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Synthesis and Cholinesterase Inhibitory Potentials of (5-formylfuran-2-yl) methyl 3,4-dimethoxy/nitro benzoates

Mehmet KOCA[1*]

ABSTRACT: Cholinesterase (ChE) inhibitors are an important group of drugs used in Alzheimer's, glaucoma, and myasthenia gravis. In recent years, cholinesterase inhibition potentials of compounds have been investigated in new drug discovery studies. In this study (5-formylfuran-2-yl) methyl 4-nitro benzoate (compound 1) and newly designed (5-formylfuran-2-yl) methyl 3,4-dimethoxybenzoate (compound 2) were synthesized. The chemical structures of the synthesized compounds were characterized by spectral data (HRMS, ^{1}H NMR, and ^{13}C NMR). The ChE inhibitory activity of the compounds was evaluated using *in vitro* colorimetric Ellman method. Compound 1 and compound 2 exhibited inhibitory activity against AChE at IC_{50} values of 3.25 µM and 8.45 µM, respectively. Compound 1 and Compound 2 showed inhibitory activity against BuChE at IC_{50} values of 8.45 µM and 14.44 µM, respectively. In Docking simulations with 1EVE and 1P0I, the binding free energy scores of compound 1 were higher than the binding free energy scores of compound 2. In this respect, *in silico* molecular docking studies overlapped with *in vitro* enzyme inhibition studies. These derivatives can be used to develop new drugs such as cholinesterase inhibitors.

Keywords: Benzoate, cholinesterase, inhibition, molecular docking, synthesis

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INTRODUCTION

Alzheimer is a disease characterized by disruption of the cholinergic system, Aβ accumulation, tau hyperphosphorylation, metal dyshomeostasis, neuroinflammation, and various cellular changes related to the pathogenesis of the disease. (Chen et al., 2022; Karran and De Strooper, 2022; Tecalco–Cruz et al., 2022) Based on these findings, various hypotheses have been proposed for the prevention of Alzheimer's and slowing the progression of Alzheimer's disease. (Contestabile, 2011; Giacobini, 2003) Acetylcholine, a neurotransmitter secreted by cholinergic neurons, is important for cognitive function.(Klinkenberg et al., 2011; Muir, 1997) . Acetylcholine is broken down or hydrolyzed to acetic acid and choline (Ch) by cholinesterases (ChEs). (Silman and Sussman, 2008) ChEs are acetylcholinesterase (AChE), which is the specific cholinesterase, and butyrylcholinesterase (BuChE), which is a non-specific cholinesterase. Acetylcholinesterase inhibitors continue to be used today to prevent the progression of Alzheimer's disease. (S. Zhou and Huang, 2022) BuChE has lower acetylcholine catalytic efficiency than AChE. (Ha et al., 2020) BChE shows protective activity against the toxicity of nerve agents. BuChE inhibitors can produce lower side effects than specific AChE inhibitors in long-term use. (S. Zhou and Huang, 2022)

There are a limited number of drugs currently used in Alzheimer's, such as Donepezil, galantamine, and rivastigmine, which are FDA-approved as cholinesterase inhibitors. (Sharma, 2019) All these drugs have side effects such as insomnia, nausea, loss of appetite, and diarrhea. In addition, these drugs may show different side effects on the cardiovascular system, which can result in death.(Ali et al., 2015)

Lipophilicity plays a role in modulating AChE inhibitor potency. (Carotti et al., 2006) The methyl and ethyl ester derivatives of phenolic acids were found to be more potent inhibitors than the corresponding free acids. (Szwajgier, 2013)

The ChEs inhibitory potentials of phenyl acetates and phenylacetamides were evaluated. According to the results, esters caused more effective ChEs inhibitory activity than their amide analogs. Among the compounds, 4-nitro phenyl derivatives showed higher activity compared to other 4-substituents.(Krátký et al., 2016)

AChE has two important active sites: the catalytic anionic domain (CAS) and the peripheral anionic domain (PAS). Acetylcholine is hydrolyzed in CAS. (Bajda et al., 2013) PAS influences the conformation of the CAS and the entry of ligands into the CAS region. (Bourne et al., 2003)

Donepezil (Figure 1) is a cholinesterase inhibitor in the structure of 3,4 dimethoxy indanone, which is the most used in mild to moderate AD. (Sugimoto et al., 2012)

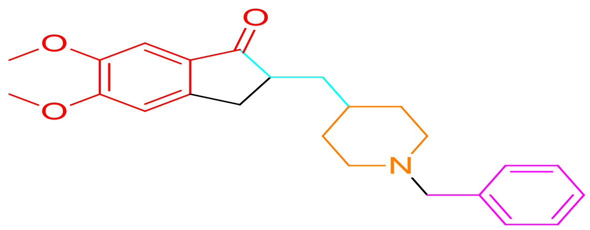


Figure 1. Structure of donepezil

In this study, two benzoate esters of 5-Hydroxymethylfurfuraldehyde were synthesized (Figure 2). From these compounds (5-formylfuran-2-yl) methyl 3,4-dimethoxybenzoate was synthesized for the first time in this study. In addition, ChE inhibitor activities of both molecules were reported for the first time in this study

