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2-Phenylbenzofuran derivatives as butyrylcholinesterase inhibitors: Synthesis, biological activity and molecular modeling

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Abstract

A series of 2-phenylbenzofurans compounds was designed, synthesised and evaluated as cholinesterase inhibitors. The biological assay experiments showed that most of the compounds displayed a clearly selective inhibition for butyrylcholinesterase (BChE), while a weak or no effect towards acetylcholinesterase (AChE) was detected. Among these benzofuran derivatives, compound **16** exhibited the highest BChE inhibition with an IC_{50} value of 30.3 μM. This compound was found to be a mixed-type inhibitor as determined by kinetic analysis. Moreover, molecular dynamics simulations revealed that compound **16** binds to both the catalytic anionic site (CAS) and peripheral anionic site (PAS) of BChE and it displayed the best interaction energy value, in agreement with our experimental data.

Keywords: Alzheimer's disease; Acetylcholinesterase; Butyrylcholinesterase; Benzofurans; Cholinesterase inhibitors; Molecular dynamics; Docking

Alzheimer's disease (AD) is an irreversible and progressive brain disorder, which is characterized by progressive memory loss and a wide range of cognitive impairments.[1] Although the precise cause of AD is not completely known, there are some factors that have been described to play a significant role in the pathogenesis of AD, such as: deficit of acetylcholine (ACh), presence of amyloid-β deposits, τ-protein aggregation, oxidative stress and metal ions imbalance. Among these distinct research approaches, the cholinergic hypothesis has been examined more extensively. In fact, low levels of ACh appear to be a critical element in the development of cognitive and neurodegenerative disorders in AD patients.[2]

Accordingly, one strategy in AD treatment is to restore the levels of ACh by inhibiting acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), which are mainly responsible for ACh hydrolysis. These enzymes belong to the superfamily of α/β-hydrolase fold proteins and they are able to hydrolyse ACh with different efficiencies.[3] They are encoded by two distinct human genes and display 65% homology in their amino acid sequences. These two proteins also show a great similarity in both their tertiary structure and their overall architecture of active sites.[4,5] Both AChE and BChE have indeed a primarily hydrophobic active gorge into which ACh diffuses and is cleaved.

Ligand binding specificity between the two enzymes has been related to differences in the residue's structural arrangement, which lead to the active site located near the bottom of a deep and narrow gorge (Figure 1, Table S1 in Supplementary Material). The gorge is characterised by (a) a peripheral site at the entrance, (b) an oxyanion hole, (c) a choline-binding site located within the entrance, and (d) the active site constituted by an acyl pocket buried near the catalytic triad.

In fact, structural analysis had revealed that these enzymes have two major substrate-binding sites. One is a peripheral anionic site (PAS) at the entrance of the gorge, acting as a regulatory site; the other is the catalytic anionic site (CAS), which is located in the bottom of the gorge and it is assigned to a Ser-His-Glu catalytic triad.[6]

AChE and BChE appear to be simultaneously active in the synaptic hydrolysis of ACh, terminating its neurotransmitter action and co-regulating levels of ACh.[7] AChE has a very high catalytic efficiency for ACh hydrolysis and it is mainly found in cholinergic synapses, while BChE has lower efficiency and it is widely distributed in tissues and plasma. In a healthy brain, AChE predominates and BChE is considered to play a minor role in regulating ACh levels. On the other hand, BChE activity increases in the temporal cortex and hippocampus during the development of AD, while at the same time AChE activity decreases.[8]

Since AD is characterized by a forebrain cholinergic neuron loss and a progressive decline in ACh, a possible therapeutic strategy involves the use of cholinesterase (ChE) inhibitors to restore the neurotransmitter levels and thus retard AD symptoms.[9-11]

These inhibitory molecules may act by binding the CAS site (competitive mechanism) or PAS (non-competitive mechanism) or may exert a dual binding enzyme inhibition – acting as mixed-type inhibitors.[12] Moreover, since the oxidative stress may be a risk factor for the initiation and progression of AD drugs, both antioxidant and inhibitory actions might be useful for either the prevention or the treatment of AD.

