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Acetylcholinesterase Inhibition Properties and Docking Studies of Compounds Based on 6-Hydrazinyl-1,3,4-Trimethyl-1H-Pyrazolo[3,4-*b*]Pyridine

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Abstract: New compounds based on 6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-b]pyridine (**3a** and **3b**) were synthesized and characterized by FTIR and $^{1}H/^{13}C$ NMR spectroscopic methods and their *in vitro* acetylcholinesterase (AChE) inhibition studies were evaluated. Compound **3b** (IC_{50} value 104.4 μM) exhibited stronger AChE inhibitory activity than the reference galantamine compound (IC_{50} value 139.4 μM). Molecular docking studies were performed to determine the key interactions and possible binding modes between AChE of compounds. The most active one, **3b**, showed a binding affinity of -10.28 kcal/mol.

Keywords: Synthesis, AChE inhibition, Docking.

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1. INTRODUCTION

Alzheimer's disease (AD) set off 60-70% of dementia cases worldwide with an approximated global incidence of 24.3 million cases. AD is a chronic syndrome that disrupts the central nervous system (CNS). The mechanism of action for AChE inhibitors is the compensation for the loss of central cholinergic neurons in AD (and thus loss of the neurotransmitter acetylcholine (ACh)) through decreased breakdown of Ach. AChE inhibitors are actively used for the effective treatment of Alzheimer's disease. AD causes deficiencies in important features of the brain such as language, learning, orientation, memory, and decision making (1). Old age comes first among the most important risk factors for AD (2). However, the exercises that can be done can cause a slight decrease in AD (3).

Among the most important therapeutic targets for AD are cholinesterases (4,5). The most important reason for the increase in cognitive disorders in AD patients is the deterioration and neurorange of cholinergic neurons in the brain. The cholinergic hypothesis postulates that Alzheimer's results from a reduced ability to synthesize (ACh) in an individual, leading to progressive neurode-generation. For this reason, AChE inhibitors have been used to reduce the degradation rate of ACh. AChE inhibitors increase the amount of ACh by preventing degradation and may increase the function of neural cells. The differential distribution of AChE and BuChE within the brain suggests that both of these enzymes may play important biological roles in their interaction. Although animal models are not transferable to human subjects due to different species-dependent systemic effects both models have proven to have a positive effect on cognitive abilities in the absence of BChE. Therefore, it has been suggested that BChE can be considered as a clinical target in the AD process, as it can take over the function of AChE in the regulation of cholinergic signalling.

There are several drugs used as cholinesterase inhibitors in the treatment of the disease. Donepezil, galantamine and rivastigmine (Figure 1) are the most widely used and have received regulatory approval for the treatment of people with mild to moderate Alzheimer's disease in all jurisdictions (6). There is also evidence to suggest that donepezil can improve the cognitive, functional and neuropsychiatric status of patients with more advanced Alzheimer's disease (7–9). However, the high side effects of these drugs prompted researchers to find new cholinesterase inhibitors.

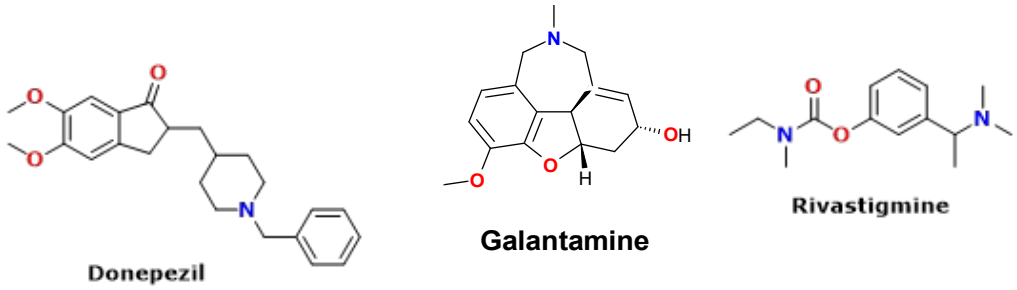


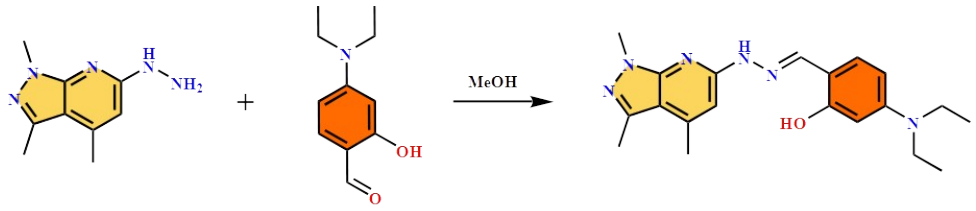
Figure 1: Drugs used for Alzheimer's.

