15, 17, 18]. It is noteworthy that in this step three stereogenic centers and two C-C bonds are formed.

The *rac-3* compound dissolved in CH<sub>2</sub>Cl<sub>2</sub> was reacted with H<sub>2</sub>SO<sub>4</sub> at room temperature to produce *rac-4* in 60% yield, with no hydrolysis products observed. The rapid dehydration under acidic conditions is explained by the fact that there is a highly reactive hydroxyl group at C-9b of *rac-3*, as it is in the benzylic position, beta to carbonyl ester, and alpha to nitrogen.

The *rac-4* diester was treated with 1.5 equivalents of NaOH in MeOH at room temperature to obtain the *rac-5* compound as the sole hydrolysis product in 81% yield, regioselective hydrolysis occurs in the ethyl ester at C-1 of

**Scheme 1** Synthesis of *N*-phthaloylphenylglycine, *rac-2*: **a)** D-(-)- $\alpha$ -phenylglycine, 165–180 °C, 30 min. **b)** MeOH, H<sub>2</sub>SO<sub>4</sub>, reflux, 3 h





**Scheme 2** Synthesis of substituted pyrrolo[2,1-*a*]isoindol-5-ones *rac-3* – *rac-9*: **a)** ethyl acrylate, LHMDS, THF, dry ice-acetone bath, 1 h. **b)** H<sub>2</sub>SO<sub>4</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 25 °C, 30 min. **c)** 1.5 eq NaOH, MeOH, 25 °C, 8 h.

**d)** 1.5 eq LiOH·H<sub>2</sub>O, THF, reflux, 10 h. **e)** 4.5 eq NaOH, MeOH, reflux, 24 h. **f)** 1.1 eq LiOH·H<sub>2</sub>O, THF/H<sub>2</sub>O, reflux, 1.5 h. **g)** 5.0 eq LiOH·H<sub>2</sub>O, THF/H<sub>2</sub>O, reflux, 1.5 h

*rac-4* because it has less steric hindrance. More basic conditions were necessary for the hydrolysis of both esters present in the *rac-4*, 4.5 equivalents of NaOH were used to obtain the *rac-7* compound with a yield of 88%. While the reaction of *rac-4* with 1.5 equivalents LiOH·H<sub>2</sub>O in refluxing THF gave *rac-6* in 55% yield, regioselective hydrolysis occurs at the sterically hindered methyl ester group [19].

Under similar hydrolysis conditions, *rac-3* was reacted with 1.1 equivalents of LiOH·H<sub>2</sub>O, resulting in the *rac-8* compound in 75% yield, regioselective hydrolysis of the ethyl ester group at C-1 [19]. Whereas the hydrolysis of both esters present in *rac-3* required 5.0 equivalents of LiOH·H<sub>2</sub>O to form the racemic compound *rac-9* in 47% yield.

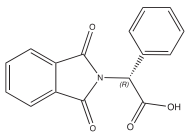
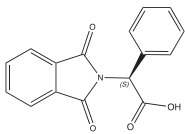
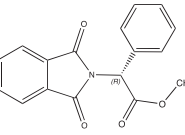
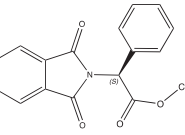
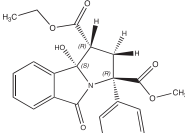
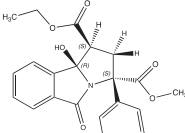
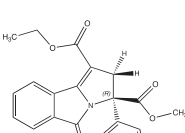
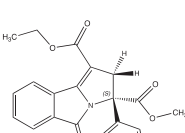
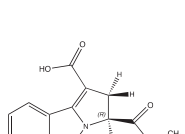
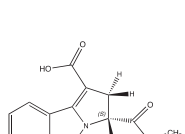
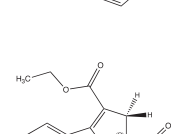
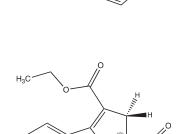
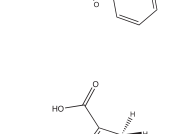
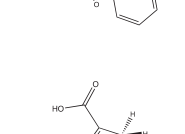
Finally, all the synthesized compounds were confirmed and analyzed by spectroscopic techniques such as <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, HRMS, and IR. With the relative stereochemistry of *rac-3* that we recently reported [16], the stereochemistry of the *rac-4* to *rac-9* derivatives could be established.

### Anticholinesterase activity assay

In this work, nine pairs of stereoisomers were evaluated. Table 1 shows the structures, the name, and the inhibition

constant (*K<sub>i</sub>*) of each ligand, as well as the 95% confidence interval and the type of AChE inhibition. The first characteristic of this type of compound is that they show a competitive inhibition for each of the pairs of molecules evaluated, just like the corresponding control or reference molecules (galantamine and neostigmine). The type of inhibition was obtained by the Lineweaver-Burk plot, while a non-linear regression gave the values of the constants of inhibition and their confidence intervals (Fig. 1). It is important to mention that the phenylglycine precursors were evaluated to observe how the addition of a new ring affects the inhibitory properties of these molecules; also, its acid form and its methyl ester form were evaluated too, and the results obtained for this pair of precursors were very similar, with a *K<sub>i</sub>* value of 141.1 and 135 μM for the acid form and for the ester, respectively. The pairs of stereoisomers that showed the best inhibitory activity on acetylcholinesterase were the compounds *rac-4*, *rac-5*, and *rac-6*, the latter being the best inhibitory compound with an inhibition constant of 77.30 μM, while the *rac-4* and *rac-5* compounds showed a *K<sub>i</sub>* of 117.5 and 90.62 μM, respectively; the structural analysis shows that *rac-4* compound is the diester form in position C-1 and C-3, in addition to having an unsaturation between C-9b and C-1; after analyzing the compound *rac-5* we visualize that it is the mono ester of *rac-4* in the C-3 position (methyl ester), while in C-1 we

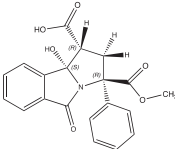
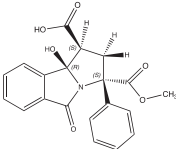
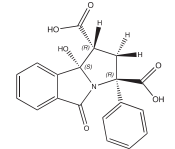
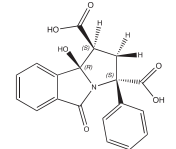
**Table 1** Chemical structures of both enantiomers that make up the racemic mixture, IDs ligands, inhibition constant values  $K_i$ , confidence interval, and type of inhibition obtained for precursors, pyrrolo[2,1-*a*]isoindol-5-ones derivatives and reference drugs with *EeAChE* in the in vitro experiments

Structure	Structure	Ligand	$K_i$ ( $\mu\text{M}$ )	Confidence interval 95%	Type of inhibition
		<i>rac-1</i>	141.1	97.68–184.6	Competitive
		<i>rac-2</i>	135.8	94.73–177.0	Competitive
		<i>rac-3</i>	306.2	202.7–409.7	Competitive
		<i>rac-4</i>	117.5	60.08–174.9	Competitive
		<i>rac-5</i>	90.62	61.55–119.7	Competitive
		<i>rac-6</i>	77.30	47.19–107.4	Competitive
		<i>rac-7</i>	849.1	653.3–1045	Competitive

can see the form of the carboxylic acid, this small structural change reduces the  $K_i$ . Similarly, compound *rac-6* is the mono ester of *rac-4*, but now in the position of C-1 (ethyl ester), while C-3 is found carboxylic acid, this structural

change was key to significantly increasing the inhibition, *rac-6* being the compound with the lowest  $K_i$  that improved the inhibitory activity of the *rac-1* precursor close to 2-fold. Regarding the other compounds, the *rac-7* ligand is the