Benzofuran scaffold (oxygen heterocycle) is a common moiety found in many biologically active natural and synthetic products. Therefore, it represents a very important pharmacophore in drug discovery.[13] It is present in many medicinally important compounds which show biological activity, including anticancer and anti-inflammatory properties.[14,15] Benzofuran scaffold has drawn considerable attention over the last few years due to its profound physiological and chemotherapeutic properties.[16] Some benzofuran derivatives are also known as monoamine oxidase and 5-lipoxygenase inhibitors, antagonists of the angiotensin II receptor, blood coagulation factor Xa inhibitors, ligands of adenosine A_1 receptor, etc.[13,17] Recent studies have also investigated their inhibitory activity towards AChE.[18-21]

In this study, a series of 2-phenylbezonfurans was synthesized and their inhibitory activity towards the ChE was investigated. To better understand the enzyme inhibition mechanisms, in relation to the

substituents and their positions in the presented compounds, molecular modeling studies were also performed.

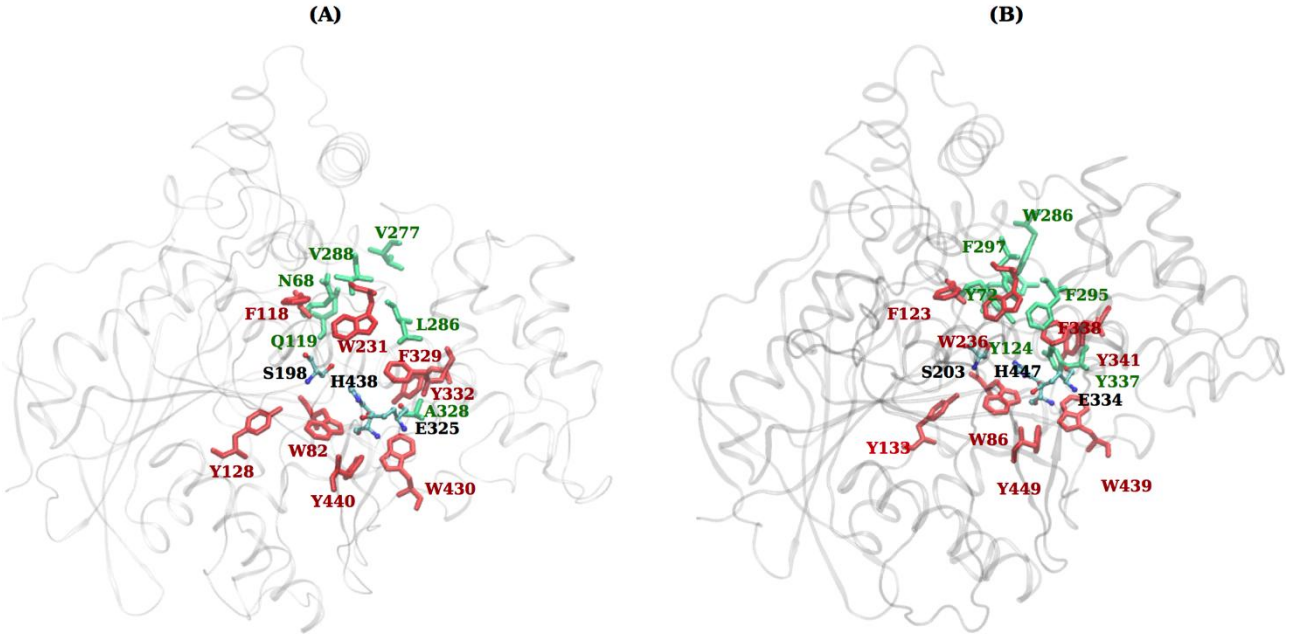
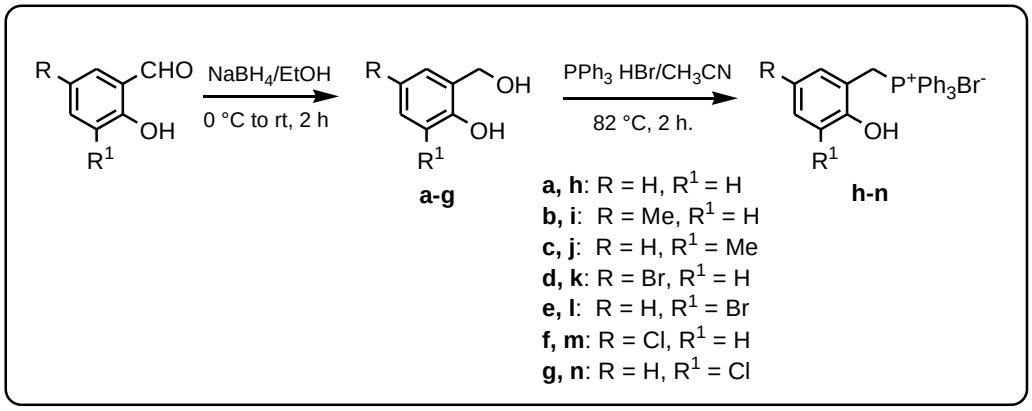
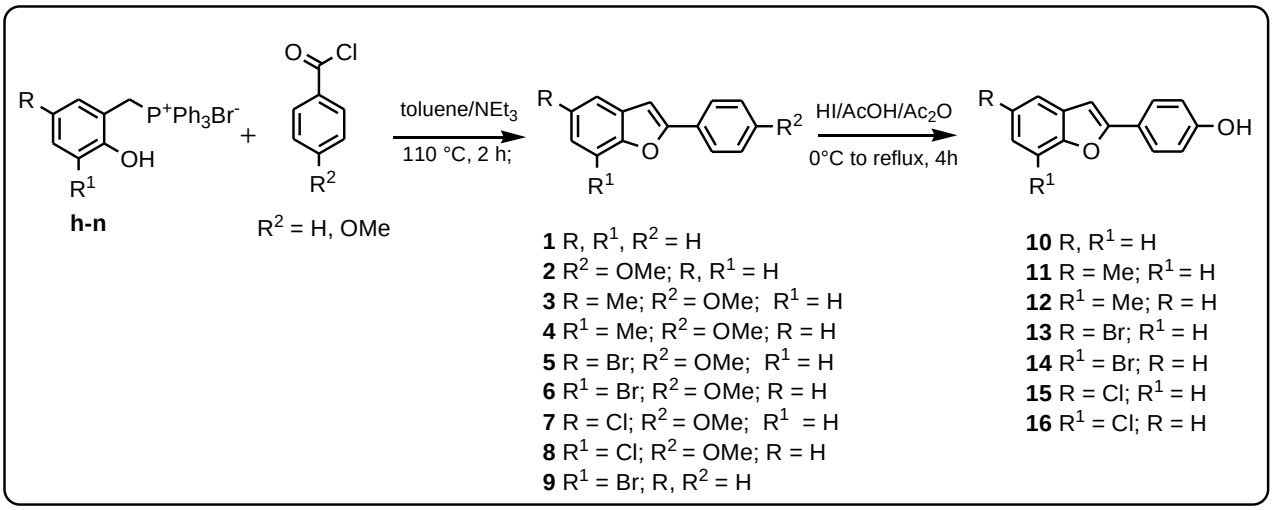


Figure 1. Cartoon representation for the enzymes under investigation. (A) *Equine serum* BChE (B) *Electrophorus electricus* AChE. The residues lining the gorge of the two enzymes are shown. The conserved residues between the two are shown in red (licorice representation) and non-conserved in green. The catalytic triad residues are shown as ball-stick representation.

Compounds **1-16** were efficiently synthesized by an intramolecular Wittig reaction (Scheme 1 and 2). The desired Wittig reagent was readily prepared from the conveniently substituted *ortho*-hydroxybenzyl alcohol **a–g** and triphenylphosphine hydrobromide (Scheme 1).[22-31] The key step for the formation of the benzofuran moiety was achieved by an intramolecular reaction between *ortho*-hydroxybenzyltriphosphonium salts **h–n** and the appropriate benzoyl chlorides (Scheme 2) (Supplementary Material).[30,32-39]



Scheme 1



Scheme 2

Hydrolysis of the methoxy groups of compound **2–8** was performed by treatment with hydrogen iodide in acetic acid/acetic anhydride, to gave the corresponding hydroxy derivatives **10–16**.[30,33,40-42] We report the ^{1}H NMR, ^{13}C NMR and mass spectrometry analysis (Supplementary Material Fig. S1-S3) of our more active compound **16**.

