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Design, Synthesis and Cholinesterase Inhibitory Activity of New Dispiro Pyrrolidine Derivatives

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Abstract

A series of new dispiro pyrrolidines were regioselectively synthesised via [3+2]-cycloaddition reactions of 3,5-bis(arylidene)-1-phenylethyl-4-piperidones with azomethine ylides generated in situ from *N*-methylglycine and appropriate isatin derivatives. The structures of the synthesised compounds were characterised by NMR, FT-IR and MS. These compounds were assayed *in vitro* for their inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), using Ellman's assay. The results demonstrate better inhibitory activity against butyrylcholinesterase as compared to acetylcholinesterase. Compound **7b** exhibits potential as a new BChE inhibitor, with an IC_{50} of $12.78 \pm 1.52 \mu\text{M}$. A kinetic study suggests **7b** as a mixed-mode inhibitor, where the active molecule can bind to the active or allosteric sites of the enzyme. An *in silico* study was performed using AutoDock Vina to identify the binding energy and conformation of **7b** with the crystal structure of BChE complexed with Thioflavin T (PDB ID: 6ESY) and the crystal structure of human AChE complexed with dihydrotanshinone 1 (PDB ID: 4M0E). The results indicate better binding properties of **7b** compared with the standard inhibitor Thioflavin T, with calculated binding energies of -13.9 and $-9.7 \text{ kcal mol}^{-1}$ for BChE and AChE, respectively.

Keywords: Dispiro pyrrolidine; Dispiro heterocycle; 1,3-Dipolar cycloaddition; Cholinesterase; Molecular docking

1. Introduction

Spiro compounds represent an important class of naturally occurring compounds found in plants and animals [1]. A number of studies have shown that various 2-indolone compounds (oxindoles) having a spiro system are of particular interest and can exhibit promising and diversified biological activities [2-4], such as anticancer [5-7], anti-Alzheimer [8-10],

antidiabetic [11], antimicrobial [12-14], antituberculosis [12-15], antiviral [16], local anesthetic [17], antileukemic and anticonvulsant [18]. The widely known biological potency of spiro compounds and oxindoles has led researchers to investigate and synthesise new spiro heterocyclic compounds comprising oxindole and pyrrolidine moieties.

Alzheimer's disease (AD) is a multifarious neurodegenerative disorder. It is the most prevalent form of dementia that may contribute 60-70% of cases. According to the World Health Organisation (WHO), the symptoms of Alzheimer's patients are manifested by progressive deterioration of cognitive function, intellectual decline, memory loss and personality change [19]. Currently, around 55 million people suffer from AD worldwide, and almost 10 million new cases are reported each year. The number of people living with dementia is estimated to reach 78 million in 2030 and 139 million by 2050 [19]. Studies have shown that women are more frequently affected by AD, with 16% prevalence at the age of 71 and above, compared to 11% of men. People with higher education years are reported to be at lower risk [20].

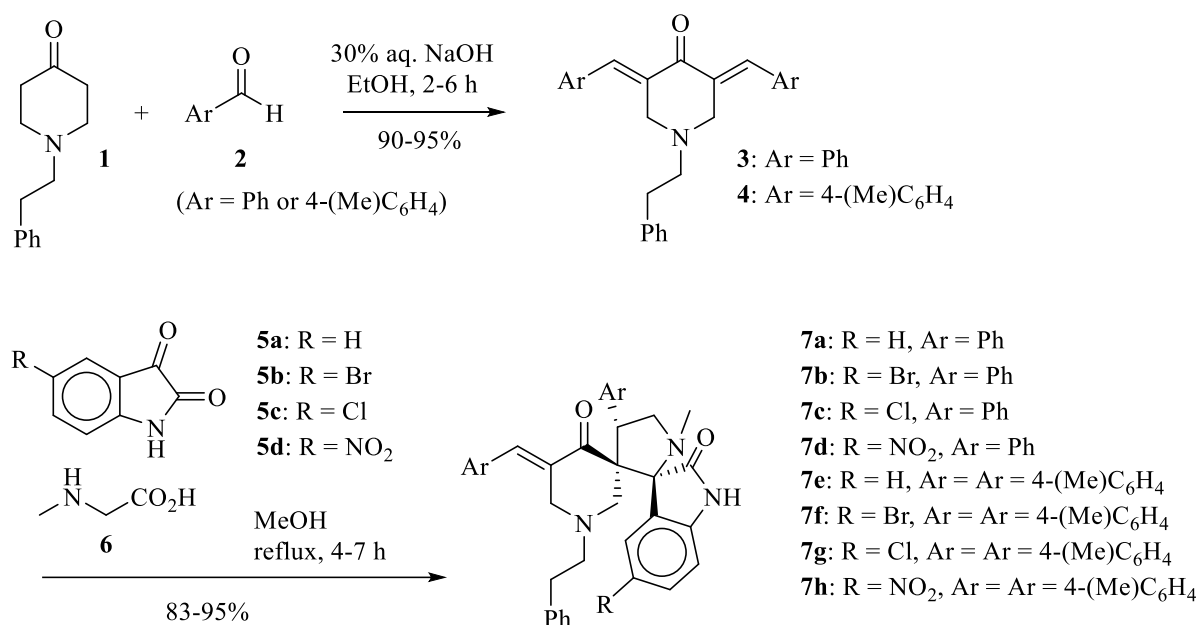
Acetylcholine (ACh) is an important neurotransmitter that helps transfer nerve messages in the human central nervous system. A low concentration of ACh arising from neurodegeneration and reduced availability of choline acetyltransferase prompts the development of the symptoms of AD [21]. Cholinesterases are the enzymes responsible for breaking down ACh. The brain contains two types of cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Both of them are hydrolytic enzymes that react with ACh and terminate its actions at the synaptic cleft by cleaving the neurotransmitter to choline and acetate ion [22-23]. Therefore, one of the strategies for the treatment of AD is to find an inhibitor that can inhibit AChE and BChE, thus prolonging the availability of ACh at the synaptic cleft. Examples of such cholinesterase inhibitors that are available on the market to treat AD patients include rivastigmine, donepezil and galantamine.

In this context and based on our previous work on this series [8-10], we are now reporting the synthesis of new dispiro pyrrolidine derivatives, as well as their cholinesterase inhibitory properties, evaluated against AChE and BChE. These experimental data are completed by molecular modelling studies that help understand the interaction of the most active inhibitor with the appropriate target active site of the cholinesterase enzymes.

2. Results and Discussions

2.1. Synthesis of dispiro pyrrolidine compounds

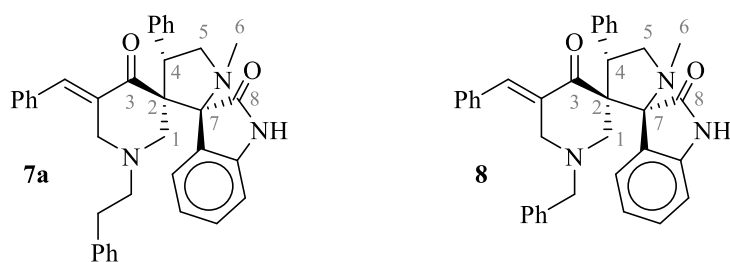
The target dispiro pyrrolidine compounds **7a-h** were synthesised by 1,3-dipolar cycloaddition reactions of azomethine ylides, generated *in situ* by decarboxylative condensation of sarcosine **6** with isatin derivatives **5a-d**, in the presence of 3,5-bis(arylidene)-1-phenethyl-4-piperidinone **3** or **4** as appropriate dipolarophiles (Scheme 1) [5-7,24, 33-34].



Scheme 1 Synthesis of spiro pyrrolidine derivatives **7a-h**.