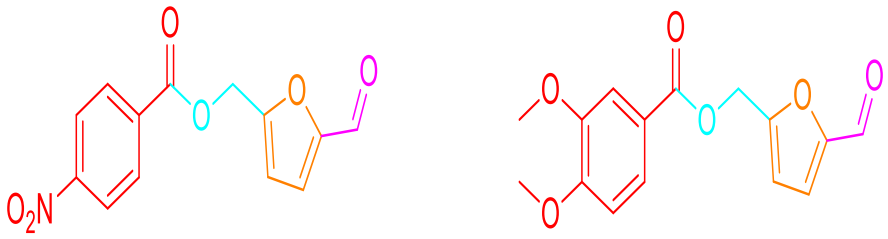


Figure 2. Structure of the benzoate esters of 5-Hydroxymethylfurfuraldehyde

MATERIALS AND METHODS

Chemistry

1 equivalent D-fructose (3,6 gr) was dissolved in DMSO (40 ml) in a 100 ml glass balloon. After that 0.1 equivalent $FeCl_3.H_2O$/Activated charcoal (135 mg/800 mg) were added as the catalyst. The mixture was stirred at 90-100 °C for 4-5 hours with a magnetic stirrer. Afterward, the mixture was filtered and removed from the activated charcoal. After that, 80 ml of water was added to the mixture and the mixture was extracted 3 times with 30 ml of ethyl acetate. Next, the ethyl acetate portion was separated and concentrated under a vacuum. The crude mixture can be used as such for the next reaction. If it is desired to obtain a pure 5-HMF completely separated from DMSO, the crude mixture can be purified using column chromatography. (Ding et al., 2018)

Synthesis of (5-formylfuran-2-yl)methyl benzoate derivatives

5-HMF (366 mg, 1 mmol) was dissolved in 10 mL of dichloromethane. Trimethylamine (606 g, 2 mmol) was then added to the reaction medium. The mixture was then cooled to 0 °C. Then, benzoyl chloride derivatives (1.5 mmol) were added slowly into the mixture. The reaction mixture was stirred at room temperature overnight (10-12 hours) (Figure 3). The product formation in the reaction was followed by TLC. The reaction was terminated by adding water to the mixture. The dichloromethane phase was separated and concentrated under a vacuum. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 3:2). (S. Zhou and Huang, 2022)

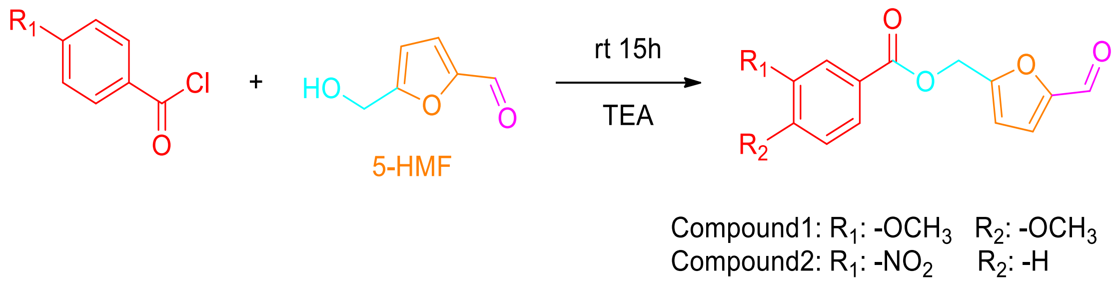


Figure 3. Synthesis of the target compounds

NMR experiments were performed and were recorded with 400 (100) MHz Bruker instruments. Interchangeable hydrogens or carbons were shown with the same letters (Figure 4 and Figure 5).