The AChE and BChE inhibitory activity[43] of all synthetized compounds was firstly evaluated at compound concentration of 100 µM (Supplementary Material). As observed, only compounds **9** and **11** exerted a very weak inhibitory activity towards AChE, while all the compounds, except compounds **2** and **10**, inhibited BChE enzymatic activity with a varying efficiency (Table 1). In

particular, compounds **12**, **14** and **16** showed the highest inhibition percentages and the lowest IC_{50} values.

Table 1. Cholinesterase inhibitory activity of compound 1, 2, 8-16.

Compound	**% Inhibition at 100 µM**		**IC_{50} ± SD[a] (µM)**
	AChE	**BChE**	**BChE**
1	-	6	> 100
2	-	-	> 100
8	-	5	> 100
9	4	12	> 100
10	-	-	> 100
11	4.8	19	> 100
12	-	58	77 ± 6.7
13	-	16.6	> 100
14	-	54	82.5 ± 7.1
15	-	15	> 100
16	-	77	30.3 ± 1.9
Galantamine			28.3 ± 2.1

[a] Data represent the mean (± standard deviation, SD) of three independent experiments

Compound **16** was found to be the best BChE inhibitor with an IC_{50} value of 30.3 µM. Thus, it has been chosen for the kinetic studies. We investigated the kinetic behaviour of BChE at different concentrations of inhibitor and substrate (BTCI). In the presence of compound **16**, the Lineweaver–Burk plots showed that this compound was a mixed-type inhibitor, since increasing the concentration of compound **16** resulted in a family of straight lines which intersected in the second quadrant (Figure 2). In this case, the inhibitor can bind not only with the free enzyme but also with the enzyme-substrate complex. The equilibrium constants for binding with the free enzyme (K_I) and

with the enzyme–substrate complex (K_{IS}) were obtained either from the slope or the V_{max} values (y-intercepts) plotted against inhibitor concentration, respectively. The values of K_I and K_{IS} of compound **16** were determined to be 5.0 and 87.4 μM, respectively.