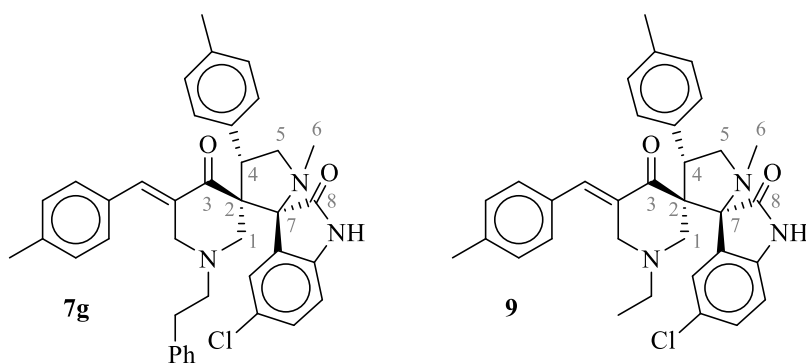
The cycloadducts were obtained in good to excellent yields (83-95%). Their structures were confirmed by spectroscopic techniques including 1D- and 2D-NMR, FT-IR and HRMS (see supplementary data). Remarkably, all these products were obtained as single diastereoisomers. The relative configurations of their chiral centres were established by comparison of key ^{13}C NMR chemical shifts with those of closely related compounds, the structures of which have been unambiguously determined by X-ray crystallography in previous work [5-7,32-33]. For instance, the chemical shifts of the carbon atoms located around the chiral centres of **7a** were all found to be different from those of the known molecule **8** by no more than 1.1 ppm (average deviation 0.5 ppm; Table 1) [32]. Furthermore, in the comparison of **7g** and the analogous compound **9**, [5] the maximal difference was found to be 0.2 ppm (Table 2). In addition, **8** and **9** were synthesised using the same route as **7a** and **7g**. Therefore, the relative configurations of the latter can be very safely assigned as shown in Scheme 1. The same ^{13}C NMR shift comparative process strongly supports identical relative configurations in **7a**, **7c** and **7e**. From there, it is reasonable to assume that all adducts **7a-h** have the same relative configurations.

Table 1 Comparison of selected ^{13}C NMR chemical shifts of product **7a** with literature compound **8** [32].



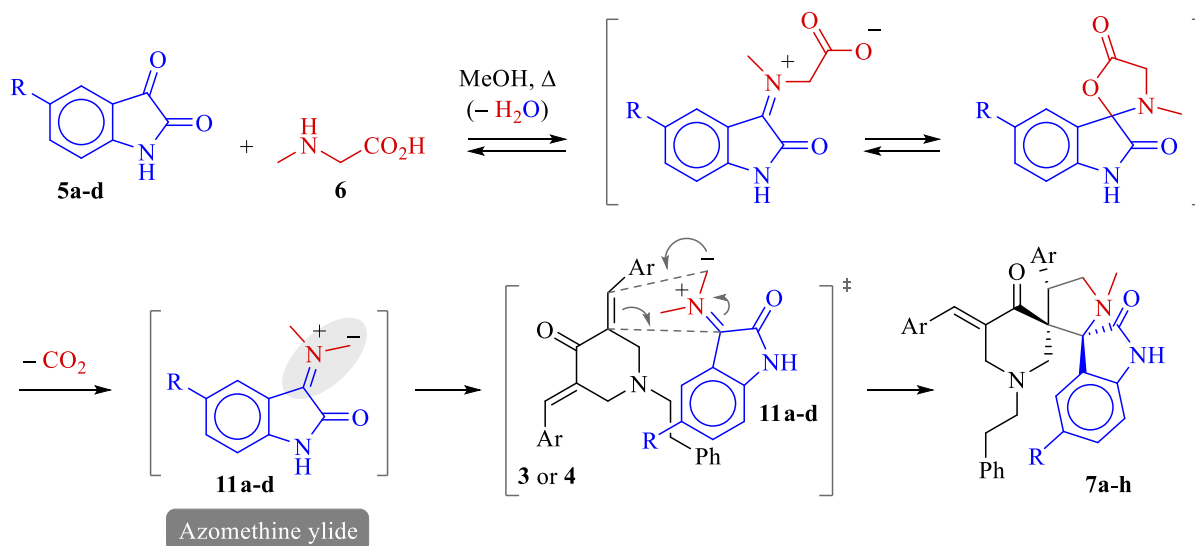
	Compound 7a δ (CDCl_3) (ppm)	Compound 8 δ (CDCl_3) (ppm) ³²
C1	57.0	57.0 or 57.1
C2	66.1	65.7
C3	198.9	199.1
C4	45.6	46.3
C5	56.2	57.0 or 57.1
C6	34.6	34.7
C7	75.0	76.1
C8	176.7	177.4

Table 2 Comparison of selected ^{13}C NMR chemical shifts of product **7g** with literature compound **9** [5].



	Compound 7g δ ($\text{DMSO}-d_6$) (ppm)	Compound 9 δ ($\text{DMSO}-d_6$) (ppm) ⁵
C1	56.8 or 56.9	56.8 or 56.9
C2	65.4	65.2
C3	198.6	198.7
C4	45.1	45.0
C5	56.8 or 56.9	56.8 or 56.9
C6	34.6	34.7
C7	75.7	75.8
C8	176.7	176.7

The proposed reaction mechanism for the formation of the dispiro pyrrolidine cycloadducts is shown in Scheme 2. First, a condensation reaction takes place between the secondary amine function of sarcosine **6** and the carbonyl group of isatin **5a-d** located at C-3. The iminium intermediate thus generated, which is thought to be in equilibrium with a cyclic hemiaminal ester, eventually loses carbon dioxide, irreversibly, to produce the key azomethine ylide species **11a-d**. Regio- and stereo-selective [3+2] cycloaddition with **3** or **4** then operates to produce the dispiro pyrrolidines **7a-h**, respectively [31]. Since the various elementary steps of this mechanism involve polar or charged species, the choice of methanol, a polar protic solvent, ensures a comparatively short reaction time, by stabilisation of the transition states involved [31,35]. The exclusive formation of *mono* adducts is most likely due to the steric hindrance of the remaining C=C exocyclic double bond, which prevents further reaction with the 1,3-dipole [36].



Scheme 2 Mechanism for the formation of the cycloadducts **7a-h**

2.2. Cholinesterase inhibitory activity of the target molecules

The new series of dispiro pyrrolidines **7a-h** were evaluated *in vitro* for their potential as inhibitors of cholinesterases (AChE or BChE), with physostigmine as a positive control. Hydrolysis of the respective substrate used in the Ellman assay by the enzymes (acetylthiocholine by AChE and butyrylthiocholine by BChE) releases yellow-coloured 5-thio-2-nitrobenzoate, with an absorption maximum at 412 nm. Recording of the decrease in absorbance thus allows measuring the inhibition of the enzyme activities, as a result of the effect of the molecules being evaluated. The inhibitions of AChE and BChE at 10 μM for series **7a-h** are summarised in Table 3. It is worthy of note that as a potent cholinesterase inhibitor, the reference drug physostigmine was used at a lower concentration in the bioassay, 0.20 μM , to avoid complete inhibition of the enzymatic activities. In summary, the compounds showed weak to moderate inhibitory activity against both cholinesterases.

Most of the compounds displayed higher inhibitory activities against BChE than against AChE. Structural features of AChE and BChE, especially at the active site gorge confer differences in their substrate specificity [37]. AChE is highly selective to Ach, while BChE can metabolize various molecules apart from Ach. Although both enzymes share 65% amino-acid sequence homology, most of the aromatic amino acid residues at the BChE active site are replaced by amino acid residues with hydrophobic aliphatic side chains such as valine (Val) and leucine (Leu) instead of phenylalanine (Phe), tryptophan (Tyr) and tyrosine (Tyr) in AChE, making BChE able to incorporate bulkier substrate and inhibitors in its active site [10]. This could provide an explanation for the higher inhibition values observed toward BChE, compared to AChE.

Out of eight new dispiro pyrrolidines, compound **7b** was the only one that showed more than 50% inhibition at 10 μM . The presence of a bromo substituent at the oxindole moiety appears to have a positive impact on the inhibition of BChE. This is to be compared to a

previous study, where the presence of a chlorine atom at the aromatic ring of the oxindole had a positive effect on BChE inhibition [27]. However, other halogen atoms (bromine or fluorine) were not evaluated in that particular study. It is interesting to note that despite being the strongest inhibitor of BChE, compound **7b** exhibited the lowest level of inhibition on AChE among the derivatives, suggesting that **7b** could be a BChE-selective inhibitor. Consequently, IC₅₀ determination against BChE was undertaken for compound **7b** only, giving a value of 12.78 ± 1.52 μM. This activity can be categorized as moderate, compared to the standard drug physostigmine, which has a reported IC₅₀ value of 0.16 μM on BChE [27].

Table 3 Cholinesterase inhibitory activity of spiro pyrrolidines **7a-h**.

Entry	Compound	Ar	R	% Inhibition at 10 μM	
				AChE	BChE
1	7a	Ph	H	21.87 ± 4.91	37.66 ± 4.33
2	7b	Ph	Br	13.49 ± 3.47	63.25 ± 7.25
3	7c	Ph	Cl	43.50 ± 5.70	47.99 ± 3.13
4	7d	Ph	NO ₂	39.49 ± 8.14	43.65 ± 4.58
5	7e	4-(Me)C ₆ H ₄	H	39.54 ± 4.84	41.64 ± 4.53
6	7f	4-(Me)C ₆ H ₄	Br	29.75 ± 4.28	44.78 ± 4.49
7	7g	4-(Me)C ₆ H ₄	Cl	37.17 ± 5.13	30.68 ± 3.94
8	7h	4-(Me)C ₆ H ₄	NO ₂	28.60 ± 9.14	48.27 ± 5.79
Reference standard	Physostigmine (tested at 0.2 μM)			70.13 ± 2.37	47.62 ± 2.23

All data are presented as mean ± standard error, SE (n=3).