In this study, two new compounds based on 6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-*b*]pyridine were synthesized and their inhibitory effects on (AChE) activity were evaluated. Docking studies were performed to identify possible binding modes of compounds with residues in the AChE active site.

2. EXPERIMENTAL

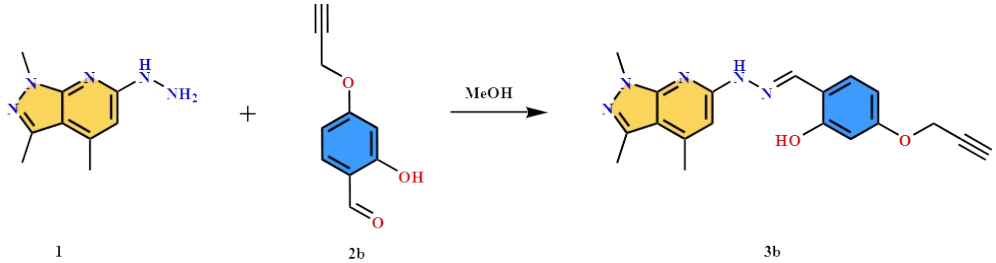
2.1. Synthesis of Compounds 3a and 3b

6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-*b*]pyridine (0.65 mmol, 0.125 g) was dissolved in MeOH. Compound **2b** was synthesized according to the literature (10,11). Then compound 2a (0.65 mmol, 0.126 g) or compound **2b** (0.65 mmol, 0.114 g) added by dissolving with MeOH (15 mL). The reaction mixture was left to reflux overnight. It was then allowed to cool to room temperature and the solvent was removed with evaporator and obtain solid.



1 2a 3a

(*E*)-5-(diethylamino)-2-((2-(1,3,4-trimethyl-1H-pyrazolo[3,4-*b*]pyridin-6-yl)hydrazinylidene)methyl)phenol (**3a**): $C_{20}H_{26}N_6O$. Yield: 93%, Color: Light Yellow.^{1}H NMR (d_6-DMSO, δ) 11.03 (s, 1H), 10.89 (s, 1H), 8.12 (s, 1H), 7.22 (d, J = 8.5 Hz, 1H), 6.48 (s, 1H), 6.24 (d, J = 8.5 Hz, 1H), 6.14 (s, 1H), 3.78 (s, 3H), 3.34 (d, J = 6.9 Hz, 4H), 2.55 (s, 3H), 2.48 (s, 3H), 1.11 (t, J = 6.7 Hz, 6H). ^{13}C NMR (d_6-DMSO, δ) 158.85, 155.84, 151.30, 149.59, 144.09, 142.81, 140.24, 130.33, 109.00, 108.16, 104.00, 102.55, 98.13, 44.24, 33.17, 19.43, 14.95, 13.00. FTIR (ν, cm^{-1}): 2969, 1631 (-C=N-), 1588, 1387, 1351, 1247, 1218, 1190, 1126, 1091, 1070, 819.



1 2b 3b

(*E*)-5-(prop-2-yn-1-yloxy)-2-((2-(1,3,4-trimethyl-1H-pyrazolo[3,4-*b*]pyridin-6-yl)hydrazinylidene)methyl)phenol (**3b**): $C_{19}H_{19}N_5O_2$. Yield: 90%, Color: Light Yellow. ^{1}H NMR (d_6-DMSO, δ) 11.11 (s, 2H), 8.22 (s, 1H), 7.62 – 7.37 (m, 1H), 6.60 (s, 1H), 6.57 – 6.44 (m, 1H), 4.81 (d, J = 2.1 Hz, 1H), 3.79 (s, 2H), 3.63 (s, 1H), 2.57 (s, 2H), 2.49 (s, 2H). ^{13}C NMR (d_6-DMSO, δ) 159.23, 158.18, 155.86, 151.17, 144.35, 140.30, 129.41, 114.41, 109.30, 107.15, 102.74, 102.58, 79.57, 78.89, 55.94, 33.23, 19.42, 14.96. FTIR (ν, cm^{-1}): 2923, 2109 (-C≡CH), 1626 (-C=N-), 1593, 1497, 1386, 1242, 1160, 1028.