**Table 1** (continued)

Structure	Structure	Ligand	$K_i$ ( $\mu\text{M}$ )	Confidence interval 95%	Type of inhibition
		<i>rac-8</i>	409.7	259.6–559.7	Competitive
		<i>rac-9</i>	827.6	377.9–1277	Competitive
		Galantamine	0.316	0.270–0.362	Competitive
		Neostigmine	22.56	20.19–24.93	Competitive

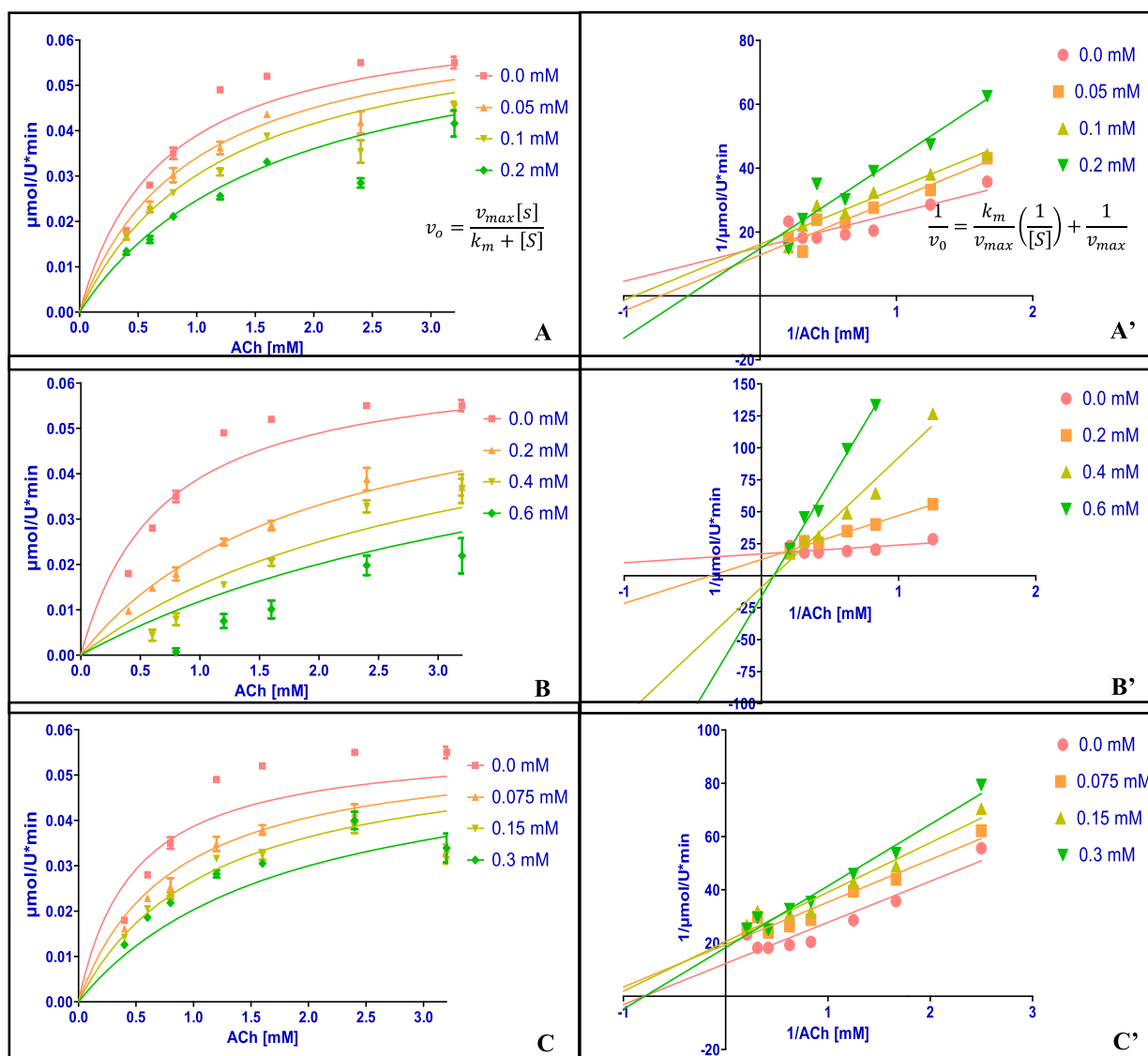
diacid form of *rac-4*. This structural change increased the inhibition constant by around 11 times the value of *rac-6*. In previous studies [18] it was reported that the addition of ester groups in the structure of the ligands improved their inhibitory activity, here again, it is demonstrated that the presence of ester groups in the structure of the ligands improves inhibitor-enzyme recognition. Another important structural aspect for the recognition of the inhibitor with AChE is the double bond, the ligands *rac-3*, *rac-8*, and *rac-9* do not have the double bond at position C-9b and C-1 as their counterparts *rac-4* to *rac-7*. However, even though the ligands *rac-3* and *rac-8* present their corresponding ester groups, this is not enough to decrease  $K_i$ . In contrast, *rac-9* does not have the double bond or any ester group, obtaining values of high  $K_i$  like *rac-7* and contrary to *rac-6*. The presence of the double bond in the newly formed ring of pyrrolo[2,1-*a*]isoindol-5-ones favors the inhibition of the enzyme, as well as the presence of ester groups in the structure. In further studies, it would be important to evaluate different types of esters and observe if this produces significant changes in the inhibition of the enzyme. The *rac-6* ligand seems to be the most selective and with the best inhibitory properties of the acetylcholinesterase, so in future work, we can synthesize compounds derived from this ligand and improve its inhibitory properties.

### Molecular docking and theoretical calculations

To better understand the interaction observed in the in vitro model between the racemic compounds as ligands and the *Electrophorus electricus* AChE enzyme, molecular docking was carried out since one of the main differences between the in vitro model and the in vivo models is the different

pH. While for the in vivo models the pH is 7.4, in the enzymatic kinetics a pH of 8 is used, which is higher than the physiological one. It is intended to demonstrate if there is an effect on the potency of the ligands due to this change in pH, through in silico experiments. The results in Table 2 show the different ligands as a racemic mixture in the first column. In contrast, the second column shows the IDs of the two enantiomers that make up the racemic mixture and with which the molecular docking was made (it is worth mentioning that this is one of the advantages of in silico studies, be able to separate mixtures virtually and assess whether their physical separation is worthwhile and test them separately in vivo and in vitro experiments). The following column shows the Gibbs free energy ( $\Delta G$ ) for each of the interactions present in the molecular approach of the different enantiomers. It is important to mention that Gibbs free energy is a thermodynamic parameter that represents the energy available to do useful work. It is commonly used to predict the spontaneity of a chemical reaction or interaction and whether a reaction or interaction will proceed in the forward or reverse direction. If  $\Delta G$  is negative ( $\Delta G < 0$ ), the reaction or interaction is spontaneous in the forward direction, meaning the reaction proceeds spontaneously, and the reaction is considered exergonic. If  $\Delta G$  is positive ( $\Delta G > 0$ ), the reaction or interaction is non-spontaneous in the forward direction, and it will not proceed without the input of additional energy. Such reactions or interactions are called endergonic. If  $\Delta G$  is zero ( $\Delta G = 0$ ), the reaction or interaction is at equilibrium.