3.3. Kinetic study of BChE inhibition

Compound **7b** was further evaluated in an enzyme kinetic study. The kinetic inhibition data are summarised in Table 4 and the Lineweaver-Burk plot is shown in Figure 1. The calculated Michaelis constant (K_m) and maximal rate of reaction (V_{max}) for BChE inhibition increase when the concentration of compound **7b** increases from 0 to 3 μM . When it increases further to 6 μM , K_m decreases and V_{max} remains unchanged. At 12 μM inhibitor concentration, both K_m and V_{max} values decline. Lineweaver-Burk plot is a reciprocal reaction velocity ($1/V$) versus reciprocal substrate concentration ($1/[S]$) graph for inhibitors at different concentrations. The plot shows an intercept occurring above the x-axis at increasing concentration of the inhibitor, suggesting mixed-mode inhibition [38]. The findings indicate that compound **7b** is a mixed-mode inhibitor of BChE, where it can bind to the enzyme's active or allosteric sites. The binding of an inhibitor to the enzyme active site prevents the insertion and breakdown of butyrylthiocholine. In contrast, an inhibitor binding to allosteric sites may alter the shape of the enzyme, making it no longer optimal to accommodate butyrylthiocholine in its active site [39]. Mixed-mode inhibition is a special case that can have both competitive and non-competitive traits. A recent study reported a BChE inhibitor, norcymserine, suppresses the breakdown of acetylcholine in the post-mortem AD brain [40]. Spiropyrrolidine compounds could potentially be good alternatives for AD patients not responding to selective AChE inhibitors.

Table 4 Kinetic parameters of butyrylcholinesterase (BChE) inhibition for compound **7b**.

Concentration (μM)	K_m (mM)	V_{max} ($\Delta\text{OD}412/\text{min}$)
0 (no inhibitor)	13.54 ± 2.28	0.066 ± 0.012
3	21.46 ± 9.04	0.077 ± 0.016
6	17.32 ± 7.65	0.077 ± 0.032
12	31.57 ± 13.25	0.077 ± 0.012

All data are presented as mean \pm standard error, SE (n=3).

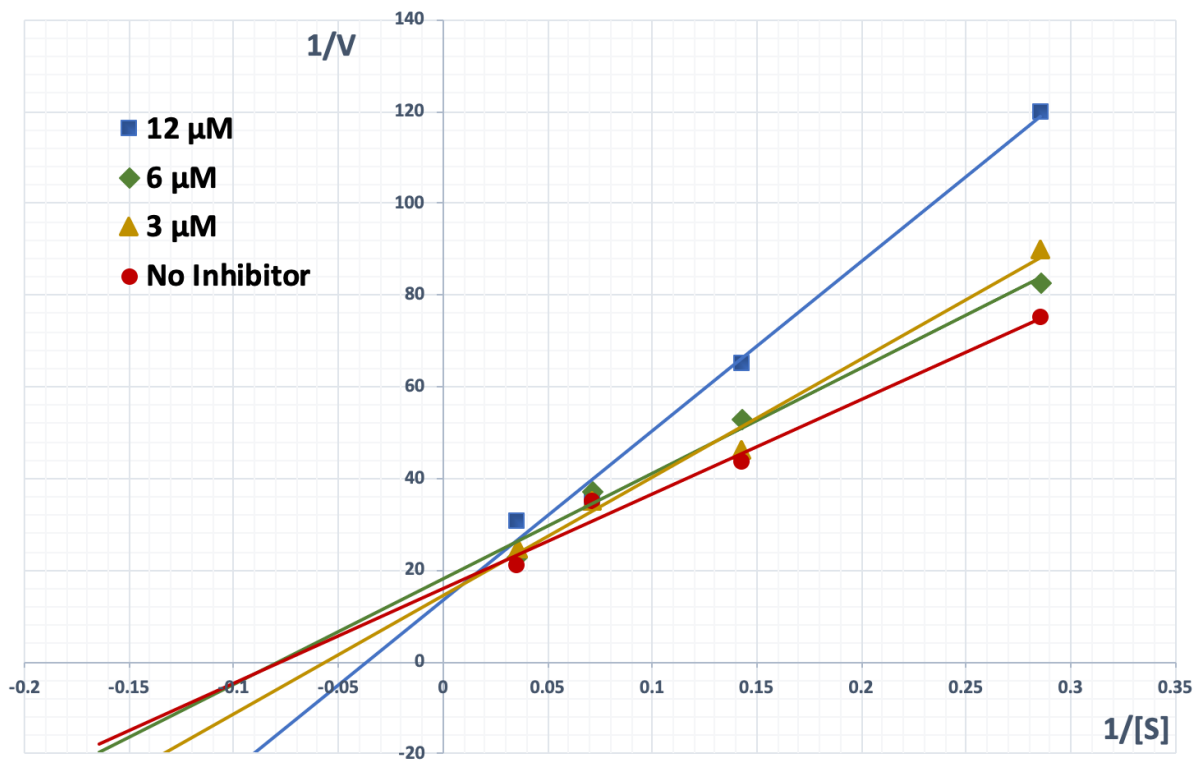


Figure 1 Lineweaver-Burk plot of butyrylcholinesterase (BChE) inhibition of compound **7b**.

3.4. Molecular docking studies

The free binding energy and interaction modes between compound **7b** and residues in the active site of either human BChE or human AChE were identified by molecular docking studies using AutoDock Vina version 1.14. The crystal structures of human BChE complexed with thioflavin T (PDB ID: 6ESY) and human AChE complexed with dihydrotanshinone 1 (PDB ID: 4M0E) were used as a reference structure for the docking process [41-42].

Since **7b** had been prepared as a racemic mixture and used as such in the biological assays, calculations were carried out with both enantiomers of the molecule. For the enantiomer (*S,S,R*)-**7b**, having the same absolute configuration as drawn in Schemes 1-2 and Tables 1-2, the docking results indicated binding energies of -13.9 and -9.7 kcal mol⁻¹ for BChE and AChE, respectively (Table 5). For the other enantiomer, (*R,R,S*)-**7b**, these binding energies were calculated to be -12.0 kcal mol⁻¹ for BChE and -8.6 kcal mol⁻¹ for AChE. These results are fully consistent with the experimentally observed higher inhibitory activity of **7b** against BChE

than against AChE. Moreover, they suggest that (*S,S,R*)-**7b** is a significantly better inhibitor towards both enzymes than its enantiomer (*R,R,S*)-**7b**. To ascertain this, separation or enantioselective synthesis of these enantiomers would be required, in order to evaluate their individual activities. This may be done on the occasion of future studies.

Table 5 Binding energies between butyrylcholinesterase or acetylcholinesterase with both enantiomers of compound **7b** and control (Thioflavin T; TFX or Dihydrotanshinone 1; 1YL), respectively

Enzyme	Compound	Binding energy (kcal mol ⁻¹)
Butyrylcholinesterase (PDB 6ESY)	(<i>S,S,R</i>)- 7b	-13.9 ± 0.11
	(<i>R,R,S</i>)- 7b	-12.0 ± 0.00
	TFX	-7.8 ± 0.26
Acetylcholinesterase (PDB 4M0E)	(<i>S,S,R</i>)- 7b	-9.7 ± 0.12
	(<i>R,R,S</i>)- 7b	-8.6 ± 0.23
	1YL	-7.5 ± 0.17

All data are presented as mean ± standard error, SE (n=3).

Interaction modes of both enantiomers of compound **7b** with BChE residues are shown in Figure 2, and details of their interaction in the active sites are listed in Table 6. The binding interactions of (*S,S,R*)-**7b** in BChE (Figure 2, left) show seven hydrophobic interactions between all three of phenyl groups and amino acids residue TYR332, TRP82, PHE329, GLY116, TRP231, LEU 286 and GLY117. The result explained that the oxindole NH group and pyrrolidine CH played an important role in the binding interaction and beneficial role of the amino acid residue ASP70 and THR120 via hydrogen bond. The bromine also shows hydrophobic interaction with HIS438 and TRP82. As displayed in Figure 2 (right) and Table 6, the enantiomer (*R,R,S*)-**7b** is the subject of hydrophobic interactions between TYR332,

TRP82, TRP231 and LEU286 with three phenyl rings. One anionic interaction was observed at phenyl group with ASP70. No other interaction was observed in oxindole. Based on this molecular docking result, we can conclude that the isatin ring played a huge contribution in inhibiting the BChE enzyme and interaction with the amino acid residue via hydrogen bond.