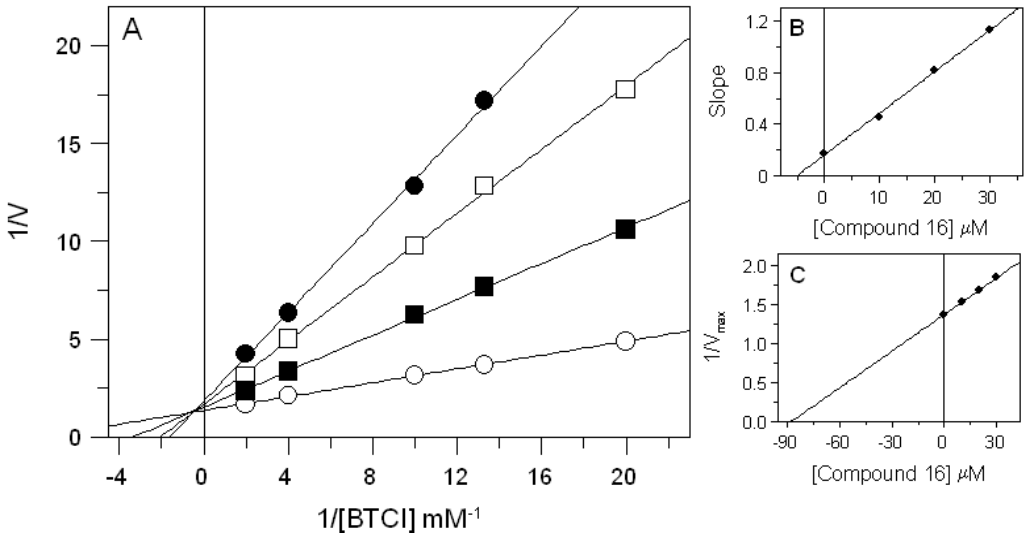


Figure 2. Kinetic study of the mechanism of BChE inhibition displayed by compound 16. In (A) Overlaid Lineweaver-Burk reciprocal plots of BChE initial velocity at increasing substrate concentration (0.05-0.50 mM) in the absence and in the presence of compound **16**. The concentrations of inhibitor were 0 (○), 10 (■), 20 (□) and 30 (●) μM; (B) Secondary plot of slopes *versus* compound **16** concentrations and (C) Secondary plot of $1/V_{max}$ values *versus* compound **16** concentrations.

Our in depth analysis showed that the simplest 2-phenylbenzofuran (compound **1**), without any substitution, displayed no inhibitory activity towards AChE and BChE. The introduction of a methoxy or a hydroxy substituent in the *para* position of the 2-phenyl ring led to compounds **2** and **10**, respectively. Both compounds proved to be inactive against both ChE enzymes.

We then studied the effect of the introduction of an electron-donating group (methyl) or an electron-withdrawing group (bromine and chlorine) at positions 5 or 7 of the benzofuran nucleus. The introduction of a methyl substituent at these positions of the 2-(4-hydroxyphenyl)benzofuran (**10**)

led to compounds **11** and **12**, respectively. Compound **12**, which only differs from compound **11** in its respective position of methyl group (position 7 compared to position 5, Scheme 2), showed an increase in its BChE inhibitory activity. Interestingly, substitution of bromine at position 7 (compound **14**), increases the enzymatic inhibition respecting to substitution at position 5 (compound **13**), which is consistent with the trend observed on methyl substitutions. Furthermore, substitution with chlorine at position 7 (compound **16**) significantly improved the inhibitory activity towards BChE, if compared with compound **15** (chlorine at position 5).

In summary, our data for 2-phenylbenzofuran derivatives with hydroxyl at *para* position and substitution with methyl, bromine and chlorine at position 7, resulted in a better inhibition value regarding to the same substitution at position 5.

Therefore, the most relevant structural feature of the synthesized 2-phenylbenzofurans is the contemporary presence of a hydroxy group at *para* position of the 2-phenyl ring and a substitution at position 7; all these compounds (**12, 14** and **16**) being active against BChE.

Since oxidative stress is closely associated with the development of AD,[44] antioxidant activity of the described compounds was evaluated by measuring radical scavenging capacity by ABTS assay (Supplementary Material).[45] As expected, compounds without hydroxylic groups in their structure (compounds **1**, **2, 8** and **9**) did not present radical scavenging activity (data not shown). Compounds **12**, **14** and **16**, which are the best BChE inhibitors, exhibited the highest antioxidant activity (Table 2). Taking into account these results, and the structural characteristics of the compounds, we observed that the concurrently presence of a hydroxyl group in the *para* position of the phenyl ring and the substitution at position 7 of the benzofuran scaffold, appeared to play an important role in determining the desired antioxidant activity.

In addition to the above biological evaluation, the potential cytotoxicity effect of compound **16** on the cell line NSC-34 (motor-neuron) was evaluated to determine the safety of this molecule (Supplementary Material). After treating the cells with this compound at different concentrations (5-100 μM) for 48 h, the cell viability was determined (Figure 3). The results indicate that this

inhibitor exhibited no considerable cytotoxic effect on NSC-34 neuron like cell at the concentration at which it inhibits the ChE activity.

Table 2. Radical scavenging activity of compounds 10-16.

Compound	ABTS scavenging	
	% Inhibition at 100 µM	**IC_{50} ± SD[a] (µM)**
10	24	-
11	31	-
12	72	53.5 ± 4.7
13	15	-
14	53	93.6 ± 6.4
15	15	-
16	62.4	67.6 ± 4.5
Trolox[b]		13 ± 1.1

[a] Data represent the mean (± standard deviation, SD) of three independent experiments

[b] Trolox was used as the positive control.