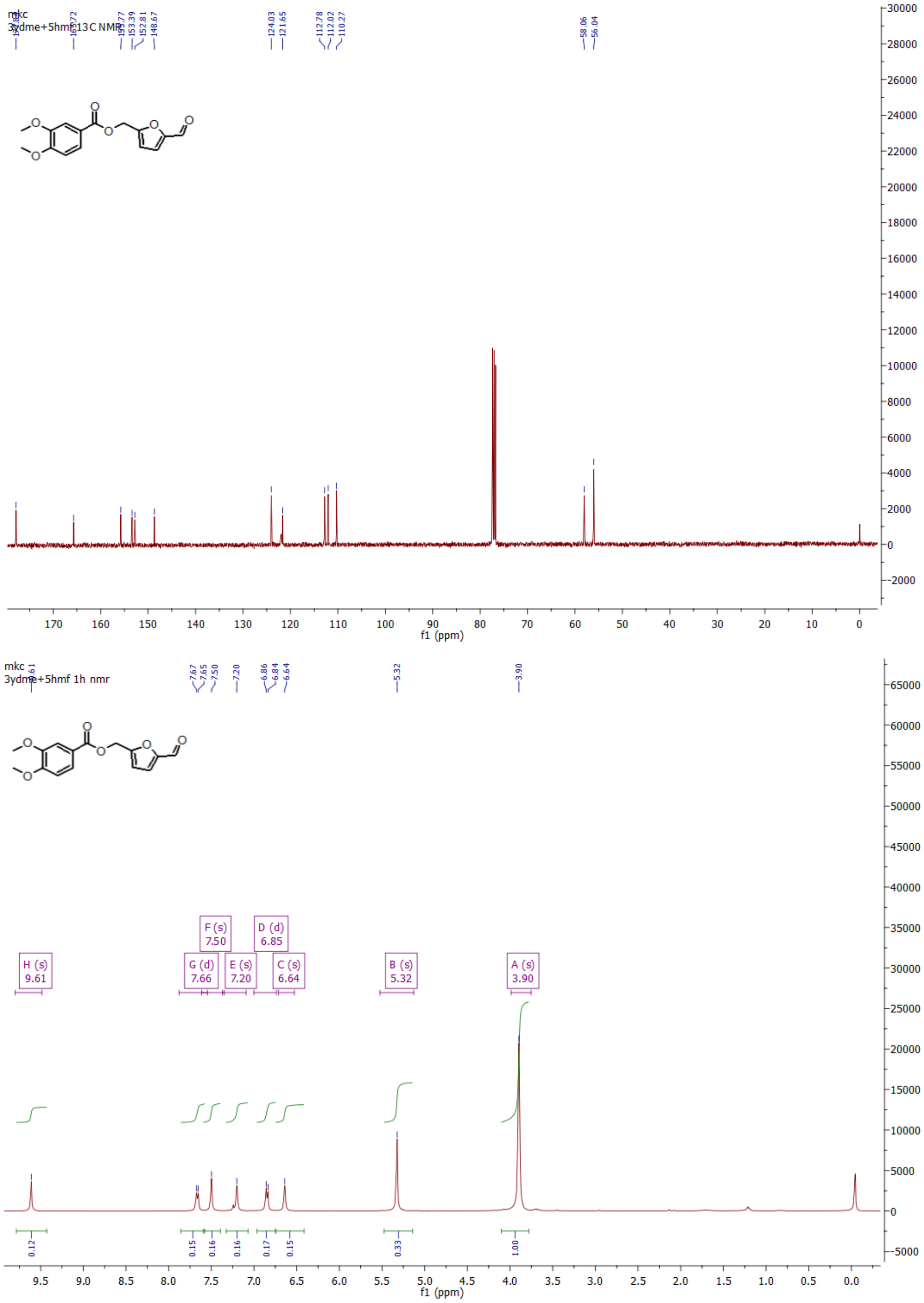


Figure 4. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra of compound 1 (CDCl3).

(5-formylfuran-2-yl) methyl 3,4-dimethoxybenzoate

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.61 (s, 1H), 7.66 (d, J = 8.3 Hz, 1H), 7.50 (s, 1H), 7.20 (s, 1H), 6.85 (d, J = 8.4 Hz, 1H), 6.64 (s, 1H), 5.32 (s, 2H), 3.90 (s, 6H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 177.84, 165.72, 155.77, 153.39, 152.81, 148.67, 124.03, 121.65, 112.78, 112.02, 110.27, 58.06, 56.04. **HRMS (Q-TOF) m/z** Calcd for $[M+Na]^+$ 313.06815, found. 313.06815

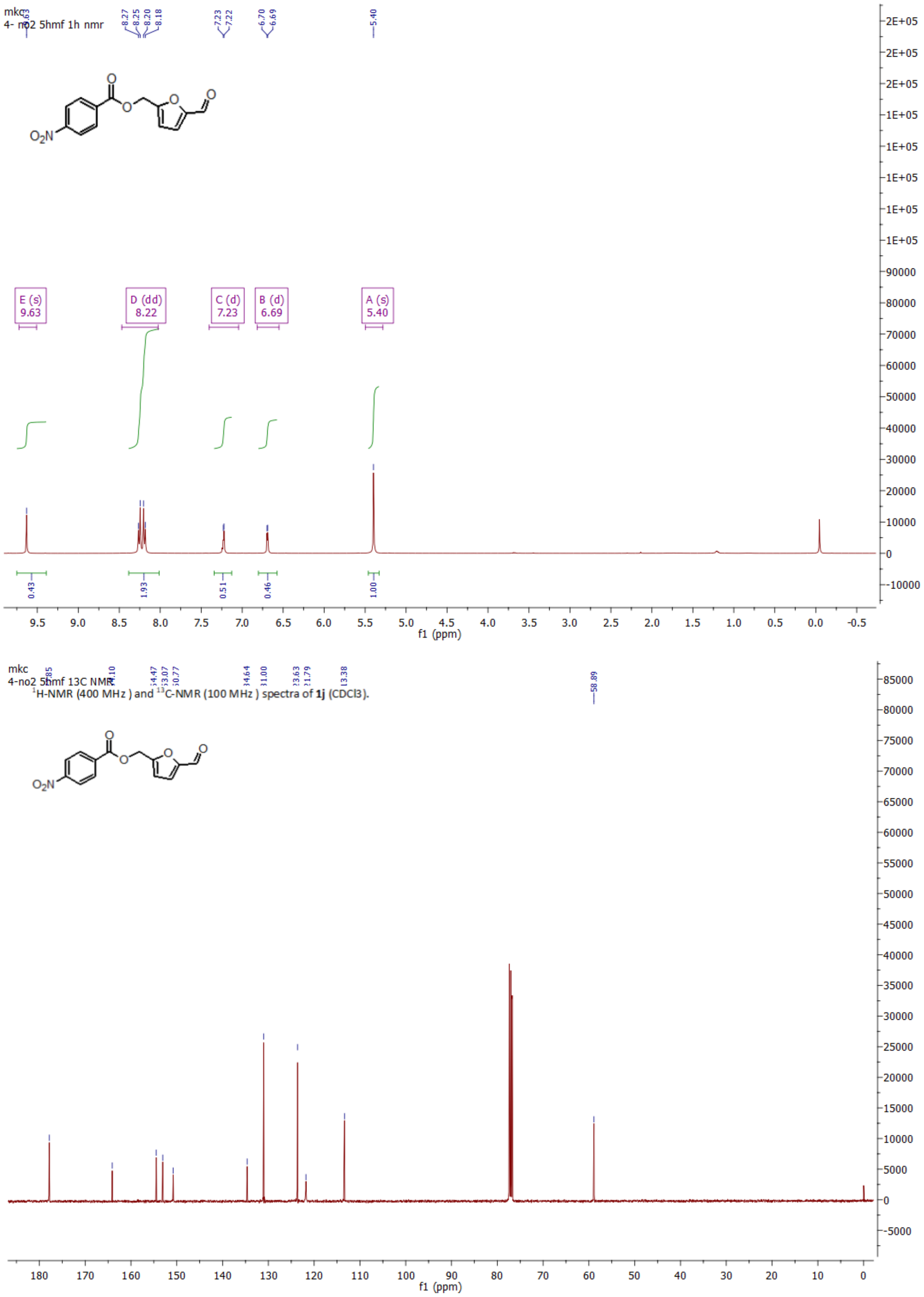
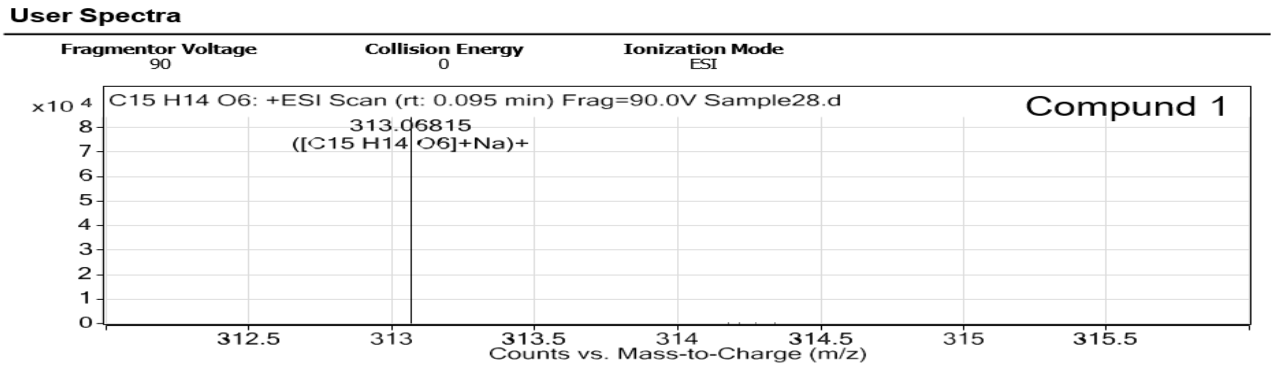