Interaction of the active molecules with these residues may block the catalysis process of BChE against its substrate, butyrylthiocholine. The docking analysis suggests that if the active molecules bind competitively with the substrate in the active pocket, both enantiomers of **7b** are able to interact with the P-site and the A-site of the enzyme (TYR322, TRP82, TRP231 and PHE329), mostly via hydrophobic interaction. Since both sites are crucial for substrate activation, inhibition of the enzyme by interactions of potential inhibitors with this pocket are promising. In addition, the interactions that formed between ASP70 with molecule (*S,S,R*)-**7b**, viewed from docking analysis suggested to eliminate substrate activation in BChE by blocking the hydrogen bond formation between ASP70 with TYR322 [41]. The molecules may comprise the mode of actions like most of the inhibitors against BChE.

Table 6 Binding interactions between both enantiomers of compound **7b** and butyrylcholinesterase (BChE)

Compound	Interacting unit	Protein residue	Type of interaction
<i>(S,S,R)</i> - 7b	CH	ASP70	H-bond
	NH	THR120	H-bond
	Br	HIS438	π -alkyl
	Br	TRP82	π - π stacked
	Ph	TYR332	π - π stacked
	Ph	TRP82	π - π stacked
	Ph	PHE329	π - π stacked
	Ph	GLY116	π - π stacked
	Ph	TRP231	π - π stacked
	Ph	LEU286	π -alkyl
	Ph	GLY117	Van der Waals
	<i>(R,R,S)</i> - 7b	Ph	ASP70
Ph		TYR332	π - π stacked
Ph		TRP82	π - π stacked
Ph		TRP231	π - π stacked
Ph		LEU286	π -alkyl

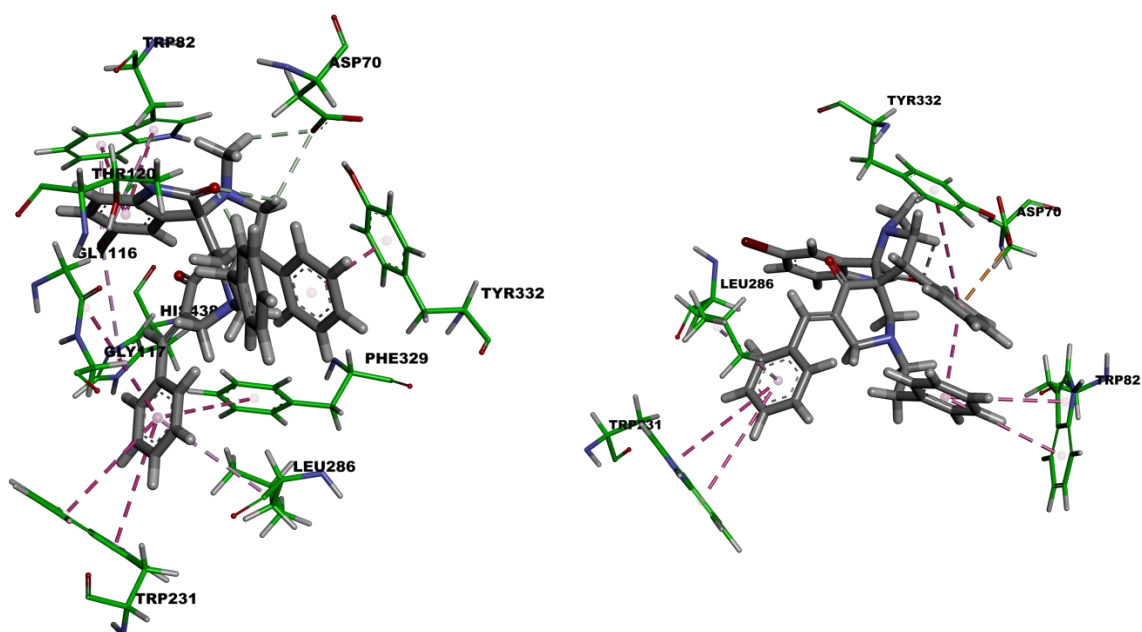


Figure 2 Three-dimensional binding modes of enantiomer *(S,S,R)*-**7b** (left) and enantiomer *(R,R,S)*-**7b** (right) in the active site of BChE

3. Conclusions

In summary, new dispiropyrolidine derivatives were synthesised by a facile one-pot, three-component [3+2]-cycloaddition protocol. The designed new cycloadducts were screened for their inhibitory activities against cholinesterase enzymes. The inhibitory activities exhibited by the tested compounds were typically higher against BChE than AChE. Compound **7b** displayed a moderate BChE inhibitory activity, with an IC_{50} of $12.78 \pm 1.52 \mu\text{M}$. Kinetic studies showed that **7b** is a mixed-mode inhibitor. It can be projected from the present study that the dispiro pyrrolidine **7b** could be further modified to improve its potential as a potent BChE selective inhibitor.

4. Materials and method

4.1. General

All chemicals reagents were obtained from Acros Organic (USA), QRec (Asia), Sigma Aldrich Chemical Co. and E. Merck (Germany). Melting points were determined on a Stuart Scientific SMP10 or SMP40 melting point apparatus. Thin-layer chromatography (TLC) plates was performed on alumina plates pre-coated with silica gel (Merck 60 F254) in the solvent system acetone-hexane with two different ratios of 3:7 or 1:9. The progress of reactions was determined by the appearance of product and disappearance of reactant spots under UV radiation lamp ($\lambda_{\text{max}} = 254 \text{ nm}$), respectively. All spectral data were obtained on the following instruments: infrared spectra were recorded on a Perkin Elmer 2000 FTIR spectrometer at wavenumbers ranging from 4000 to 600 cm^{-1} . ^1H (400.2 or 500 MHz) and ^{13}C (100.6 or 125 MHz) nuclear magnetic resonance spectra were recorded with an AVANCE 400 or an AVN 500 Bruker spectrometer (Bruker Biospin, Billerica, MA, U.S.A.). Data were analysed using NMRnotebook 2.80 (NMRTEC S.A.S.). Chemical shifts δ are reported in parts per million

(ppm), referenced to the peak of tetramethylsilane, defined at $\delta = 0.00$ (^1H and ^{13}C NMR), or the residual solvent peak, defined at $\delta = 2.50$ (^1H NMR, DMSO- d_6), 7.26 (^1H NMR, CDCl_3), 39.5 (^{13}C NMR, DMSO- d_6) or 77.0 (^{13}C NMR, CDCl_3). Multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), br (broad). Coupling constants J are given in Hz and are rounded to the closest multiple of 0.5. Mass spectrometry experiments were performed using a Waters Xevo QTOF MS system or, alternatively, on a tims-TOF mass spectrometer (Bruker) in the *Laboratoire de Chimie Moléculaire* (Ecole polytechnique, CNRS, Palaiseau, France). The electrospray source was used in positive mode. Samples were prepared in acetonitrile with 0.1% of formic acid, introduced at a 5 $\mu\text{L}/\text{min}$ flow rate into the interface of the instrument. Capillary and end plate voltages were set at 4.5 kV and 0.7 kV. Nitrogen was used as the nebulizer and drying gas at 2.5 bar and 3 L/min, respectively, with a drying temperature of 180°C. Tuning mix (Agilent) was used for calibration. The elemental compositions of all ions were determined with the instrument software Data Analysis; the precision of mass measurement was less than 3 ppm.

For the assays the chemicals and enzymes used in the cholinesterase inhibition investigation are as follows: Acetylthiocholine iodide (Sigma Aldrich, USA); 5,5'-Dithiobis-2-benzoic acid (Sigma Aldrich, USA); Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) (Sigma Aldrich, USA); Butyrylthiocholine iodide (Sigma Aldrich, USA); Butyrylcholinesterase (BChE) from equine serum (Sigma Aldrich, USA).

4.2. Chemistry

4.2.1. General procedure for synthesis of (3E,5E)-3,5-bis(arylidene)-1-phenethyl-4-piperidinone (**3-4**)

The intermediate compounds, 3,5-bis(arylidene)-1-phenylethyl-4-piperidones (**3-4**) were synthesised following the synthesis protocol reported in the literature [24]. A mixture of

1-phenylethyl-4-piperidone **1** (1.00 mmol) and appropriate aryl aldehyde **2** (2.00 mmol) was dissolved in ethanol (10 mL) and 30% sodium hydroxide (5.0 mL) and stirred for 2-6 hours at room temperature until completion of the reaction (as evidenced by TLC). The mixture was then poured onto crushed ice. The solid precipitate was filtered off and washed with water to afford the product, which was then purified by recrystallisation.