These first results indicate that the approach is an exergonic type for each of the ligands, on average the  $\Delta G$  is  $-8.31$  Kcal/mol, these results were compared with the reference molecules donepezil, galantamine, rivastigmine,



**Fig. 1** Non-linear regression (left) and Lineweaver-Burk (right) plots for the best three inhibitors of *EeAChE*. **A** and **A'** for *rac-4*; **B** and **B'** for *rac-5*; and **C** and **C'** for *rac-6*

and tacrine, being donepezil the reference molecule with the lowest  $\Delta G$  with a value of  $-10.57$  Kcal/mol. On average, the best racemic mixtures obtained in the enzymatic kinetics were *rac-4*, *rac-5*, and *rac-6*, which correspond to the ligands (*R*)-**4**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*R*)-**6**, and (*S*)-**6** obtained a  $\Delta G$  of  $-8.98$  Kcal/mol, which is slightly better than the average obtained by the reference molecules, which was  $-8.76$  Kcal/mol. Likewise, these same ligands present the best  $\Delta G$  of the inhibitors studied, which agrees with the results of the enzymatic kinetics model. The results obtained from the dissociation constant ( $K_d$ ) and  $\text{p}K_d$  show a pattern identical to that obtained by the Gibbs free energy. This value ( $K_d$ ) should not be confused with  $K_i$ , since the former is a specific type of equilibrium constant that measures the propensity of two molecules (the inhibitor and the

enzyme) to reversibly dissociate into smaller components, and the latter ( $K_i$ ) refers to the inhibition constant obtained in the in vitro experiments and is an indication of how potent an inhibitor is.

On the other hand, no structural relationship was observed with the different *R* or *S* enantiomers and their affinity for the AChE enzyme as previously reported, so based on in silico studies, an enantiomeric separation would not be worthwhile to test them individually. Finally, the conformational analysis of the enantiomers that make up the racemic mixtures with AChE was carried out. The results for the three mixtures that best inhibited the enzyme in vitro (*rac-4*, *rac-5*, and *rac-6*) are shown in Table 3 and Fig. 2, the conformational analysis indicates that these ligands bind directly at the catalytic site of the enzyme, peripheral



**Table 2** Racemic mixture IDs, enantiomers IDs, Gibbs free energy ( $\Delta G$ ), the dissociation constant ( $K_d$ ), and  $-\log_{10}$  dissociation constant ( $pK_d$ ) for the molecular docking between *EeAChE* (enzyme) and test compounds (references, precursors, and pyrrolo[2,1-*a*]isoindol-5-ones)

Ligand	Docking ID	$\Delta G$ (Kcal/mol)	$K_d$ ( $\mu M$ )	$pK_d$
<i>rac</i> -1	( <i>R</i> )- <b>1</b>	-7.480	3.280	5.48
	( <i>S</i> )- <b>1</b>	-7.330	4.200	-7.330
<i>rac</i> -2	( <i>R</i> )- <b>2</b>	-8.490	0.599	6.22
	( <i>S</i> )- <b>2</b>	-8.530	0.558	6.25
<i>rac</i> -3	(1 <i>R</i> ,3 <i>R</i> ,9 <i>bS</i> )- <b>3</b>	-8.190	0.558	6.01
	(1 <i>S</i> ,3 <i>S</i> ,9 <i>bR</i> )- <b>3</b>	-8.840	0.325	6.49
<i>rac</i> -4	( <i>R</i> )- <b>4</b>	-9.900	0.055	7.26
	( <i>S</i> )- <b>4</b>	-9.540	0.101	6.99
<i>rac</i> -5	( <i>R</i> )- <b>5</b>	-8.160	1.040	5.98
	( <i>S</i> )- <b>5</b>	-9.040	0.236	6.63
<i>rac</i> -6	( <i>R</i> )- <b>6</b>	-8.390	0.711	6.15
	( <i>S</i> )- <b>6</b>	-8.850	0.325	6.49
<i>rac</i> -7	( <i>R</i> )- <b>7</b>	-7.450	3.470	5.46
	( <i>S</i> )- <b>7</b>	-7.630	11.590	4.94
<i>rac</i> -8	(1 <i>R</i> ,3 <i>R</i> ,9 <i>bS</i> )- <b>8</b>	-8.170	1.020	5.99
	(1 <i>S</i> ,3 <i>S</i> ,9 <i>bR</i> )- <b>8</b>	-7.840	1.800	5.74
<i>rac</i> -9	(1 <i>R</i> ,3 <i>R</i> ,9 <i>bS</i> )- <b>9</b>	-7.670	2.370	5.63
	(1 <i>S</i> ,3 <i>S</i> ,9 <i>bR</i> )- <b>9</b>	-6.340	22.710	4.64
	Donepezil	-10.57	0.170	6.77
	Galantamine	-9.34	0.141	6.85
	Rivastigmine	-7.90	0.163	6.79
	Tacrine	-7.23	0.502	6.30

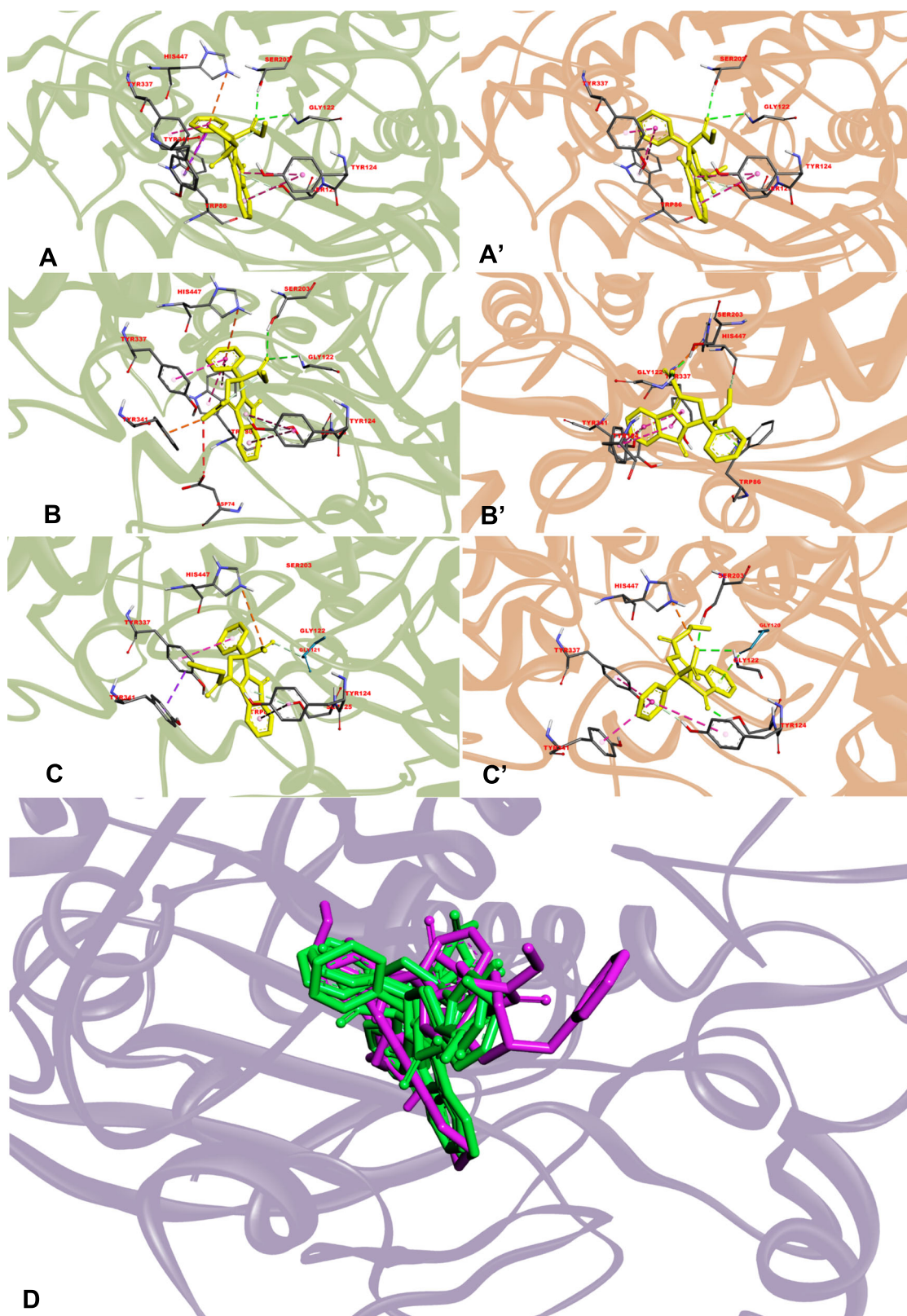
anionic site, the acyl pocket, the aromatic patch, and the oxyanion hole of the enzyme, interacting with amino acid residues important for enzyme-substrate recognition. These amino acid residues with which the ligands interact are Ser203, His447, Tyr124, Trp286, Try341, Trp86, Trp236, Gly121, Tyr337, etc. Having an average of up to 8 interactions, the main types of interactions in these ligands are hydrophobic and  $\pi$ - $\pi$ , mainly. This is due to the formation of a third ring in the chemical structure of the newly synthesized compounds (pyrrolo[2,1-*a*]isoindol-5-ones). Although the results of the molecular approach cannot predict the type of enzymatic inhibition, we can observe where each of the ligands is interacting and compare it with the reference molecules, which in turn allows us to validate our molecular docking model (RMSD donepezil 0.9744, galantamine 0.0780, rivastigmine 0.1002 and tacrine 0.0487 (27,28)), with the data of the type of interaction, the conformation in the ligand-enzyme coupling, amino acid residues present in the interaction, we could predict that the type of inhibition would be competitive, which agrees with the data obtained in the in vitro experiments.