Figure 5. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra of compound 2 (CDCl3)

(5-formylfuran-2-yl) methyl 4-nitro benzoate

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.63 (s, 1H), 8.22 (dd, *J* = 25.0, 8.7 Hz, 4H), 7.23 (d, *J* = 3.5 Hz, 1H), 6.69 (d, *J* = 3.4 Hz, 1H), 5.40 (s, 2H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 177.85, 164.10, 154.47, 153.07, 150.77, 134.64, 131.00, 123.63, 121.79, 113.38, 77.38, 77.07, 76.75, 58.89. **HRMS (Q-TOF) m/z** Calcd for $[M+Na]^+$ 298.03218, found. 298.03208

HRMS spectra were recorded with an Agilent 6530 LC-MS QTOF (Figure 6).

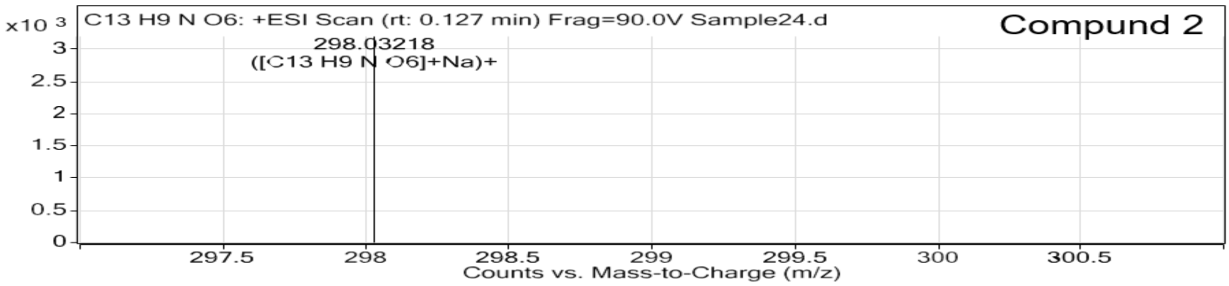


Formula Calculator Element Limits

Element	Min	Max
C	3	15
H	0	14
O	0	6

Formula Calculator Results

Formula	Best	Mass	Tgt Mass	Diff (ppm)	Ion Species	Score
C15 H14 O6	TRUE	290.079	290.079	0.14	C15 H14 Na O6	98.64



Formula Calculator Element Limits

Element	Min	Max
C	3	13
H	0	9
O	0	6
N	0	1

Formula Calculator Results

Formula	Best	Mass	Tgt Mass	Diff (ppm)	Ion Species	Score
C13 H9 N O6	TRUE	275.0431	275.043	-0.47	C13 H9 N Na O6	84.17

Figure 6. Q-TOF Analysis Results of Compound 1 and Compound 2

ChEs inhibitory activity

The inhibitory effect of (5-formylfuran-2-yl)methyl (4-nitro/3,4-dimethoxy)benzoates on AChE and BuChE activities was performed according to the spectrophotometric method of Ellman et al. (1961) (Ellman et al., 1961) with slight modifications as described previously. (Koca and Bilginer, 2022; Koca et al., 2015) AChE (E.C.3.1.1.7) and BuChE (E.C.3.1.1.8) were obtained from Sigma-Aldrich. Donepezil was used as the reference compound. All test compounds were prepared in dimethylsulfoxide at 4 different concentrations ranging from 0.05 to 4.30 µM. The solutions of ChEs (0.2 U/mL), 5,5′-Dithio-bis(2-nitro-benzoic)acid (DTNB) (3 mM), tris buffer solution (50 mM, PH 8.0), acetylthiocholine iodide (ATCI) / butyrylthiocholine iodide (BTCI) (15 mM) were prepared in deionized water. The absorbance of the reaction mixture was then measured three times at 412 nm every 45 s using a microplate reader (Bio-Tek ELx800, Winooski, VT). IC_{50} values were obtained from activity (%) versus compounds plots (Figure 7).