4.2.1.1 3,5-Dibenzylidene-1-phenylethyl-4-piperidinone (**3**)

Yellow crystals. $R_f \approx 0.2$ [UV-active, EtOAc/petroleum ether 20%, orange spot (anisaldehyde)]. M.p. 88–92 °C (EtOH) (Lit. 165 °C) [24]. FTIR (ATR, neat): ν 3026 (w, C-H), 1669 (s, C=O), 1608 (s, C=C), 1181 (m, C-N) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ 2.74 (2H, m), 2.81 (2H, m), 3.90 (4H, d, $J=1.5$), 7.11 (2H, dd, $J=8.0, 1.5$), 7.15 (1H, tt, $J=7.5, 1.5$), 7.23 (2 H, br dd, $J=8.0, 7.5$), 7.34-7.46 (10H, m), 7.83 (2H, t, $J=1.5$). ^{13}C NMR (100.6 MHz, CDCl_3): δ 34.0 (C2), 54.7 (C7), 58.6 (C1), 126.1 (C6), 128.4 (C4), 128.6 (C5), 128.6 (C13), 129.0 (C14), 130.4 (C12), 133.2 (C8), 135.2 (C11), 136.7 (C10), 139.8 (C3), 187.4 (C9). ^1H NMR (500 MHz, DMSO-d_6): δ 2.70 (2H, t, $J=7.5$), 2.81 (2H, t, $J=7.5$), 3.88 (4H, s), 7.14-7.23 (5H, m), 7.42-7.51 (10H, m), 7.58 (2H, s). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 32.8 (C2), 54.0 (C7), 57.8 (C1), 125.8 (C6), 128.1 (C4), 128.5 (C5), 128.7 (C13), 129.2 (C14), 130.5 (C12), 133.8 (C8), 134.6 (C11), 134.8 (C10), 140.0 (C3), 186.9 (C9). HRMS (TOF-ES⁺): m/z 380.2016 ($\text{MH}^+ \text{C}_{27}\text{H}_{26}\text{NO}^+$ requires 380.2009).

4.2.1.2 3,5-Bis(4-methylbenzylidene)-1-phenylethyl-4-piperidinone (**4**)

Yellow solid. $R_f \approx 0.78$ [UV-active, Acetone:hexane 10%]. M.p. 118–121 °C. FTIR (ATR, neat): ν 3024 (w, C-H), 1668 (s, C=O), 1606 (m, C=C), 1175 (m, C-N) cm^{-1} . ^1H NMR (500 MHz, DMSO-d_6): δ 2.35 (6H, s), 2.70 (2H, distorted t, $J=7.5$), 2.80 (2H, distorted t, $J=7.5$), 3.85 (4H, br s), 7.14 (1H, tt, $J=7.5, 1.5$), 7.15 (2H, d, $J=7.5$), 7.22 (2H, br t, $J=7.5$), 7.28

(4H, d, $J=8.0$), 7.39 (4H, d, $J=8.0$), 7.57 (2H, br s). ^{13}C NMR (125.8 MHz, DMSO- d_6): δ 21.0 (C15), 32.8 (C2), 54.1 (C7), 57.9 (C1), 125.8 (C6), 128.2 (C4), 128.5 (C5), 129.4 (C13), 130.5 (C12), 131.9 (C11), 133.0 (C8), 134.7 (C10), 139.1 (C14), 140.0 (C3), 186.8 (C9). HRMS (TOF-ES $^+$): m/z 408.2324 (MH^+ $\text{C}_{29}\text{H}_{30}\text{NO}^+$ requires 408.2322).

4.2.2. General procedure for synthesis of spiropyrrolidines derivatives **7a-h**

(3*E*,5*E*)-3,5-Bis(arylidene)-1-phenethyl-4-piperidinone **3** or **4** (1.50 mmol) was refluxed together with the appropriate isatin **5a-d** (1.50 mmol) and sarcosine **6** (3.00 mmol) in methanol (20 mL) for 4-7 hours. After completion of the reaction, the mixture was allowed to cool to room temperature. Most of the solvent was removed under reduced pressure and the residue was poured onto crushed ice. The solid product obtained was filtered off and washed with water. In some cases, further purification by recrystallisation was carried out.

4.2.2.1 1-Methyl-4(phenyl)pyrrolo-(spiro[2.3']oxindole)-spiro[3.3']-5'-(phenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7a**)

Pale yellow crystal. $R_f \approx 0.15$ [UV-active, EtOAc:petroleum ether 20%]. M.p. 200-202 °C. ^1H NMR (400 MHz, CDCl_3): δ 1.73 (1H, d, $J=12.5$), 2.07 (3H, s), 2.34 (1H, ddd, $J=12.0, 6.5, 5.0$), 2.53 (2H, AB part of an ABXY system, δ_A 2.48, δ_B 2.57, $J_{AB}=14.0$, $J_{AX}=6.5$, $J_{AY}=9.0$, $J_{BX}=5.0$, $J_{BY}=7.0$), 2.78 (ddd, $J=12.0, 9.0, 7.0$), 2.78 (1H, dd, $J=14.0, 2.0$), 3.25 (1H, dd, $J=8.5, 7.0$), 3.34 (1H, dd, $J=12.5, 2.0$), 3.65 (1H, ddd, $J=14.5, 2.0, 1.5$), 3.88 (1H, dd, $J=11.5, 8.5$), 4.80 (1H, dd, $J=11.5, 7.0$), 6.04 (1H, br s, NH), 6.38 (1H, br d, $J=7.5$), 6.93 (1H, td, $J=7.5, 1.0$), 6.97 (1H, dd, $J=2.0, 1.5$), 6.98 (2H, br dd, $J=7.5, 2.0$), 7.01 (2H, br dd, $J=8.0, 1.5$), 7.05 (1H, br dd, $J=7.5, 1.5$), 7.07 (1H, ddd, $J=8.0, 7.5, 1.5$), 7.20-7.34 (9H, m), 7.37 (2H, br dd, $J=7.5, 1.5$). ^{13}C NMR (100.6 MHz, CDCl_3): δ 32.5 (C2), 34.6 (C22), 45.6 (C12), 53.4 (C7), 56.2 (C13), 57.0 (C11), 58.8 (C1), 66.1 (C10), 75.0 (C14), 108.7 (C19), 121.7 (C17), 125.8

(C6), 126.7 (C26), 127.5 (C15), 127.6 (C16), 128.17, 128.19 (C4, C25), 128.3, 128.5 (C5, C30), 129.0 (C18), 129.0 (C31), 129.3 (C24), 129.8 (C29), 133.5 (C8), 135.2 (C28), 137.5 (C27), 138.3 (C23), 140.8 (C3), 141.9 (C20), 176.7 (C21), 198.9 (C9). ¹H NMR (500 MHz, DMSO-d₆): δ 1.76 (1H, d, *J*=12.5), 1.99 (3H, s), 2.30–2.55 (4H, m), 3.06 (1H, dd, *J*= 15.0, 2.5), 3.21 (1H, dd, *J*= 8.5, 7.5), 3.23 (1H, d, *J*= 12.5), 3.34 (1H, br d, *J*= 15.0), 3.83 (1H, dd, *J*= 11.0, 8.5), 4.65 (1H, dd, *J*= 11.0, 7.5), 6.64 (1H, br d, *J*= 7.5), 6.85–6.90 (2H, m), 7.05 (1H, ddd, *J*= 7.5, 6.0, 2.5), 7.09 (1H, m), 7.11 (2H, d, *J*= 7.5), 7.11 (2H, d, *J*= 7.0), 7.11 (1H, t, *J*= 7.0), 7.15 (1H, br t, *J*= 7.5), 7.22 (2H, dd, *J*= 7.5, 7.0), 7.30–7.36 (7H, m), 10.38 (1H, br s, NH). ¹³C NMR (125.8 MHz, DMSO-d₆): δ 32.0 (C2), 34.1 (C22), 45.2 (C12), 53.9 (C7), 56.2, 56.3 (C11, C13), 59.1 (C1), 64.6 (C10), 75.2 (C14), 108.7 (C19), 120.7 (C17), 125.8 (C6), 126.75 (C16 or C26), 126.85 (C15), 126.91 (C16 or C26), 128.18 128.20 (C4, C25), 128.46, 128.47 (C5, C30), 128.6 (C18), 128.9 (C31), 129.1 (C24), 130.0 (C29), 132.9 (C8), 134.5 (C28), 136.6 (C27), 138.4 (C23), 140.0 (C3), 143.4 (C20), 176.6 (C21), 198.2 (C9). HRMS (TOF-ES⁺): *m/z* 554.2801 (MH⁺ C₃₇H₃₆N₃O₂⁺ requires 554.2802).