## Conclusions

New racemic compounds derived from phenylglycine (including two precursors) were synthesized and tested by in vitro and in silico models achieved that way the primary

**Table 3** Best ligands and references analysis for the type of interactions with the *EeAChE*

Ligand	Hydrophobic	H-bond	$\pi$ - $\pi$ interaction	$\pi$ -cation	$\pi$ -anion	$\pi$ -donor	Electrostatic
( <i>R</i> )- <b>4</b>	Tyr341	Ser203, Gly122	Trp86, Tyr337, Tyr124	His447	-	Ser125	-
( <i>S</i> )- <b>4</b>	-	Ser203, Gly122	Trp86, Tyr337, Tyr124	-	-	Ser125	-
( <i>R</i> )- <b>5</b>	-	Ser203, Gly122	Trp86, Tyr337, Tyr124	His447	Tyr341	Ser125	Asp74
( <i>S</i> )- <b>5</b>	-	Ser203, Gly122	Tyr124, Tyr341, Tyr337	-	Trp86	Tyr124	His447
( <i>R</i> )- <b>6</b>	Tyr341, Gly121	-	Tyr337, Tyr124	-	-	Ser125	His447
( <i>R</i> )- <b>6</b>	-	Ser125, Gly122, Ser203	Tyr337, Tyr124, Tyr341	-	-	Tyr124	His447
Donepezil	Tyr341 Phe338 Tyr337 Gly120 Gly202 Tyr133	-	Trp86	-	-	-	-
Galantamine	Tyr337 Trp86	Tyr133 Glu202		-	-	-	-
Rivastigmine	Tyr341 Tyr337 Trp86 His447 Glu202	Tyr124	Phe338	-	-	-	-
Tacrine	-	Trp86, Ser125	Trp86	-	-	-	-



**Fig. 2** Binding modes and amino acid residues in the interaction of the ligands and the site of *Electrophorus electricus* AChE: (A) enantiomer (R)-4 in complex with AChE; (A') enantiomer (S)-4 in complex with AChE; (B) enantiomer (R)-5 in complex with AChE; (B')

**(S)-5 in complex with AChE; (C) enantiomer (R)-6 in complex with AChE; (C') enantiomer (S)-6 in complex with AChE; (D) Isoindolones (green) and reference compounds (fuchsia) interacting with the active site of AChE**



objective of this research, which was identifying new compounds capable of inhibiting the acetylcholinesterase enzyme (AChE). The synthesized pyrrolo[2,1-*a*]isoindol-5-ones derived from phenylglycine, especially *rac*-**6** with an inhibition constant of 77.3  $\mu\text{M}$ , exhibited promising inhibitory activity against AChE. The significance of these new racemic compounds lies in their potential therapeutic impact on diseases associated with cholinergic dysfunction, such as Alzheimer's disease and other neurodegenerative disorders. The findings from molecular docking studies provided valuable insights into the structural features responsible for the enhanced inhibitory activity of these compounds since it was found that the presence of ester groups, particularly the ethyl ester, and a double bond at C-9b and C-1 were critical factors contributing to the effectiveness of the compounds as AChE inhibitors. Moreover, the identification of hydrophobic and  $\pi$ - $\pi$  interactions as the main binding forces strengthens our understanding of the ligand-receptor interactions that influence inhibitory potency. Additionally, our molecular docking studies revealed that the *R* or *S* configuration does not significantly affect the inhibitory properties, highlighting the robustness of the inhibitory effect irrespective of chirality. The results of this study have the potential to significantly impact related fields, particularly in drug discovery and medicinal chemistry. The knowledge gained from this research can guide the rational design and synthesis of new compounds with improved AChE inhibitory activity by understanding the specific structural requirements for effective enzyme inhibition, researchers can focus on developing more potent and selective inhibitors. Despite these promising findings, further studies are warranted to gain a comprehensive understanding of the biological properties of the *rac*-**6** mixture. Investigating its pharmacokinetic and pharmacodynamic profiles, as well as conducting *in vivo* studies, will be essential to validate its therapeutic potential and safety. In summary, the discovery of these new racemic pyrrolo[2,1-*a*]isoindol-5-ones derived from phenylglycine and their potent inhibitory activity against AChE holds great promise for the development of novel treatments for diseases associated with cholinergic dysfunction. The insights gained from this research can pave the way for more targeted drug design approaches, ultimately contributing to advancements in the field of drug discovery and potentially improving the lives of patients affected by neurological disorders.

## Materials and methods

### Synthesis and characterization

All reagents obtained from Sigma-Aldrich were used without additional purification, except for tetrahydrofuran (THF), which was distilled with sodium prior to use. The reactions

were conducted in oven-dried round-bottomed flasks, pre-fitted with a magnetic stirrer bar and with sufficient volume capacity to hold the reaction and obtain products in the stated amounts, which were concentrated using a standard rotary evaporator under reduced pressures once the reaction finished. All the organic phases obtained from each extraction of the reactions were washed with brine and subsequently dried over anhydrous  $\text{Na}_2\text{SO}_4$  to obtain the crude product. Thin-layer chromatography (TLC) on silica gel 60 plates (precoated with Merck F254) was routinely performed to monitor the progress of the reaction. Visualization of starting materials and products was achieved by exposing the TLC plates to UV lamp irradiation (254 nm). Silica gel (230–400 mesh) was utilized for column chromatography. Melting points were measured using an uncorrected Melt-Temp “Electrothermal” apparatus. FT-IR spectra were obtained with an ATR accessory on a Perkin-Elmer spectrometer (PC16, Spectrum GX).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded using either a Bruker ASCEND 400 for  $^1\text{H}$  and 101 MHz for  $^{13}\text{C}$ , and Bruker ASCEND Ultrashield 750 for  $^1\text{H}$  and 188 MHz for  $^{13}\text{C}$ . The chemical shifts were referenced to the internal deuterated solvent. Electrospray ionization high-resolution mass spectrometry (ESI-HRMS) was conducted on a Bruker microOTOF-Q instrument. HPLC analyses were performed on a Waters HPLC Alliance 2695 equipped with a UV/Visible detector and a Waters 2996 diode array. The separation conditions for HPLC involved a Chiralpak AD-H column, a mobile phase consisting of 90% hexane and 10% IPA, and a flow rate of 1.0 mL/min.

To prepare the following racemic compounds, minor modifications were made to the protocol reported in previous work [18].