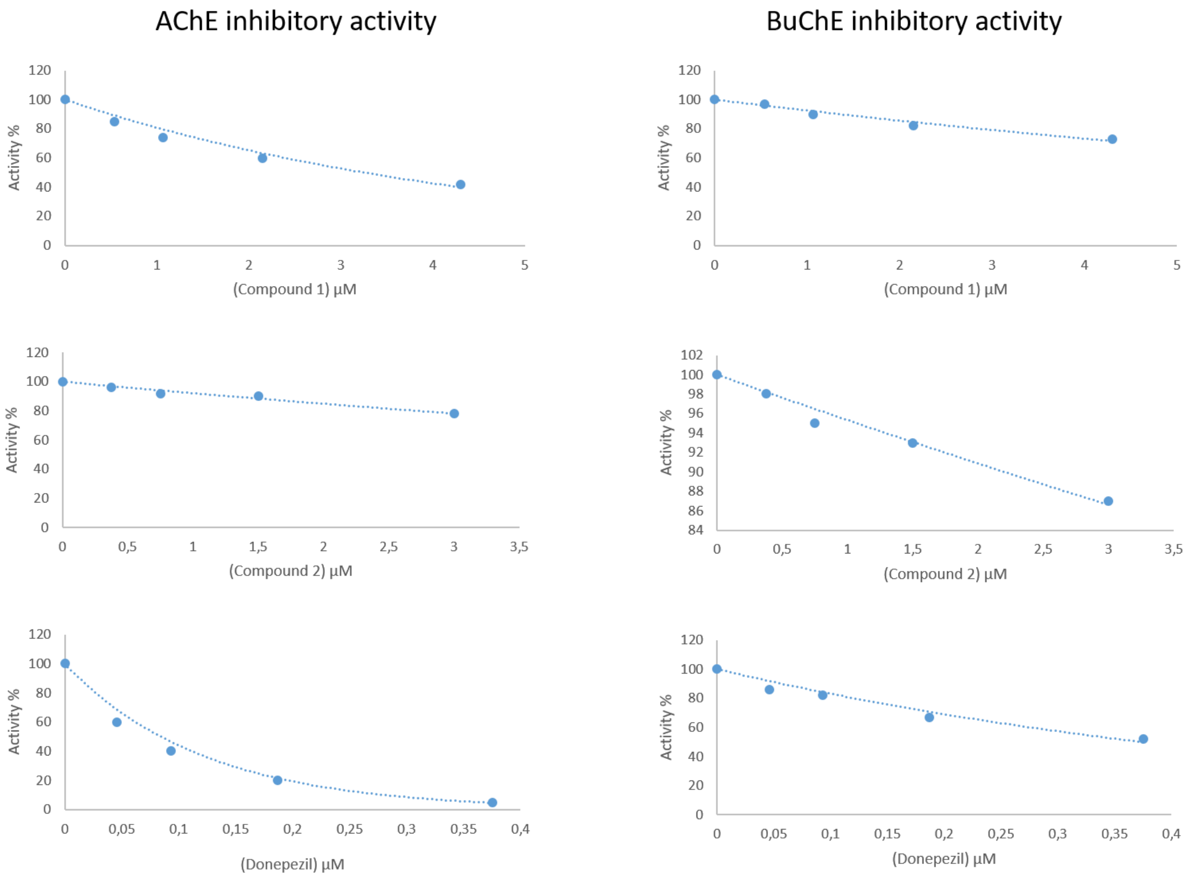


Figure 7. Activity % – [inhibitor] plots of compounds on ChEs**.**

Enzyme kinetic studies

Catalytic evaluation of ChEs was performed in the presence or absence of inhibitor compound at 5 different substrate concentrations 100 mM, 50 mM, 25 mM, 12.5 mM, and 6.25 mM. ATCI was used as the substrate for AChE, while BTCI was used as the substrate for BuChE. The K_i value of compound 1 was tested at 5 µM,10 µM, and 15 µM concentrations, the K_i value of compound 2 was tested at 7 µM,14 µM, 21 µM concentrations, and the K_i value of donepezil was tested at 0.08 µM, 0.16 µM, 0.32 µM concentrations. While measuring the enzymatic reactions, the conditions mentioned in the ChEs inhibitory activity section were applied. The K_i values were calculated by plotting the Lineweaver-Burk curves using excel (Figure 8). (Lineweaver and Burk, 1934)