4.2.2.2 1-Methyl-4(phenyl)pyrrolo-(spiro[2.3"]-5"-bromooxindole)-spiro[3.3']-5'-(phenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7b**)

Pale yellow solid. *R_f* ≈ 0.62 [UV-active, Acetone:hexane 10%]. M.p. 123–126 °C. FTIR (ATR, neat): ν 3387 (w, N–H), 3026 (w, C–H), 1711 (s, C=O), 1614 (m, C=C), 1181 (m, C–N) cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ 1.75 (1H, d, *J*= 12.5), 2.01 (3H, s), 2.32–2.53 (4H, m), 3.10 (1H, dd, *J*= 15.0, 3.0), 3.22 (1H, dd, *J*= 8.5, 7.5), 3.22 (1H, dd, *J*= 12.5, 1.5), 3.38 (1H, br d, *J*= 15.0), 3.81 (1H, dd, *J*= 11.0, 8.5), 4.64 (1H, dd, *J*= 11.0, 7.5), 6.61 (1H, d, *J*= 8.0), 6.94 (1H, d, *J*= 2.0), 7.11 (2H, br d, *J*= 7.5), 7.12 (1H, t, *J*= 7.5), 7.13 (1H, m), 7.14 (1H, t, *J*= 7.5), 7.19 (2H, dd, *J*= 8.0, 2.0), 7.23 (2H, t, *J*= 7.5), 7.24 (1H, dd, *J*= 8.0, 2.0), 7.30–7.38 (7H, m), 10.56 (1H, br s, NH). ¹³C NMR (125.8 MHz, DMSO-d₆): δ 31.8 (C2), 34.1 (C22), 45.0

(C12), 53.9 (C7), 56.2, 56.3 (C11, C13), 59.0 (C1), 65.0 (C10), 75.0 (C14), 110.7 (C19), 112.6 (C17), 125.8 (C6), 126.8 (C26), 128.16 (C4), 128.24 (C25), 128.4 (C5), 128.5 (C30), 129.08 (C24), 129.10 (C31), 129.4 (C15), 129.5 (C16), 130.0 (C29), 131.2 (C18), 133.0 (C8), 134.3 (C28), 137.0 (C27), 138.1 (C23), 139.9 (C3), 142.7 (C20), 176.0 (C21), 198.1 (C9). HRMS (TOF-ES⁻) *m/z* 630.0966 ([M-H]⁻ C₃₇H₃₃⁷⁹BrN₃O₂⁻ requires 630.1761), 632.0932 ([M-H]⁻ C₃₇H₃₃⁸¹BrN₃O₂⁻ requires 632.1741).

4.2.2.3 1-Methyl-4(phenyl)pyrrolo-(spiro[2.3']-5"-chlorooxindole)-spiro[3.3']-5'- (phenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7c**)

Pale yellow solid. *R_f* ≈ 0.63 [UV-active, Acetone:hexane 10%]. M.p. 131-134 °C. FTIR (ATR, neat): ν 3244 (w, N-H), 3027 (w, C-H), 1698 (s, C=O), 1615 (m, C=C), 1182 (m, C-N) cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ 1.75 (1H, d, *J* = 12.5), 2.01 (3H, s), 2.32–2.53 (4H, m), 3.10 (1H, dd, *J* = 15.0, 2.5), 3.23 (1H, dd, *J* = 8.5, 7.5), 3.23 (1H, dd, *J* = 12.5, 1.5), 3.38 (1H, br d, *J* = 15.0), 3.81 (1H, dd, *J* = 11.0, 8.5), 4.65 (1H, dd, *J* = 11.0, 7.5), 6.66 (1H, d, *J* = 8.0), 6.82 (1H, d, *J* = 2.0), 7.11 (2H, br d, *J* = 7.5), 7.12 (1H, dd, *J* = 8.0, 2.0), 7.14 (1H, m), 7.15 (1H, t, *J* = 7.5), 7.13-7.18 (3H, m), 7.23 (2H, t, *J* = 7.5), 7.30–7.38 (7H, m), 10.56 (1H, s, NH). ¹³C NMR (125.8 MHz, DMSO-d₆): δ 32.0 (C2), 34.2 (C22), 45.1 (C12), 54.0 (C7), 56.3, 56.4 (C11, C13), 59.1 (C1), 65.1 (C10), 75.2 (C14), 110.3 (C19), 125.0 (C17), 125.9 (C6), 126.8 (C26), 126.9 (C16), 128.27 (C4), 128.34 (C25), 128.5 (C5), 128.6 (C18), 128.7 (C30), 129.1 (C15), 129.23 (C31), 129.1 (C15), 130.1 (C29), 133.1 (C8), 134.3 (C28), 137.2 (C27), 138.1 (C23), 140.0 (C3), 142.4 (C20), 176.3 (C21), 198.2 (C9). HRMS (TOF-ES⁺): *m/z* 588.2390 (MH⁺ C₃₇H₃₅³⁵ClN₃O₂⁺ requires 588.2413).

4.2.2.4 1-Methyl-4(phenyl)pyrrolo-(spiro[2.3']-5'-nitrooxindole)-spiro[3.3']-5'-(phenylmethylidene)-1'-phenylethyl piperidin-4'-one (**7d**)

Dark brown solid. $R_f \approx 0.47$ [UV-active, Acetone:hexane 10%]. M.p. 127–130 °C. FTIR (ATR, neat): ν 3248 (w, N–H), 3026 (w, C–H), 1718 (s, C=O), 1599 (m, C=C), 1182 (m, C–N) cm^{-1} . ^1H NMR (500 MHz, DMSO- d_6): δ 1.76 (1H, d, $J= 12.5$), 2.03 (3H, s), 2.35–2.52 (4H, m), 3.11 (1H, dd, $J= 15.0, 2.5$), 3.19 (1H, dd, $J= 12.5, 1.5$), 3.29 (1H, dd, $J= 8.5, 7.5$), 3.35 (1H, br d, $J= 15.0$), 3.81 (1H, dd, $J= 10.5, 8.5$), 4.71 (1H, dd, $J= 10.5, 7.5$), 6.86 (1H, d, $J= 8.5$), 6.98 (2 H, m), 7.12 (2H, d, $J= 7.0$), 7.14 (1H, m), 7.14 (1H, t, $J= 8.0$), 7.15 (1H, t, $J= 8.0$), 7.23 (2H, dd, $J= 8.0, 7.0$), 7.31–7.35 (7H, m), 7.71 (1H, d, $J= 2.5$), 8.04 (1H, dd, $J= 8.5, 2.5$), 11.22 (1H, br s, NH). ^{13}C NMR (125.8 MHz, DMSO- d_6): δ 31.9 (C2), 34.2 (C22), 44.7 (C12), 53.8 (C7), 56.2, 56.5 (C11, C13), 59.0 (C1), 65.3 (C10), 75.0 (C14), 109.1 (C19), 122.6 (C16), 125.9 (C6), 126.1 (C18), 127.0 (C26), 127.8 (C15), 128.2 (C4), 128.36 (C25), 128.44 (C5), 128.7 (C30), 129.1 (C24), 129.3 (C31), 129.9 (C29), 132.8 (C8), 134.0 (C28), 137.3 (C27), 137.8 (C23), 139.8 (C3), 141.5 (C17), 150.0 (C20), 176.9 (C21), 197.7 (C9). HRMS (TOF-ES $^+$): m/z 599.2764 ($\text{MH}^+ \text{C}_{37}\text{H}_{35}\text{N}_4\text{O}_4^+$ requires 599.2653).