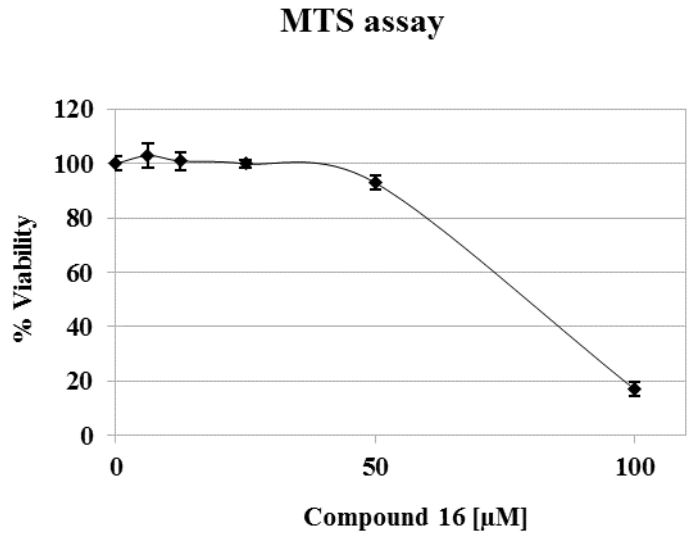


Figure 3. The effect of compound 16 on NSC-34 cell viability. Cells were treated with different concentrations of compound (5-100 µM) and studied by MTS reagent. Data is expressed as a percentage of the control.

To better understand the impact of substitutions (benzofuran moiety and 2-phenyl ring) on enzyme inhibitory ability, compounds **8**, **15** and **16** were selected for computational modeling (Fig. S5 Supplementary Material). Molecular docking results showed that the three compounds were able to interact with CAS residues in BChE and with PAS in AChE with very similar docking energies (~6-7 kcal/mol). To investigate the structural and dynamical aspects upon ligand binding we simulated the two enzymes for 50 ns in the free and ligand bound configurations. In the case of AChE, all the compounds (**8**, **15** and **16**) did not interact with any CAS residues. Therefore no enzymatic inhibition was observed. On the other hand, in the case of BChE, the three compounds interacted with at least one of the three CAS residues, displaying enzymatic inhibition. Therefore, the simulations of our models suggested the interaction of 2-phenylbenzofurans with CAS residues to be crucial for enzymatic inhibition. However, the levels of BChE inhibition varied in the three compounds, displaying compound **16** the highest value (77%). Based on these results, we decided to focus our attention on the interaction of these three compounds with BChE.

Enzyme dynamics was investigated in the presence of these compounds using Prody software.[46] The results obtained (Fig. S6, Supplementary Material) highlighted the importance of the substitution at position 7 in enzyme dynamics. We further estimated the binding strength between the enzyme residues and the three compounds. Interestingly, compound **16** displayed the best interaction energy value and also possessed the highest percentage of its total surface area buried in the gorge (Table S2, Supplementary Material). To better understand the origin of these differences, we carefully inspected the binding mode of the ligands in complex with BChE (Figure 4), and with AChE (Figure 5, shown for comparison) using Ligplot.[47]