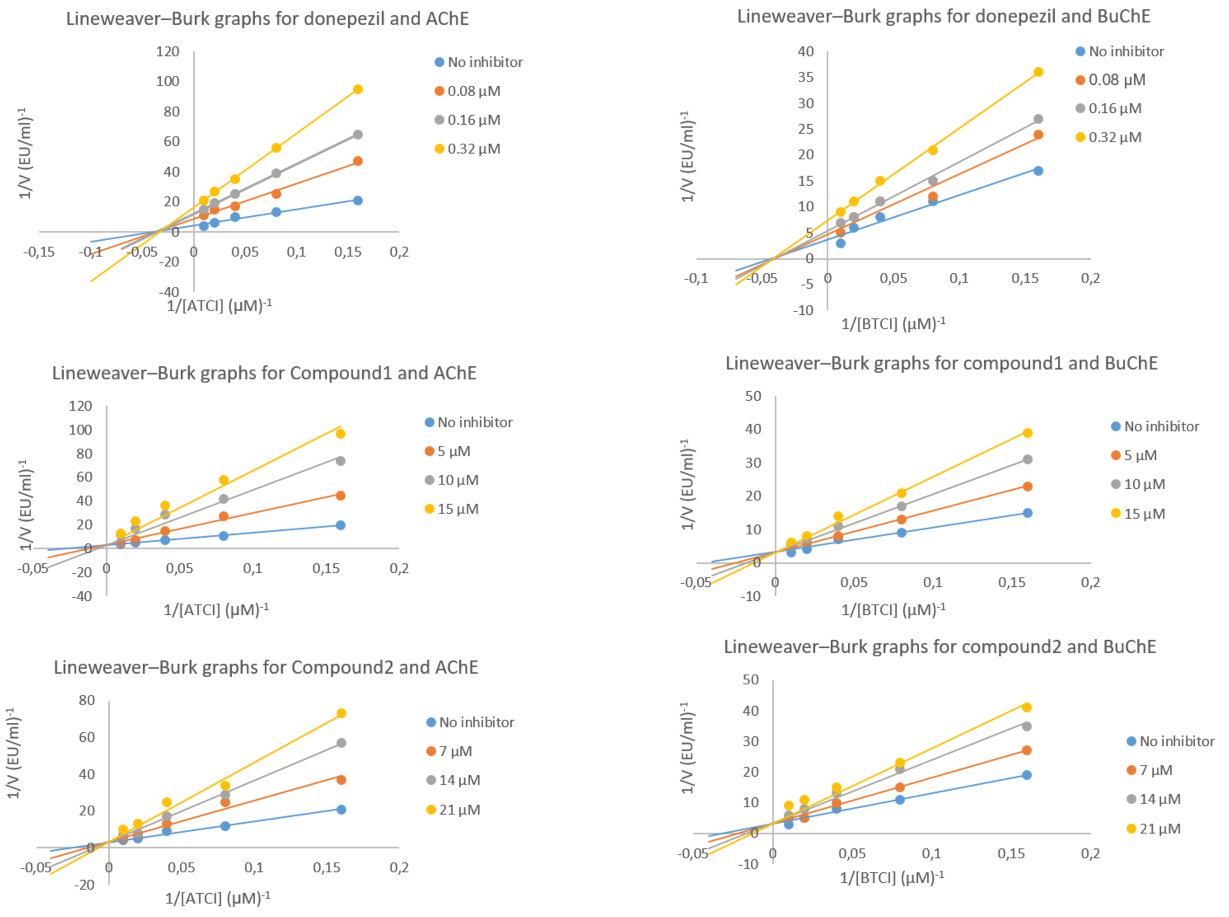


Figure 8. Lineweaver-Burg graphs for inhibitors and ChEs

Molecular docking studies

The crystal structure of AChE (1EVE) and the crystal structure of BuChE (1P0I) were attained from the Protein Data Bank. Ligand and receptor structures were prepared for molecular docking using Autodock4.2 tools. (Morris et al., 2009)

The grid dimensions were 40 × 40 × 40. The spacing between grid points was separated by 0.375 Å. Docking studies were performed on the binding sites of specific ligands for receptors. The binding positions of the ligands were determined using the Lamarckian genetic algorithm. A maximum of 10 conformers were considered during the docking process for each compound. Clustering conformations were analyzed with RMSD tolerance of less than 2.0 Å. The binding free energy scores were ranked by the lowest energy representative of each cluster. Protein-ligand interactions were visualized by using Discovery Studio Visualiz

RESULTS AND DISCUSSION

(5-formylfuran-2-yl) methyl 3,4-dimethoxy benzoate (compound 1) and (5-formylfuran-2-yl) methyl 4-nitro benzoate (compound 2) were obtained in good yields, 92%, and 83%, respectively. While the benzoyl chloride used in the synthesis of the first molecule has a strong electron-withdrawing group such as nitro at the para position, the benzoyl chloride used in the synthesis of the second molecule has a strong electron-donating group such as methoxy at the para position. Therefore, the first molecule was synthesized with a higher yield than the second molecule. (Krygowski and Stępień, 2005) The reaction took place at room temperature.

The ^{1}H NMR spectrum of compounds showed a singlet at δ 5.32/5.40 due to methylene (CH_2) protons. δ The two doublets in 7.6 and 6.6 are due to furan protons. A singlet at δ 9.61/9.63 is due to aldehyde (CHO) further confirms the structure. The ^{1}H NMR spectrum of compound 1 methoxy protons (2xOCH_3) appeared as a singlet at 3.90. The mass spectrum of compound 1 and compound 2 showed natrium ion peaks at m/z = 313.06815 (M+Na) and m/z = 298.03218 (M+Na) respectively which is in agreement with the molecular formulas ($C_{15}H_{14}O_6$ and $C_{13}H_9O_6$).

Tacrine is a competitive/non-competitive reversible ChEs inhibitor approved by the FDA for use in Alzheimer's. (Osmaniye et al., 2022) Tacrine, which has IC_{50} values of low micromolar concentrations against ChEs, is used as a reference inhibitor in many studies on *in vitro* ChE inhibition. (Özbey et al., 2016; Yılmaz et al., 2016) However, due to the hepatotoxic effects of tacrine, its clinical use is very limited.(Uğur Güller, Pınar Güller, 2021) In this study, donepezil was used as the reference inhibitor. Results of *in vitro* inhibitory and molecular docking studies of (5-formylfuran-2-yl) methyl benzoate derivatives on AChE and BuChE are summarized in Table 1. According to Table 1, IC_{50} values of the reference drug donepezil were 0.08 µM and 0.37 µM against AChE and BuChE. Compound 1 and Compound 2 had IC_{50} values against AChE of 3.25 and 8.45 µM, respectively, while IC_{50} values of Compound 1 and Compound 2 were 8.88 µM and 14.44 µM against BuChE, respectively. In enzyme kinetic studies, donepezil showed a K_i value of 0.070±0.002 µM against AChE, while a K_i value of 0.29±0.003 µM against BuChE. Compound 1 exhibited a K_i value of 3.03±0.12 µM against AChE and a K_i value of 7.28±0.07 µM against BuChE. Compound 2 performed a K_i value of 7.2±0.14 µM against AChE and a K_i value of 13.78±0.62 µM against BuChE. The inhibitory potential of compound 1 against both ChEs was approximate twice the inhibitory potential of compound 2 against both ChEs.

In a study in the literature, nitrobenzoate derivatives were synthesized by reacting the hydroxyl group of salicylaldehyde with nitro substituted benzoyl chloride. In the study, the effect of the position of the electron-withdrawing nitro group in the benzene ring on the choline esterase inhibitory activity of the molecules was investigated. Acetylcholine esterase inhibitory activity of 4-Nitrobenzoate derivative was found to be higher than the inhibitory activity of 2-Nitrobenzoate and 3-Nitrobenzoate derivatives at micromolar concentrations.(Çakmak et al., 2021)

According to the results of dockings with both ChEs while donepezil's binding free energy scores were higher than compound 1's binding free energy scores, compound 1's binding free energy scores were higher than compound 2's binding free energy scores. The fact that compound 1 has a higher inhibition potential than compound 2 may be due to the presence of a group that provides electrons to the ring with resonance in compound 1, and the presence of a group that withdraws electrons by resonance from the ring in compound 2.