2.2. AChE Inhibitors' Activities

Inhibitory activities of compounds based on 6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-*b*]pyridine (**3a** and **3b**) on acetylcholinesterase enzyme (AChE) were determined using the Ellman's method (12,13). *Electrophorus electricus* (electric eel AChE) is well known that the fish enzyme from was used due to lower price, availability, and high compatible to human AChE. The enzyme solution was prepared as 0.22 units/mL. Compounds **3a** and **3b** were the dissolved in water (1×10^{-4}–1×10^{-7} M). 100 µL of phosphate buffer (pH: 6.7) was added to each well of 96 well-plates. Afterwards, different concentrations of the tested compounds (20 µL) and AChE (20 µL/well) were added and incubated at 25 °C for 10 min. Chromatographic reagent 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) (3 mM, 50 µL/well) and substrates acetylthiocholine iodine (ATCI) (3 mM, 50 µL/well) was added to the enzyme-inhibitor mixture. Yellow anion (2-nitro-5-thiobenzoic acid) formation was recorded at 412 nm for 10 min. It was determined by preparing an identical solution of the enzyme in the absence of tested compounds (as a control). Control and inhibitor readings were corrected with a blank read. All operations were repeated three times. Concentrations of samples that inhibit degradation of substrate (acetylcholine) by 50% (IC_{50}) were determined by linear regression analysis between percent inhibition and sample concentration using Excel program. Galantamine was used as a standard and results were compared.

2.3. Docking Studies

The binding modes and interactions of **3a** and **3b** compounds, whose ACh inhibitory effects of examined by in vitro method were investigated molecular docking studies using AutoDock 4.2 software (14). For this purpose, crystal structure of acetylcholinesterase (AChE) (pdb code: 4EY6) were downloaded from the RCSB Protein Data Bank. Then using AutoDock Tools version 1.5.6 ligands, anions and water molecules were removed from the enzymes and polar hydrogen atoms, Kollman and Gasteiger charges were added to amino acid residues in the protein structure (15). The number of points in the x, y and z dimensions was 60 × 60 × 60. The root mean squared deviation (RMSD) of the atomic position between the original orientation of the co-crystal ligand and the re-docked ligand is computed. If the RMSD value is less than or equal to 2.0 Å is acceptable. To visualize molecular docking results of conformations with the lowest binding energy, Discovery Studio 2021 (16) and PyMOL (17) were used.

3. RESULTS AND DISCUSSION

3.1. FTIR Analyses of Compounds 3a and 3b

FT-IR analysis with ATR was performed to investigate the characteristic vibrational bands of compounds **3a** and **3b**. In the FT-IR spectra of the compounds, the carbonyl (C=O) group peaks of the starting compounds did not appear and the vibration bands of the imine (C=N) group were 1631 cm^{-1} for **3a** and 1626 cm^{-1} for **3b** was observed. In addition, vibrational band of the characteristic propargyl (C≡C) group of **3b** was observed at 2109 cm^{-1}.

3.2. NMR Analyses of Compounds 3a and 3b

^{1}H/^{13}C NMR spectra of compounds **3a** and **3b** were recorded in d_6-DMSO and data are presented in the experimental part. ^{1}H/^{13}C NMR spectra of the compounds are given in Figures 1 and 2. When the ^{1}H-NMR spectra of **3a** and **3b** are examined, the methyl group protons on the pyrazole and pyridine rings can be attributed to peaks at 2.48 ppm, 2.55 ppm and 3.78 ppm (NCH_3), respectively. The observed peaks in the range of 6.14-7.53 ppm belong to the aromatic ring protons. Imine group (HC=N) protons were observed at 8.12 and 8.22 ppm, respectively. The hydroxyl group (OH) protons of **3a** were observed at 11.03 ppm and the NH protons at 10.89 ppm, while the hydroxyl and NH protons of **3b** overlapped at 11.11 ppm. The methyl (CH_3) protons of the *N,N*-diethyl group of **3a** were detected at 1.09-1.12 ppm and the protons of the NCH_2 group at 3.33-3.35 ppm. In the ^{1}H-NMR spectrum of **3b**, the peak at 3.63 ppm the propargyl group proton and the peaks observed at 4.80-4.81 ppm to the OCH_2 protons are attributed. When the ^{13}C-NMR spectra of **3a** and **3b** were examined, the imine group carbon atom signals were observed at 158.85 ppm and 159.23 ppm, respectively. Proton signals of aromatic ring carbon atoms were observed in the range of 98.13-158.18 ppm. The methyl group carbon atoms on the pyrazole and pyridine rings are attributed to signals observed around 14.95 ppm, 19.42 ppm and 33.17 ppm, respectively. The methyl (CH_3) and NCH_2 group carbon atom signals of the *N,N*-diethyl group of **3a** were observed at 13.00 ppm and 44.24 ppm, respectively. The propargyl group carbon atom signals of **3b** were observed at 78.89-79.57 ppm and the OCH_2 carbon atom signal was at 55.94 ppm.