4.2.2.5 1-Methyl-4(-4-methylphenyl)pyrrolo-(spiro[2.3']oxindole)-spiro[3.3']-5'-(4-methylphenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7e**)

Pale yellow solid. $R_f \approx 0.40$ [UV-active, Acetone:hexane 10%]. M.p. 192–195 °C. FTIR (ATR, neat): ν 3397 (w, N–H), 3023 (w, C–H), 1694 (s, C=O), 1605 (m, C=C), 1177 (m, C–N) cm^{-1} . ^1H NMR (500 MHz, DMSO- d_6): δ 1.76 (1H, d, $J= 12.5$), 1.98 (3H, s), 2.26 (3H, s), 2.27 (3H, s), 2.28–2.55 (4H, m), 3.04 (1H, dd, $J= 15.0, 2.5$), 3.16 (1H, dd, $J= 8.5, 7.5$), 3.20 (1H, dd, $J= 12.5, 1.5$), 3.33 (1H, dd, $J= 15.0, 2.0$), 3.79 (1H, dd, $J= 11.0, 8.5$), 4.60 (1H, dd, $J= 11.0, 7.5$), 6.63 (1H, br d, $J= 7.5$), 6.82–6.89 (2H, m), 7.01 (2H, d, $J= 8.0$), 7.03 (1H, ddd, $J= 7.5, 7.0, 2.0$), 7.06 (1H, dd, $J= 2.5, 2.0$), 7.11 (2H, br d, $J= 8.0$), 7.12 (2H, br d, $J= 7.0$), 7.14

(2H, br d, $J= 8.0$), 7.15 (1H, t, $J= 8.0$), 7.19 (2H, br d, $J= 8.0$), 7.23 (2H, dd, $J= 8.0, 7.0$), 10.35 (1H, br s, NH). ^{13}C NMR (125.8 MHz, DMSO- d_6): δ 20.6 (C27), 20.9 (C33), 32.1 (C2), 34.1 (C22), 44.8 (C12), 54.0 (C7), 56.3, 56.4 (C11, C13), 59.1 (C1), 64.5 (C10), 75.2 (C14), 108.6 (C19), 120.6 (C17), 125.8 (C6), 126.88 (C16), 126.93 (C15), 128.2 (C4), 128.5 (C18), 128.4 (C5), 128.8 (C25), 129.0 (C31), 129.1 (C24), 130.2 (C30), 131.7 (C29), 132.1 (C8), 135.3 (C23), 135.7 (C26), 136.6 (C28), 138.8 (C32), 140.1 (C3), 143.4 (C20), 176.6 (C21), 198.2 (C9). HRMS (TOF-ES $^+$): m/z 582.3081 (MH $^+$ C $_{39}$ H $_{40}$ N $_3$ O $_2^+$ requires 582.3115), 604.2894 (MNa $^+$ C $_{39}$ H $_{39}$ N $_3$ NaO $_2^+$ requires 604.2935).

4.2.2.6 1-Methyl-4-(4-methylphenyl)pyrrolo-(spiro[2.3 $''$]-5 $''$ -bromooxindole)-spiro[3.3 $''$]-5 $''$ -(4-methylphenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7f**)

Pale yellow solid. $R_f \approx 0.66$ [UV-active, Acetone:hexane 10%]. M.p. 205–208 °C. FTIR (ATR, neat): ν 3347 (w, N–H), 3024 (w, C–H), 1690 (s, C=O), 1604 (m, C=C), 1180 (m, C–N) cm^{-1} . ^1H NMR (500 MHz, DMSO- d_6): δ 1.75 (1H, d, $J= 12.5$), 2.00 (3H, s), 2.26 (3H, s), 2.29 (3H, s), 2.29–2.53 (4H, m), 3.08 (1H, dd, $J= 15.0, 2.5$), 3.17 (1H, dd, $J= 8.5, 7.0$), 3.19 (1H, dd, $J= 12.5, 1.5$), 3.37 (1H, br d, $J= 15.0$), 3.76 (1H, dd, $J= 11.0, 8.5$), 4.59 (1H, dd, $J= 11.0, 7.0$), 6.60 (1H, d, $J= 8.0$), 6.92 (1H, d, $J= 2.0$), 7.09 (2H, d, $J= 8.0$), 7.09–7.12 (1H, m), 7.12 (2H, br d, $J= 7.0$), 7.12 (2H, br d, $J= 8.0$), 7.15 (1H, t, $J= 8.0$), 7.17 (2H, br d, $J= 8.0$), 7.18 (2H, br d, $J= 8.0$), 7.22 (1H, dd, $J= 8.0, 2.0$), 7.23 (2H, dd, $J= 8.0, 7.0$), 10.54 (1H, br s, NH). ^{13}C NMR (125.8 MHz, DMSO- d_6): δ 20.7 (C27), 20.9 (C33), 32.0 (C2), 34.2 (C22), 44.7 (C12), 54.1 (C7), 56.351, 56.430 (C11, C13), 59.1 (C1), 65.0 (C10), 75.1 (C14), 110.7 (C19), 112.6 (C17), 125.9 (C6), 128.2 (C4), 128.5 (C5), 128.9 (C25), 129.0 (C31), 129.2 (C24), 129.6, 129.6 (C15, C16), 130.2 (C30), 131.2 (C18), 131.6 (C29), 132.2 (C8), 135.0 (C23), 135.9 (C26), 137.2 (C28), 139.2 (C32), 140.0 (C3), 142.7 (C20), 176.1 (C21), 198.0 (C9). HRMS

(TOF-ES⁺): m/z 660.2328 (MH⁺ C₃₉H₃₉⁷⁹BrN₃O₂⁺ requires 660.2221), 662.2327 (MH⁺ C₃₉H₃₉⁸¹BrN₃O₂⁺ requires 662.2200).

4.2.2.7 1-Methyl-4(-4-methylphenyl)pyrrolo-(spiro[2.3']-5''-chlorooxindole)-spiro[3.3']-5'-(4-methylphenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7g**)

Pale yellow solid. $R_f \approx 0.66$ [UV-active, Acetone:hexane 10%]. M.p. 186–189 °C. FTIR (ATR, neat): ν 3180 (w, N–H), 3025 (w, C–H), 1691 (s, C=O), 1607 (m, C=C), 1182 (m, C–N) cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ 1.75 (1H, d, $J= 12.5$), 2.00 (3H, s), 2.26 (3H, s), 2.29 (3H, s), 2.31–2.52 (4H, m), 3.08 (1H, dd, $J= 15.0, 2.5$), 3.18 (1H, dd, $J= 8.5, 7.5$), 3.20 (1H, dd, $J= 12.5, 1.5$), 3.37 (1H, br d, $J= 15.0$), 3.77 (1H, dd, $J= 11.0, 8.5$), 4.59 (1H, dd, $J= 11.0, 7.5$), 6.64 (1H, d, $J= 8.5$), 6.80 (1H, d, $J= 2.0$), 7.06 (2H, d, $J= 8.0$), 7.09 (1H, dd, $J= 8.5, 2.0$), 7.11 (1H, m), 7.12 (2H, br d, $J= 7.0$), 7.12 (2H, br d, $J= 7.5$), 7.15 (1H, t, $J= 8.0$), 7.17 (2H, br d, $J= 8.0$), 7.19 (2H, br d, $J= 7.5$), 7.23 (2H, dd, $J= 8.0, 7.0$), 10.53 (1H, br s, NH). ¹³C NMR (125.8 MHz, DMSO-d₆): δ 20.6 (C27), 20.9 (C33), 32.0 (C2), 34.1 (C22), 44.6 (C12), 54.0 (C7), 56.3, 56.4 (C11, C13), 59.0 (C1), 64.9 (C10), 75.2 (C14), 110.1 (C19), 124.8 (C17), 125.8 (C6), 126.8 (C16), 128.2 (C4), 128.3 (C18), 128.4 (C5), 128.8 (C25), 128.9 (C31), 129.1 (C15), 129.2 (C24), 130.1 (C30), 131.5 (C29), 132.2 (C8), 135.0 (C23), 135.8 (C26), 137.1 (C28), 139.1 (C32), 139.9 (C3), 142.3 (C20), 176.2 (C21), 198.1 (C9). HRMS (TOF-ES⁺): m/z 616.2735 (MH⁺ C₃₉H₃₉³⁵ClN₃O₂⁺ requires 616.2726), 638.2595 (MNa⁺ C₃₉H₃₈³⁵ClN₃NaO₂⁺ requires 638.2545).