Table 1. Results of inhibitory activity and docking scores

Enzyme	Compounds	R1	R2	IC_{50} (µM)	Types of Inhibition	K_i (µM)	Estimated Free Energy of Binding (kcal/mol)
hAChE	Compound 1	$-OCH_3$	$-OCH_3$	3.25	Competitive	3.03±0.12	-7.60
	Compound 2	$-NO_2$	-H	8.45	Competitive	7.2±0.14	-7.14
	Donepezil			0.08	Non-competitive	0.070±0.002	-10.44
hBuChE	Compound 1	$-OCH_3$	$-OCH_3$	8.88	Competitive	7.28±0.07	-6.90
	Compound 2	$-NO_2$	-H	14.44	Competitive	13.78±0.62	-6.45
	Donepezil			0.37	Non-competitive	0.29±0.003	-8.88

Binding interactions of the compounds and AChE were presented in Figure 9. The catalytic triad, also known as the estartatic portion of the enzyme, is where acetylcholine is hydrolyzed to choline and acetic acid. (Y. Zhou et al., 2010) CAS consists of Ser200, His440, and Glu327 residues called catalytic triad in Torpedo californica (TcAChE). In docking studies, hydrogen bond formation was observed between both compounds and the catalytic residues of 1EVE (HIS440 and SER200).

The peripheral anionic domain (PAS) is located at the entrance of AChE and is important for the molecule to reach the catalytic part of the enzyme.(Colletier et al., 2006; Silman and Sussman, 2008) PAS consists of residues Tyr 70, Asp 72, Tyr 121, Trp 279, and Tyr 334 in Torpedo California. (Johnson and Moore, 2006) While hydrogen bond formation was observed between the benzoyl carbonyl of Compound 1 and the residue of TYR121 in the PAS of the enzyme, no bond formation was observed between the benzoyl carbonyl of Compound 2 and the enzyme. In addition, Compound 1 showed a pi-alkyl hydrophobic interaction with Trp279 residue of PAS. Another hydrogen bond was formed between the 3-OCH_3 moiety of compound 1 and PHE 288 in the acyl pocket of the enzyme.