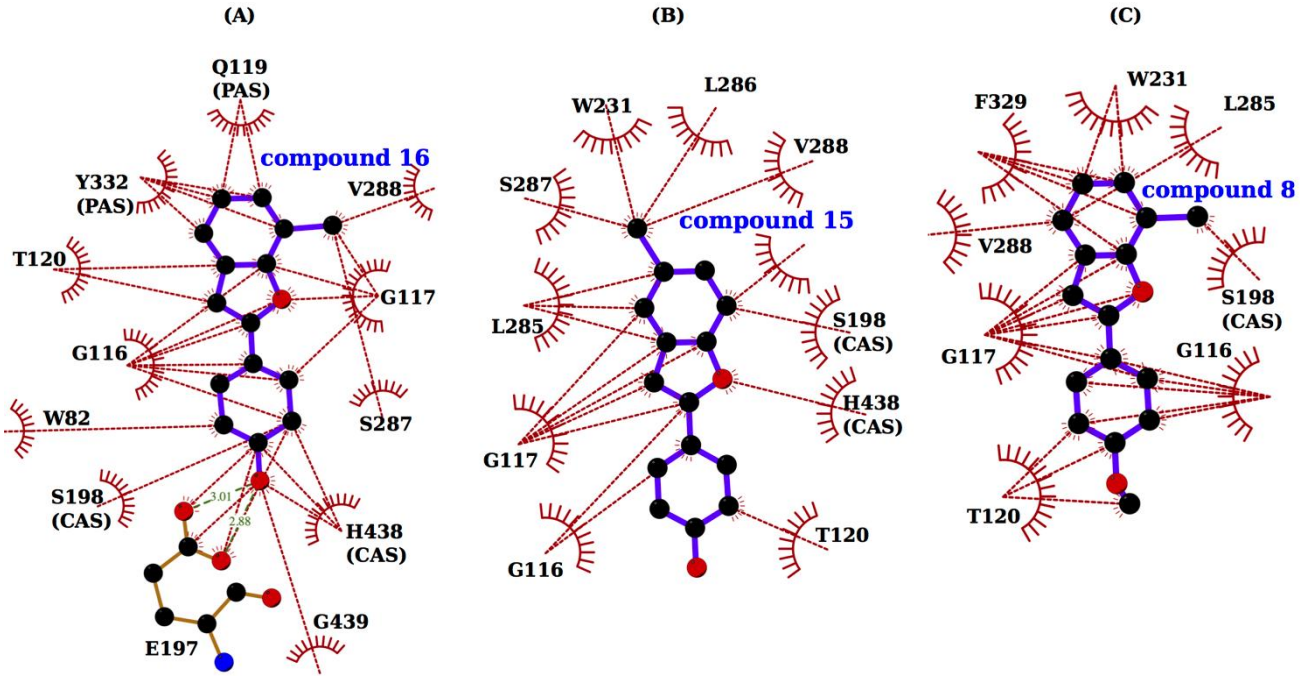


Figure 4. Molecular interaction picture of *equine serum* BChE bound to the three compounds. In (A) compound **16** (B) compound **15** and (C) compound **8**.