4.2.2.8 1-Methyl-4(-4-methylphenyl)pyrrolo-(spiro[2.3']-5''-nitrooxindole)-spiro[3.3']-5'-(4-methylphenylmethylidene)-1'-phenylethyl-4'-piperidinone (**7h**)

Dark yellow solid. $R_f \approx 0.41$ [UV-active, Acetone:hexane 10%]. M.p. 191–194 °C. FTIR (ATR, neat): ν 3294 (w, N–H), 3028 (w, C–H), 1701 (s, C=O), 1601 (m, C=C), 1177 (m, C–N)

cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ 1.76 (1H, d, *J*= 12.5), 2.02 (3H, s), 2.27 (6H, br s), 2.34–2.53 (4H, m), 3.09 (1H, dd, *J*= 15.0, 2.5), 3.16 (1H, dd, *J*= 12.5, 1.5), 3.25 (1H, dd, *J*= 8.5, 7.5), 3.34 (1H, br d, *J*= 15.0), 3.77 (1H, dd, *J*= 11.0, 8.5), 4.66 (1H, dd, *J*= 11.0, 7.5), 6.84 (1H, d, *J*= 8.5), 6.89 (2H, d, *J*= 8.0), 7.11 (1H, m), 7.13 (2H, br d, *J*= 7.0), 7.13 (2H, br d, *J*= 8.0), 7.14 (2H, br d, *J*= 8.0), 7.16 (1H, t, *J*= 8.0), 7.20 (2H, br d, *J*= 8.0), 7.24 (2H, dd, *J*= 8.0, 7.0), 7.70 (1H, d, *J*= 2.5), 8.02 (1H, dd, *J*= 8.5, 2.5), 11.19 (1H, br s, NH). ¹³C NMR (125.8 MHz, DMSO-d₆): δ 20.6, 20.9 (C27, C33), 31.9 (C2), 34.1 (C22), 44.3 (C12), 53.9 (C7), 56.2, 56.5 (C11, C13), 59.0 (C1), 65.2 (C10), 75.0 (C14), 109.0 (C19), 122.6 (C16), 125.9 (C6), 126.0 (C18), 127.9 (C15), 128.2 (C4), 128.4 (C5), 128.91, 128.95 (C25, C31), 129.3 (C24), 130.0 (C30), 131.9 (C29), 132.3 (C8) 134.7 (C23), 135.9 (C26), 137.3 (C28), 139.4 (C32), 139.8 (C3), 141.4 (C17), 149.9 (C20), 176.9 (C21), 197.7 (C9). HRMS (TOF-ES⁺): *m/z* 627.3000 (MH⁺ C₃₉H₃₉N₄O₄⁺ requires 627.2966).

4.3. Biology

4.3.1 Assay for Cholinesterase Inhibitions

4.3.1.1 Preparation of samples, reagents and enzyme solutions.

Physostigmine was used as a positive control. The test samples and physostigmine were initially prepared in dimethylsulfoxide (DMSO) solution at a concentration of 1 mg/mL. The reaction mixture was diluted with phosphate buffer until the concentration of DMSO was 10%. The concentration of DMSO in the reaction mixture was further diluted with the addition of reagents and enzyme solutions until the final reaction mixture was 1%. The concentration of DMSO of 1% gives no inhibitory effect towards both acetylcholinesterase and butyrylcholinesterase enzymes.

The 0.1 M phosphate buffer with pH 8.0 was used as an aqueous medium for enzymatic reaction in the cholinesterase inhibitory assay. The 0.01 M of dithiobisnitrobenzoic

acid (DTNB) was prepared by dissolved 39.6 mg of DTNB in 10 mL of phosphate buffer (pH 8.0). Two substrate solutions, acetylthiocholine iodide (AChI) and butyrylthiocholine iodide (BChI) were prepared at a concentration of 0.014 M by dissolving 40.4 mg of AChI and 31.6 mg of BChI respectively in 10 mL deionized water. Acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase (BChE) from equine serum was used at a concentration of 0.09 u/mL enzyme solution in buffer pH 7.4 as enzyme working solution.

4.3.1.2 Ellman's Assay and IC₅₀ determination

The activities of AChE and BChE were conducted based on Ellman's method using a 96-wells microplate plate assay [26]. The reaction mechanism of this assay is based on the hydrolysis of AChI or BChI by AChE or BChE to thiocholine and acetate entities, respectively. Assay of acetylcholinesterase (AChE) activity was started by adding 140 µL of 0.1 M phosphate buffer (pH 8.0) into each well of a 96-well microplate. Test samples were added and followed with 20 µL of 0.09 u/mL acetylcholinesterase. The microplate was incubated for 15 minutes at 25 °C (in dark) before 10 µL of 10 mM DTNB were added into each well and followed with 10 µL of 14 mM acetylthiocholine iodide. The concentration of 10% DMSO in 20 µL reaction mixture was diluted to 1% DMSO with the addition of 140 µL reagents and enzyme solutions. BioTek PowerWave X 340 microplate spectrophotometer was used to measure the absorbance of the coloured end-product, 5-thio-2-nitrobenzoate at 412 nm after the enzymatic reaction initiated for 30 minutes. The BChE activity assay was conducted using the same procedure described above with different enzyme and substrate, butyrylthiocholine esterase from equine serum and butyryl-choline iodide.

Each test was performed in triplicate. The blank absorbance is the absorbance of test samples in DMSO with substrate and DTNB, but without enzyme. The test samples'

absorbance was corrected by subtracting the absorbance of their respective blank. The following formula was used to calculate the percentage of inhibition (Eq. 1)

$$\% \text{ Inhibition} = (\text{Abs. control} - \text{Abs. sample}) / \text{Abs control} \times 100 \quad (\text{Eq. 1})$$

Compound with more than 50% inhibition potential during the screening was further tested to determine the half-maximal inhibitory concentration (IC_{50}). The procedure was conducted using same procedure described above with enzyme butyrylcholine esterase from equine serum, butyrylthiocholine iodide as substrate and a set of five concentrations test sample, 1.25, 2.5, 5, 10, and 20 μM .

4.3.2 Kinetic study and inhibition mode

Determinations of enzyme kinetic and inhibition mode were done by using Lineweaver-Burk analysis. The Lineweaver-Burk or double-reciprocal plot (Eq. 2) is a reciprocal rearrangement of the Michaelis-Menten equation (Eq. 3) to give a linear dependent $1/v$ upon $1/[S]$ displaying the kinetic behaviour of the inhibitor.

$$v = ([S]V_{max}) / (K_m + [S]) \quad (\text{Eq. 2})$$

$$1/v = (K_m/V_{max})(1/[S]) + (1/V_{max}) \quad (\text{Eq. 3})$$

The enzyme inhibition kinetic was conducted in the absence and presence of sample compound at concentration of 0 μM , 3 μM , 6 μM and 12 μM at a different concentration of substrate 3.5 mM, 7 mM, 14 mM and 28 mM. Kinetic parameters of inhibition, Michealis-

Menten (K_m) constant and maximum velocity (V_{max}) were derived from Lineweaver-Burk plot [27].

4.3.4 Molecular docking

The three-dimensional coordinates of reference structure such as human butyrylcholinesterase, BChE complexed with Thioflavin T (PDB ID: 6ESY) and human acetylcholinesterase, AChE complexed with Dihydrotanshinone 1 (PDB ID: 4M0E) were fetched by ID from the Protein Data Bank (PDB) database in UCSF Chimera version 1.14 /1.15 [28]. Before docking, both crystal PDBs were processed using Dock Prep tool, starting by removing of water molecules and unrelated hetam, followed with separation of receptor and inhibitor from the complexes into individual structures and finally minimization of individual structures by steepest descent steps (polar hydrogens and Gasteiger charges were added) [29]. The two enantiomers of compound **7b** were built using ChemDraw and subsequently converted from cdx format to PDB.

To recognize the binding sites in BChE, the grid size was set to 65, 74, and 69 along the X-, Y-, and Z-axes with a 0.375Å grid spacing. The grid centre along the X-, Y-, Z-axes was set to -2, -9, and -15Å. Meanwhile, to recognize the binding sites in AChE, the grid size was set to 90, 81, and 74 along with the X-, Y-, and Z-axes with a 0.375Å grid spacing. The grid centre along the X-, Y-, Z-axes was set to 33, 46, and 131Å. The AutoDock Vina tool was finally used to calculate possible bindings and energies [30]. The output from AutoDock Vina was further analyzed using Biovia Discovery Studio Visualizer Client 2020 (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

Conflict of Interest

The authors declare no potential conflict of interest.

Authors Contributions

Nadia Mohamed Yusoff: Methodology, Formal Analysis, Investigation, Data Curation, Writing–Original Draft Preparation, Visualization. **Hasnah Osman:** Conceptualization, Methodology, Resources, Supervision, Project Administration, Funding Acquisition. **Valentia Katemba:** Methodology, Formal Analysis, Investigation, Data Curation. **Muhammad Solehin Abd Ghani:** Formal Analysis, Investigation, Data Curation, Writing–Original Draft Preparation. **Unang Supratman:** Investigation, Data Curation, Writing–Review and Editing. **Mohammad Tasyriq Che Omar:** Software, Formal Analysis, Writing–Original Draft Preparation. **Vikneswaran Murugaiyah:** Methodology, Resources, Writing–Review and Editing, Supervision. **Xiang Ren:** Investigation, Data Curation. **Yvan Six:** Methodology, Validation, Writing–Original Draft Preparation, Writing–Review and Editing, Visualization. **Mohamad Nurul Azmi:** Conceptualization, Methodology, Validation, Resources, Writing–Original Draft Preparation, Writing–Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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