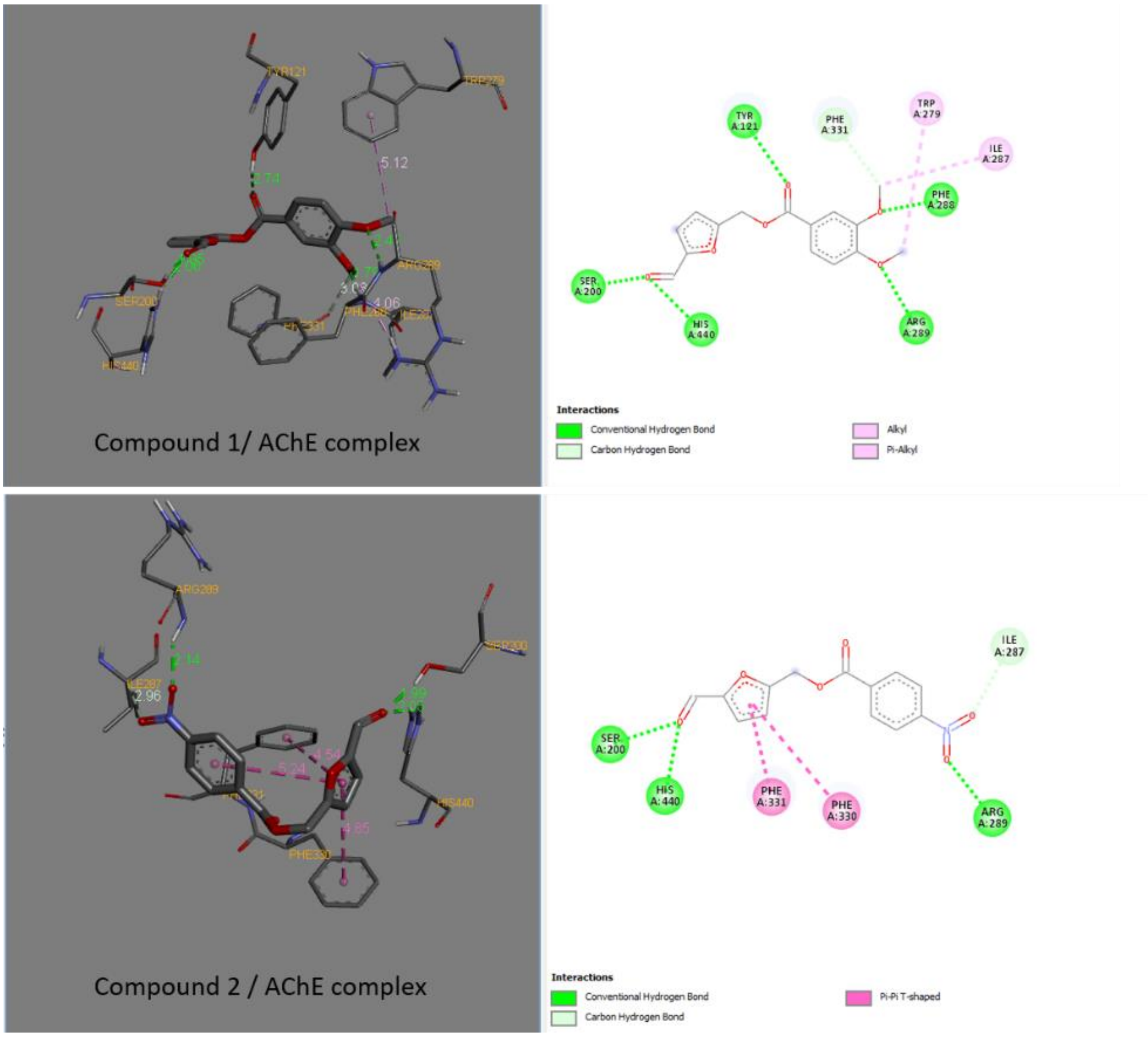


Figure 9. Binding interactions of the compounds and AChE

Binding interactions of the compounds and BuChE were presented in Figure 10. The CAS of human BuChE consists of residues Ser198, His438, and Glu325. (Xing et al., 2021) PAS of human BuChE is constructed from residues Asp70 and Tyr332. (Szwajgier, 2013) In docking studies, Compound 1 formed more hydrogen bonds with the catalytic residues of 1P0I (SER198 and HIS438) than Compound 2. Hydrogen bonding and pi-pi interactions occurred between both compounds and the anionic residues of the enzyme (PHE329, TRP82, TYR128). An unfavorable interaction occurred between compound 2 and the residue of pro285. This may also affect the fact that the activity in compound 2 is lower than in compound 1. (Dhorajiwala et al., 2019)

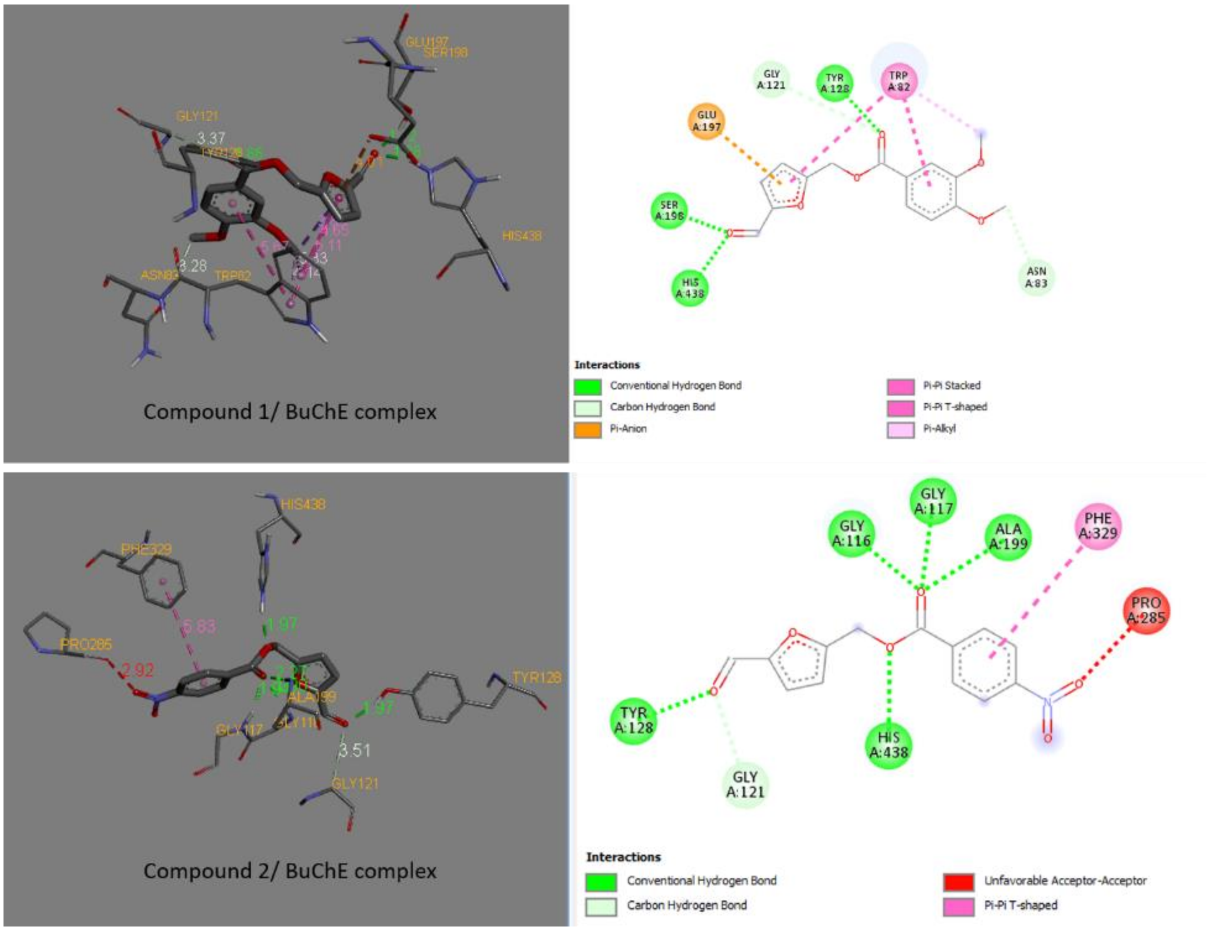


Figure 10. Binding interactions of the compounds and BuChE

CONCLUSION

(5-formylfuran-2-yl) methyl 4-nitro benzoate and the newly designed (5-formylfuran-2-yl) methyl 3,4-dimethoxybenzoate were synthesized and purified successfully. Potential inhibitory effects of the compounds on AChE and BuChE enzymes were evaluated for the first time in this study. The compounds inhibited both ChEs at micromolar levels. In molecular docking studies, it was observed that the furfural carbonyl of the compounds has the potential to form hydrogen bonds with the catalytic residues of ChEs. In both *in vitro* ChE inhibition studies and molecular docking studies, the 3,4 dimethoxy benzoate derivative showed higher cholinesterase inhibitory activity than the 4-nitrobenzoate derivative.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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