### **(*RS*)-2-(1,3-Dioxoisindolin-2-yl)-2-phenylacetic acid, [*rac*-1]**

A flask pre-fitted with a reflux condenser was charged with a mixture of phthalic anhydride (16.2 g, 109.4 mmol) and D-(-)- $\alpha$ -phenylglycine (15.9 g, 105.2 mmol) and, without adding solvent it was allowed to react for 30 min by heating at 165–180  $^\circ\text{C}$ , and then it was allowed to cool to room temperature. The crude product was purified by recrystallization from EtOH/ $\text{H}_2\text{O}$  (2:1). *rac*-**1** was obtained as a white solid; yield 28.4 g (96%); HPLC, column Chiralpak AD-H; 90:10 hexane-IPA, and Flux 1.0 mL/min; retention time 24.6 and 29.3 min; mp 170–171  $^\circ\text{C}$  (Lit. [15] mp 170.5–171.5  $^\circ\text{C}$ ); TLC:  $R_f = 0.31$  (hexane/EtOAc, 7:3); ATR-FTIR  $\nu_{\text{max}}$  1706 (C=O), 1379  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (Chloroform-*d*, 301 MHz)  $\delta$  7.85 (2H, dd,  $J = 5.5, 3.1$  Hz, H-Phth), 7.71 (2H, dd,  $J = 5.5, 3.0$  Hz, H-Phth), 7.58 (2H, dd,  $J = 7.7, 1.9$  Hz,  $\text{H}_{\text{ortho-Ph}}$ ), 7.35 (3H, m, H-Ph), 6.09 (1H, s, NCHPh);  $^{13}\text{C}$  NMR (Chloroform-*d*, 76 MHz)  $\delta$  173.2 (C, HOC=O), 167.0 (2 C, NC=O), 134.3 (2 CH,

Phth), 133.7 (C, Ph), 131.7 (2C, Phth), 129.7 (2CH<sub>ortho</sub>, Ph), 128.8 (CH<sub>para</sub>, Ph), 128.6 (2CH<sub>meta</sub>, Ph), 123.7 (2CH, Phth), 55.5 (CH, NCH); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>16</sub>H<sub>12</sub>NO<sub>4</sub> 282.0766, found 282.0768 (M + H<sup>+</sup>).

### Methyl (*RS*)-2-(1,3-dioxoisindolin-2-yl)-2-phenylacetate, [*rac*-2]

A flask pre-fitted with a reflux condenser was charged with a solution of *rac*-1 (12.1 g, 43.0 mmol) in MeOH (100 mL) before adding H<sub>2</sub>SO<sub>4</sub> (1.4 mL). The mixture was allowed to react for 3 h to reflux temperature and then was cooled to room temperature. The product was concentrated before the addition of water (200 mL) and neutralization with Na<sub>2</sub>CO<sub>3</sub>·H<sub>2</sub>O (3.1 g). The mixture was dissolved in water (200 mL) and EtOAc (100 mL), and the aqueous layer was extracted with EtOAc (3 × 30 mL). The organic layers were combined, washed, dried, and concentrated. It was obtained as colorless crystals; yield 10.9 g (85%); mp 94–96 °C (Lit. [15] mp 99.6–100.5 °C); TLC: R<sub>f</sub> = 0.36 (hexane/EtOAc, 8:2); **ATR-FTIR**  $\nu_{max}$  1713 (C = O), 1735 (C = O ester). **<sup>1</sup>H NMR** (Chloroform-*d*, 301 MHz)  $\delta$  7.85 (2H, dd, *J* = 5.5, 3.1 Hz, H-Phth), 7.72 (2H, dd, *J* = 5.5, 3.0 Hz, H-Phth), 7.55 (2H, dd, *J* = 7.7, 1.9 Hz, H<sub>ortho</sub>-Ph), 7.35 (3H, m, Ph), 6.03 (1H, s, NCHPh), 3.81 (3H, s, OCH<sub>3</sub>); **<sup>13</sup>C NMR** (Chloroform-*d*, 76 MHz)  $\delta$  168.5 (C, MeOC=O), 167.1 (2C, NC=O), 134.4 (C, Ph), 134.2 (2CH, Phth), 131.8 (2C, Phth), 129.7 (2CH<sub>ortho</sub>, Ph), 128.6 (CH<sub>para</sub>, Ph), 128.5 (2CH<sub>meta</sub>, Ph), 123.6 (2CH, Phth), 55.8 (C, NCHPh), 53.0 (C, OCH<sub>3</sub>); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>17</sub>H<sub>14</sub>NO<sub>4</sub> 296.0923, found 296.0918 (M + H<sup>+</sup>).

### 1-Ethyl 3-methyl (1*R*,3*R*,9*bS*)- and (1*S*,3*S*,9*bR*)-9*b*-hydroxy-5-oxo-3-phenyl-2,3,5,9*b*-tetrahydro-1*H*-pyrrolo[2,1-*a*]isoindole-1,3-dicarboxylate, [*rac*-3]

The following reaction was carried out under an inert atmosphere. Once the flask was charged with *rac*-2 (16.3 g, 55.2 mmol), a rubber septum was attached to the flask to seal it, and it was inserted syringe needles through it to allow entry of nitrogen and displacement of oxygen. After 30 min, dry THF (270 mL) and ethyl acrylate (7.0 mL, 66.0 mmol) were added via cannula in order, slowly and stirring constantly. Subsequently, the mixture was brought to a temperature of −75 °C by immersing it in a dry ice-acetone bath to then add lithium bis(trimethylsilyl)amide (1 M in THF, LHMDs, 56.2 mL, 56.2 mmol) with a syringe through the rubber septum to keep oxygen out of the system. The resulting mixture was stirred for 1 h at the same temperature before adding aq. NH<sub>4</sub>Cl (100 mL) and the reaction mixture were extracted with EtOAc (3 × 100 mL). The organic extracts were combined, washed with brine

(100 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Subsequently, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (hexane/EtOAc, 75:25) and recrystallization from CH<sub>2</sub>Cl<sub>2</sub>/hexane (1:3). It was obtained as colorless crystals; yield 21.3 g (97%); mp 163–165 °C (Lit. [15] mp 164–166 °C); TLC: R<sub>f</sub> = 0.21 (hexane/EtOAc, 7:3); **ATR-FTIR**  $\nu_{max}$  3439 (OH), 1704 (C = O), 1744 (C = O ester); **<sup>1</sup>H NMR** (Chloroform-*d*, 400 MHz)  $\delta$  7.85 (1H, d, *J* = 7.7 Hz, H-6), 7.79 (1H, d, *J* = 7.7 Hz, H-9), 7.65 (1H, td, *J* = 7.6, 1.2 Hz, H-7), 7.56 (1H, td, *J* = 7.5, 0.4 Hz, H-8), 7.51 (2H, dd, *J* = 7.1, 1.7 Hz, H<sub>ortho</sub>-Ph), 7.34 (3H, m, Ph), 4.33 (2H, m, OCH<sub>2</sub>CH<sub>3</sub>), 3.65 (3H, s, OCH<sub>3</sub>), 3.58 (1H, dd, *J* = 12.4, 7.2 Hz, H<sub>a</sub>-1), 3.40 (1H, t, *J* = 12.7 Hz, H<sub>b</sub>-2 diastereotopic proton), 3.35 (1H, s br, OH), 3.26 (1H, dd, *J* = 13.0, 7.2 Hz, H<sub>c</sub>-2 diastereotopic proton), 1.36 (3H, t, *J* = 7.1 Hz, OCH<sub>2</sub>CH<sub>3</sub>); **<sup>13</sup>C NMR** (Chloroform-*d*, 101 MHz)  $\delta$  172.0 (C, MeOC=O), 169.9 (C, EtOC=O), 169.0 (C, NC=O), 145.6 (C, C-9a), 140.8 (C, Ph), 133.3 (CH, C-8), 131.6 (C, C-5a), 130.3 (CH, C-7), 128.0 (2CH<sub>meta</sub>, Ph), 127.7 (CH<sub>para</sub>, Ph), 127.1 (2CH<sub>ortho</sub>, Ph), 124.1 (CH, C-6), 123.9 (CH, C-9), 95.8 (C, C-9b), 68.5 (C, C-3), 61.6 (CH<sub>2</sub>, OCH<sub>2</sub>CH<sub>3</sub>), 53.1 (CH, C-1), 49.2 (CH<sub>3</sub>, OCH<sub>3</sub>), 44.6 (CH<sub>2</sub>, C-2), 14.2 (CH<sub>3</sub>, OCH<sub>2</sub>CH<sub>3</sub>); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>22</sub>H<sub>22</sub>NO<sub>6</sub> 396.1447, found 396.1444 (M + H<sup>+</sup>).