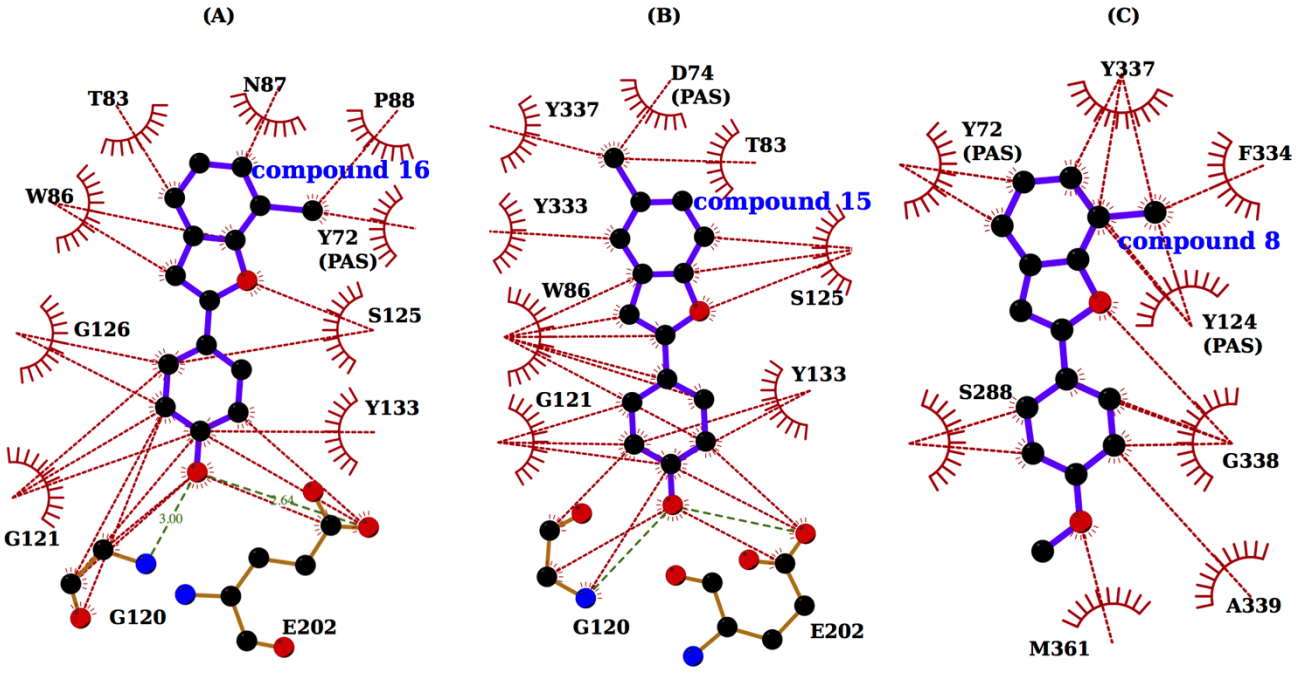


Figure 5. Molecular interaction picture of *Electrophorus electricus* AChE bound to the three compounds. In (A) compound **16** (B) compound **15** and (C) compound **8**.

Previous biochemical and molecular studies[48] found the difference in the inhibitory property of E2020 towards AChE and BChE to be related to simultaneous participation of CAS and PAS binding residues (present only for AChE). Our modeling results confirm a similar effect for 2-phenylbenzofurans (compound **16**, Figure 4). Interestingly, in all three complexes, the compounds globally interacted with CAS residues (S198, H438). However, only in compound **16** we found benzofuran moiety to interact with PAS residues (Q119, Y332) and 2-phenyl ring moiety to interact with CAS residues and with residues W82, E197 and G439. Therefore, explaining the high interaction energy (Table S2, Supplementary Material) and the related high inhibition (Table 1). In a recent study, residue Q119 was described to be important towards selective inhibition of mouse BChE by two biscarbamates compounds[49] and residue W82 (W86 in AChE) as a crucial component of the anionic site[6] and as a controller for opening and closing of CAS.[50] Other experimental studies further analyzed the relevance of residue E197 (E202 in AChE) in substrate inhibition[51] and of residue Y332 (Y341 in AChE) in substrate binding.[52] Overall, jointly with all these previous proposals and findings, our current experimental and modeling results confirm and explain the highest BChE inhibitory activity noticed for compound **16**.

In conclusion, in this study a series of 2-phenylbenzofurans has been designed, synthesized and evaluated for its ChE inhibitory activity. These compounds showed no inhibition against AChE, while inhibiting BChE with various levels of efficiency. Compounds **12**, **14** and **16** proved to be the most potent inhibitors. These compounds also displayed the highest antioxidant activity as well. According to our results, the contemporary presence of a hydroxy group in the *para* position of the phenyl ring and a substitution at position 7 of the benzofuran scaffold, improved the inhibitory activity, regarding to the other synthesized compounds. In particular, compound **16** exhibited the highest BChE inhibition. In addition, kinetic analysis indicated that compound **16** is a mixed-type inhibitor with no considerable cytotoxic effect on NSC-34 cells.

Molecular modeling demonstrated that the interaction of 2-phenylbenzofurans with CAS residues is crucial for the enzymatic inhibition. Our simulation also revealed that compound **16** binds both

CAS and PAS sites in BChE, in accordance to the experimental data, which showed that this compound acts as a mixed-type inhibitor.

We can therefore conclude that the combination of biological assays and molecular dynamics simulations allowed highlighting the molecular basis of the selective BChE inhibitory activity by the benzofuran scaffold.

Since the BChE activity progressively increases in patients with AD, while AChE activity remains unchanged or declines during the disease course, the use of molecules that selectively interact with BChE might have a relevant role in treatment of patients with advanced AD. In this scenario, our findings could be extended to design and develop new potentially useful selective therapeutic molecules.

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