### 1-Ethyl 3-methyl (*RS*)-5-oxo-3-phenyl-2,5-dihydro-3*H*-pyrrolo[2,1-*a*]isoindole-1,3-dicarboxylate, [*rac*-4]

A solution of *rac*-3 (5.7 g, 14.4 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (60 mL) was reacted with H<sub>2</sub>SO<sub>4</sub> (1.9 mL) at room temperature for 30 min. Having finished the reaction, water (30 mL) was added, and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 60 mL). The organic extracts were combined, washed with brine (60 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Subsequently, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (hexane/EtOAc, 85:15) and recrystallization from MeOH. It was obtained as colorless crystals; yield 3.3 g (60%); mp 90–92 °C; TLC: R<sub>f</sub> = 0.70 (hexane/EtOAc, 7:3); **ATR-FTIR**  $\nu_{max}$  1656 (C = C); **<sup>1</sup>H NMR** (Chloroform-*d*, 400 MHz)  $\delta$  8.63 (1H, dd, *J* = 7.4, 1.1 Hz, H-9), 7.88 (1H, dt, *J* = 7.0, 0.8 Hz, H-7), 7.69 (1H, td, *J* = 7.5, 1.3 Hz, H-6), 7.63 (1H, td, *J* = 7.5, 1.2 Hz, H-8), 7.51 (2H, dd, *J* = 7.1, 1.7 Hz, H<sub>ortho</sub>-Ph), 7.37 (2H, m, H<sub>meta</sub>-Ph), 7.32 (1H, m, H<sub>para</sub>-Ph), 4.33 (2H, c, *J* = 7.1 Hz, OCH<sub>2</sub>CH<sub>3</sub>), 4.15 (1H, d, *J* = 17.8 Hz, H-2 diastereotopic proton), 3.83 (3H, s, OCH<sub>3</sub>), 3.74 (1H, d, *J* = 17.8 Hz, H-2 diastereotopic proton), 1.38 (3H, t, *J* = 7.1 Hz, OCH<sub>2</sub>CH<sub>3</sub>); **<sup>13</sup>C NMR** (Chloroform-*d*, 101 MHz)  $\delta$  171.2 (C, MeOC=O), 164.0 (C, EtOC=O), 163.6 (C, NC=O), 148.6 (C, C-9b), 138.1 (C, Ph), 135.7 (C, C-5a), 132.7 (CH, C-7), 131.6 (CH, C-8), 129.5 (C, C-9a), 128.6 (2CH<sub>meta</sub>, Ph), 128.3 (CH<sub>para</sub>, Ph),

126.9 (CH, C-9), 126.4 (2CH<sub>ortho</sub>, Ph), 124.0 (CH, C-6), 107.1 (C, C-1), 69.7 (C, C-3), 60.9 (CH<sub>2</sub>, OCH<sub>2</sub>CH<sub>3</sub>), 53.5 (CH<sub>3</sub>, OCH<sub>3</sub>), 51.0 (CH<sub>2</sub>, C-2), 14.4 (CH<sub>3</sub>, OCH<sub>2</sub>CH<sub>3</sub>); HRMS (ESI<sup>+</sup>) *m/z* calculated for C<sub>22</sub>H<sub>20</sub>NO<sub>5</sub> 378.1341, found 378.1341 (M + H<sup>+</sup>).

**(*RS*)-3-(Methoxycarbonyl)-5-oxo-3-phenyl-2,5-dihydro-3H-pyrrolo[2,1-*a*]isoindole-1-carboxylic acid, [*rac*-5]**

To a mixture of *rac*-4 (396 mg, 1.05 mmol), and NaOH (65 mg, 1.6 mmol) dissolved in MeOH (15 mL) was added water (1.0 mL), allowed to react for 8 h, at room temperature and then the solvent was evaporated. The product was mixed with water (10 mL) and EtOAc (10 mL) and, once the phases were separated, the aqueous phase was carefully acidified to pH 1 with aqueous 1 N HCl. The product was extracted with EtOAc (3 × 15 mL), the organic extracts were combined, washed with brine (10 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The crude product was purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 100:2). It was obtained as pale yellow solid; yield 297 mg (81%); mp 184–188 °C; TLC: R<sub>f</sub> = 0.29 (hexane/EtOAc, 7:3); ATR-FTIR  $\nu_{max}$  2955 (OH), 1730 (C = O ester), 1692 (C = O acid), 1647 (C = O lactam), 1587 (C = C); <sup>1</sup>H NMR (Chloroform-*d*, 301 MHz)  $\delta$  8.55 (1H, dt, *J* = 7.7, 1.0 Hz, H-9), 7.83 (1H, dt, *J* = 7.6, 1.0 Hz, H-6), 7.69 (1H, td, *J* = 7.6, 1.2 Hz, H-7), 7.60 (1H, td, *J* = 7.5, 1.1 Hz, H-8), 7.27 (3H, m, Ph), 7.14 (2H, m, H<sub>ortho</sub>, Ph), 4.44 (1H, d, *J* = 18.5 Hz, H-2 diastereotopic proton), 3.76 (3H, s, OCH<sub>3</sub>), 3.35 (1H, d, *J* = 18.5 Hz, H-2 diastereotopic proton); <sup>13</sup>C NMR (Chloroform-*d*, 101 MHz)  $\delta$  169.6 (C, MeOC(=O)), 167.1 (C, HOC(=O)), 163.8 (C, NC(=O)), 146.1 (C, C-9b), 137.4 (C, Ph), 134.1 (C, C-9a), 133.9 (CH, C-7), 132.0 (CH<sub>para</sub>, Ph), 129.5 (2CH<sub>meta</sub>, Ph), 129.0 (CH, C-8 y C, C-5a), 127.3 (CH, C-9), 124.4 (CH, C-6), 124.2 (2CH<sub>ortho</sub>, Ph), 111.2 (C, C-1), 72.2 (C, C-3), 52.1 (CH<sub>3</sub>, OCH<sub>3</sub>), 50.0 (CH<sub>2</sub>, C-2); HRMS (ESI<sup>+</sup>) *m/z* calculated for C<sub>20</sub>H<sub>16</sub>NO<sub>5</sub> 350.1028, found 350.1041 (M + H<sup>+</sup>).

**(*RS*)-1-(Ethoxycarbonyl)-5-oxo-3-phenyl-2,5-dihydro-3H-pyrrolo[2,1-*a*]isoindole-3-carboxylic acid, [*rac*-6]**

A flask with a reflux condenser was charged with a solution of *rac*-4 (593 mg, 1.57 mmol) and LiOH·H<sub>2</sub>O (180 mg, 2.3 mmol) in dry THF (50 mL). The mixture was allowed to react for 10 h to reflux temperature and then was cooled to room temperature. Subsequently, the solvent was evaporated, and the product was mixed with water (10 mL) and EtOAc (10 mL). After that, the phases were separated, the

aqueous phase was carefully acidified to pH 3 with aqueous 1 N HCl, and the product was extracted with EtOAc (3 × 15 mL), the organic extracts were combined, washed with brine (10 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane/EtOAc, 7:3). It was obtained as pale yellow solid; yield 311.9 mg (55%); mp 194–196 °C; TLC: R<sub>f</sub> = 0.36 (hexane/EtOAc, 7:3); ATR-FTIR  $\nu_{max}$  2937 (OH), 1732 (C = O ester), 1689 (C = O acid), 1650 (C = O), 1584 (C = C); <sup>1</sup>H NMR (Chloroform-*d*, 400 MHz)  $\delta$  8.67 (1H, d, *J* = 7.7 Hz, H-9), 7.94 (1H, dt, *J* = 7.5, 1.0 Hz, H-6), 7.79 (1H, td, *J* = 7.6, 1.2 Hz, H-8), 7.71 (1H, td, *J* = 7.8, 1.1 Hz, H-7), 7.38 (3H, m, Ph), 7.24 (2H, m, 2H<sub>ortho</sub>-Ph), 4.56 (1H, d, *J* = 18.5 Hz, H-2 diastereotopic proton), 4.32 (2H, m, OCH<sub>2</sub>CH<sub>3</sub>), 3.45 (1H, d, *J* = 18.5 Hz, H-2 diastereotopic proton), 1.36 (3H, t, *J* = 7.1 Hz, OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (Chloroform-*d*, 101 MHz)  $\delta$  169.7 (C, HOC(=O)), 167.2 (C, EtOC(=O)), 163.4 (C, NC(=O)), 145.8 (C, C-9b), 137.5 (C, Ph), 134.1 (C, C-9a), 133.8 (CH, C-7), 131.9 (CH, C-8), 129.5 (2CH<sub>meta</sub>, Ph), 129.1 (C, C-5a), 129.0 (CH<sub>para</sub>, Ph), 127.4 (CH, C-9), 124.4 (CH, C-6), 124.2 (2CH<sub>ortho</sub>, Ph), 111.8 (C, C-1), 72.3 (C, C-3), 61.3 (CH<sub>2</sub>, OCH<sub>2</sub>CH<sub>3</sub>), 50.1 (CH<sub>2</sub>, C-2), 14.2 (CH<sub>3</sub>, OCH<sub>2</sub>CH<sub>3</sub>); HRMS (ESI<sup>+</sup>) *m/z* calculated for C<sub>21</sub>H<sub>18</sub>NO<sub>5</sub> 364.1185, found 364.1194 (M + H<sup>+</sup>).

**(*RS*)-5-Oxo-3-phenyl-2,5-dihydro-3H-pyrrolo[2,1-*a*]isoindole-1,3-dicarboxylic acid, [*rac*-7]**

A flask with a reflux condenser was charged with a solution of *rac*-4 (592 mg, 1.58 mmol) and NaOH (280 mg, 7 mmol) in MeOH (70 mL) before the addition of water (6.7 mL). The mixture was allowed to react for 24 h to reflux temperature and then was cooled to room temperature. Subsequently, the solvent was evaporated, and the product was mixed with water (10 mL) and EtOAc (10 mL). After that, the phases were separated, the aqueous phase was carefully acidified to pH 1 with aqueous 1 N HCl, the product was recovered by filtration, and the crude product was purified by column chromatography (hexane/acetone, 7:3), and recrystallization MeOH/H<sub>2</sub>O (5:1). It was obtained as a white solid; yield 465 mg (88%); mp 205–207 °C; R<sub>f</sub> = 0.49 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH, 100:5:0.5); ATR-FTIR  $\nu_{max}$  3027–2575 (OH), 1712 (C = O acid), 1622 (C = O), 1585 (C = C); <sup>1</sup>H NMR (Methanol-*d*<sub>4</sub>, 400 MHz)  $\delta$  8.62 (1H, d, *J* = 7.6 Hz, H-9), 7.80 (1H, dt, *J* = 7.2, 1.0 Hz, H-6), 7.73 (1H, td, *J* = 7.6, 1.4 Hz, H-7), 7.67 (1H, td, *J* = 7.5, 1.2 Hz, H-8), 7.54 (2H, dd, *J* = 7.1, 1.6 Hz, H<sub>ortho</sub>-Ph), 7.35 (3H, m, Ph), 4.09 (1H, d, *J* = 17.9 Hz, H-2 diastereotopic proton), 3.67 (1H, d, *J* = 17.9 Hz, H-2 diastereotopic proton); <sup>13</sup>C NMR (Methanol-*d*<sub>4</sub>, 101 MHz)  $\delta$  173.3 (C, HOC(=O)), 166.8 (C, HOC(=O)), 165.6 (C, NC(=O)), 149.0 (C, C-9b), 139.7 (C, Ph), 136.9 (C, C-9a), 133.9 (CH, C-7), 132.9

(CH, C-8), 130.7 (C, C-5a), 129.5 (2CH<sub>meta</sub>, Ph), 129.2 (CH<sub>para</sub>, Ph), 128.1 (CH, C-9), 127.7 (2CH<sub>ortho</sub>, Ph), 124.5 (CH, C-6), 109.9 (C, C-1), 71.1 (C, C-3), 52.4 (CH<sub>2</sub>, C-2); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>19</sub>H<sub>14</sub>NO<sub>5</sub> 336.0872, found 336.0883 (M + H<sup>+</sup>).

**(1R,3R,9bS)- and (1S,3S,9bR)-9b-Hydroxy-3-(methoxycarbonyl)-5-oxo-3-phenyl-2,3,5,9b-tetrahydro-1H-pyrrolo[2,1-a]isoindole-1-carboxylic acid, [rac-8]**

A flask with a reflux condenser was charged with a mixture of *rac-3* (225 mg, 0.57 mmol) and LiOH·H<sub>2</sub>O (26 mg, 0.62 mmol) dissolved in dry THF (15 mL) before the addition of water (0.9 mL). The mixture was allowed to react for 1.5 h to reflux temperature and then was cooled to room temperature. The solvent was evaporated, and subsequently, the product was mixed with water (10 mL) and EtOAc (10 mL), the phases were separated, and the aqueous phase was carefully acidified to pH 1 with aqueous 1 N HCl to be later extracted with more EtOAc (3 × 10 mL). The organic extracts were combined, washed with brine (10 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Subsequently, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 100:5). It was obtained as a white solid; yield 165 mg (75%); mp 123–128 °C; TLC: R<sub>f</sub> = 0.51 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH, 100:5:0.5); **ATR-FTIR**  $\nu_{max}$  2957–2524 (OH), 1713 (C = O ester), 1703 (C = O acid), 1657 (C = O), 1610 (C = C); **<sup>1</sup>H NMR** (Methanol-*d*<sub>4</sub>, 400 MHz)  $\delta$  8.00 (1H, dt, *J* = 7.5, 1.0 Hz, H-6), 7.73 (1H, dt, *J* = 9.1, 1.2 Hz, H-9), 7.69 (1H, dd, *J* = 7.5, 1.2 Hz, H-7), 7.60 (1H, td, *J* = 7.5, 1.0 Hz, H-8), 7.50 (2H, m, H<sub>ortho</sub>-Ph), 7.35 (2H, m, H<sub>meta</sub>-Ph), 7.29 (1H, m, H<sub>para</sub>-Ph), 3.61 (3H, s, OCH<sub>3</sub>), 3.46 (dd, *J* = 12.2, 7.1 Hz, 1H, H<sub>a</sub>-1), 3.36 (t, *J* = 12.5 Hz, 1H, H<sub>b</sub>-2 diastereotopic proton), 3.25 (dd, *J* = 12.9, 7.1 Hz, 1H, H<sub>c</sub>-2 diastereotopic proton); **<sup>13</sup>C NMR** (Methanol-*d*<sub>4</sub>, 101 MHz)  $\delta$  173.5 (C, HOC = O), 172.3 (C, MeOC = O), 171.2 (C, NC = O), 148.1 (C, C-9a), 142.7 (C, Ph), 134.4 (CH, C-8), 133.0 (C, C-5a), 131.2 (CH, C-7), 128.8 (2CH<sub>meta</sub>, Ph), 128.4 (CH<sub>para</sub>, Ph), 128.4 (2CH<sub>ortho</sub>, Ph), 126.0 (CH, C-6), 124.3 (CH, C-9), 97.6 (C, C-9b), 69.6 (C, C-3), 53.3 (CH, C-1), 50.7 (CH<sub>3</sub>, OCH<sub>3</sub>), 46.1 (CH<sub>2</sub>, C-2); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>20</sub>H<sub>16</sub>NO<sub>5</sub> 350.1028, found 350.1039 (M + H<sup>+</sup> - H<sub>2</sub>O).

**(1R,3R,9bS)- and (1S,3S,9bR)-9b-Hydroxy-5-oxo-3-phenyl-2,3,5,9b-tetrahydro-1H-pyrrolo[2,1-a]isoindole-1,3-dicarboxylic acid, [rac-9]**

A flask with a reflux condenser was charged with a mixture of *rac-3* (298 mg, 0.75 mmol) and LiOH·H<sub>2</sub>O (158 mg, 3.75 mmol) dissolved in dry MeOH (9 mL) before the

addition of water (1.0 mL). The mixture was allowed to react for 1.5 h to reflux temperature and then was cooled to room temperature. The solvent was evaporated, and subsequently, the product was mixed with water (10 mL) and EtOAc (10 mL). After that, the phases were separated, and the aqueous phase was carefully acidified to pH 1 with aqueous 1 N HCl to be later gently extracted with more EtOAc (3 × 10 mL), the organic extracts were combined, washed with brine (10 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Subsequently, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 100:5). It was obtained as a white solid; yield 125 mg (47%); mp 165–168 °C; TLC: R<sub>f</sub> = 0.35 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH, 100:5:0.5); **ATR-FTIR**  $\nu_{max}$  3062–2918 (OH), 1736 (C = O), 1656 (C = O acid), 1612 (C = C); **<sup>1</sup>H NMR** (Methanol-*d*<sub>4</sub>, 400 MHz)  $\delta$  7.99 (1H, d, *J* = 7.6 Hz, H-8), 7.74 (1H, d, *J* = 7.5 Hz, H-7), 7.69 (1H, td, *J* = 7.6, 1.2 Hz, H-6), 7.59 (1H, td, *J* = 7.6, 1.1 Hz, H-9), 7.54 (2H, dd, *J* = 7.1, 1.7 Hz, H<sub>ortho</sub>-Ph), 7.34 (2H, dd, *J* = 8.3, 6.5 Hz, H<sub>meta</sub>-Ph), 7.27 (1H, m, H<sub>para</sub>-Ph), 3.48 (1H, dd, *J* = 12.3, 7.0 Hz, H<sub>a</sub>-1), 3.31 (1H, m, H<sub>b</sub>-2 diastereotopic proton), 3.23 (1H, dd, *J* = 12.9, 7.0 Hz, H<sub>c</sub>-2 diastereotopic proton); **<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 101 MHz)  $\delta$  172.4 (C, HOC = O), 170.5 (C, HOC = O), 168.5 (C, NC = O), 147.0 (C, C-9a), 142.5 (C, Ph), 133.0 (CH, C-8), 132.2 (C, C-5a), 130.0 (CH, C-7), 127.6 (2CH<sub>meta</sub>, Ph), 127.5 (2CH<sub>ortho</sub>, Ph), 127.0 (CH<sub>para</sub>, Ph), 124.8 (CH, C-6), 123.0 (CH, C-9), 95.9 (C, C-9b), 67.9 (C, C-3), 49.4 (CH, C-1), 44.6 (CH<sub>2</sub>, C-2); **HRMS (ESI<sup>+</sup>)** *m/z* calculated for C<sub>18</sub>H<sub>14</sub>NO<sub>3</sub> 292.0974, found 292.0969 (M + H<sup>+</sup> - CO<sub>2</sub> - H<sub>2</sub>O).

## Solutions

The required solutions were prepared following our previous reports [20–23]. Phosphate buffer solution 0.1 M and pH 8, sodium hydroxide solution 4.2 M, hydroxylamine hydrochloride solution 2.4 M; The alkaline hydroxylamine solution was prepared with equal volumes of the aforementioned sodium hydroxide and hydroxylamine solutions, which were mixed immediately before use; 0.75 M ferric chloride solution was afforded using 7.5 M HCl solution as a diluent; 256 mM acetylthiocholine (Ach) stock solution (to prepare Ach 48, 32, 24, 16, 12, 8, 6 and 4 mM), the AChE stock solution (1 U/mL, *Electrophorus electricus* AChE, Sigma Chemical C3389). The test compounds and reference drugs (neostigmine and galantamine) were prepared by adding DMSO (<1%) or distilled water as the dissolution medium.

## Acetylcholinesterase activity assay

We followed the modified Bonting and Featherstone method previously reported by our workgroup [21, 24],



briefly, 20  $\mu\text{L}$  of each solution (AChE, inhibitor, and ACh) was placed in a 96-well microplate with buffer solution to afford a final volume of 160  $\mu\text{L}$  for the inhibition curve (for standard curve the inhibitor was substituted for distilled water). Then, 96-well microplate was incubated for 20 min at 37  $^{\circ}\text{C}$  in a water bath and stopped by adding 40  $\mu\text{L}$  of alkaline hydroxylamine solution. Finally, 100  $\mu\text{L}$  of  $\text{FeCl}_3$  solution was added and after premixing for 30 s, the optical density was read at 540 nm in a microplate reader (Accuris MR9600). All assays were performed in triplicate.

## Molecular docking

To better understand the results obtained in the in vitro experiments and find out if there is a significant difference between the enantiomers that make up the different racemic mixtures, molecular docking was performed with the enzyme *Electrophorus electricus* Acetylcholinesterase (*EeAChE*), which was obtained from Protein Data Bank (PDB) and whose code is 1C2O [25]. This enzyme corresponds to the one used in the in vitro model to have a better comparison with the experiments carried out. The methodology used was the one that we have previously reported in our work group [18, 21, 22, 26, 27], briefly, the optimization of the geometry of each ligand was carried out using Gaussian 16 and GaussView 6 software [28], the theoretical level used was PM3 “# opt freq pm3 geom=connectivity” which corresponds to a semi-empirical level in the molecular mechanic, the ionized form was also considered at a physiological pH (7.4) for each ligand. The preparation of the molecular approach was carried out with AutoDock tools 1.5.6 and Raccoon [29, 30], the configuration used was the following; for the enzyme, the Kollman partial charges were established and the polar hydrogens were added; For the ligands and reference molecules, the number of rotational bonds, the degree of torsional freedom, and Gasteiger charges were established, the center of the grid box was established at the coordinates  $X = 42.27$ ,  $Y = 66.809$ , and  $Z = -81.47$  with a box size of 60  $\text{\AA}$  per side and a mesh separation of 0.375  $\text{\AA}$  [21], for the molecular approach the hybrid Lamarckian genetic algorithm was used with 100 conformations for the initial population. Subsequently, Fedora 22 was used as the operating system, and AutoDock4 [30] as the software. Finally, the results were obtained as Gibbs free energy  $\Delta G$ ,  $K_d$ ,  $pK_d$ , type of interaction, and amino acids involved in recognition using AutoDock Tools 1.5.6 and BIOVIA Discovery Studio 2020 [30], with the latter obtaining the images of the different interactions. To validate the docking, the reference molecules donepezil, galantamine, rivastigmine, and tacrine were used as previously reported [18, 21].

## Statistical analysis

GraphPad Prism software was used to obtain  $K_i$  by using non-linear regression and the type of inhibition was obtained by Lineweaver-Burk plot. The results are the mean  $\pm$  95% confidence intervals for all the assays.

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1007/s00044-023-03141-8>.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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