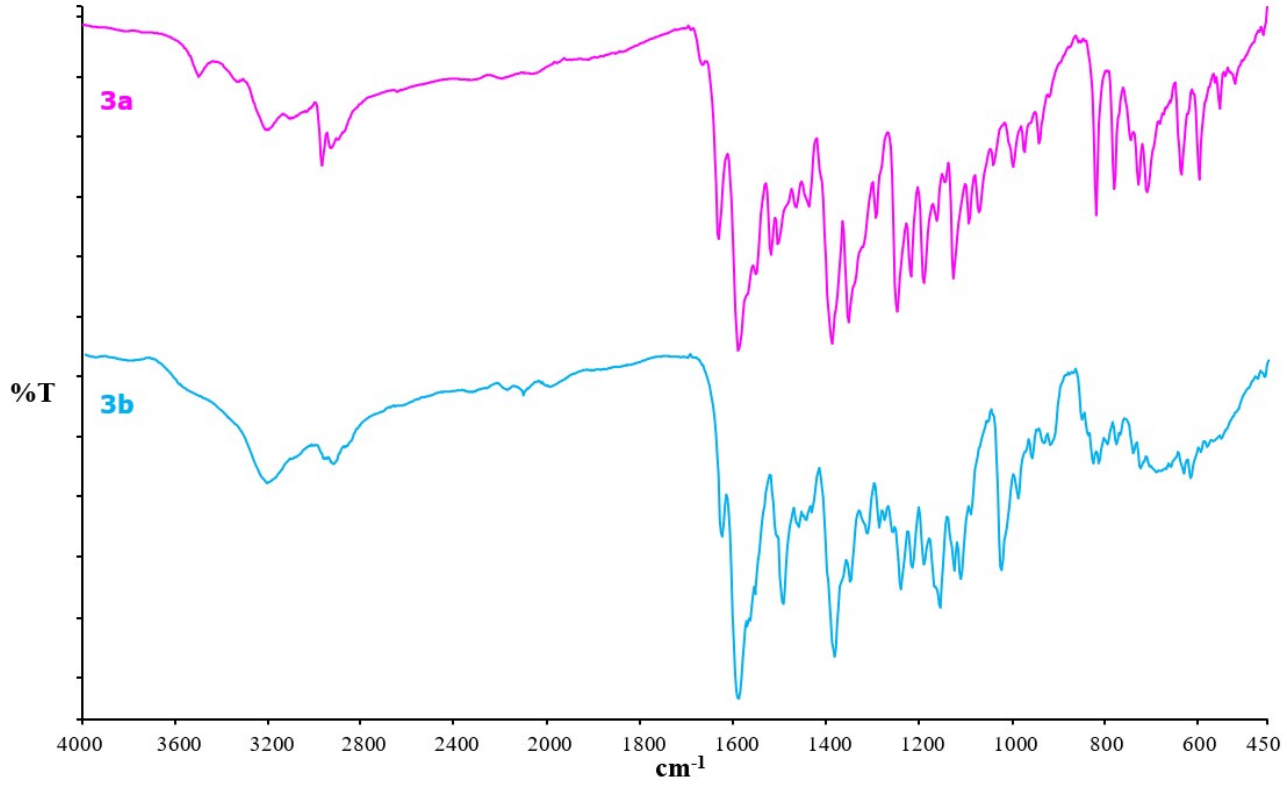
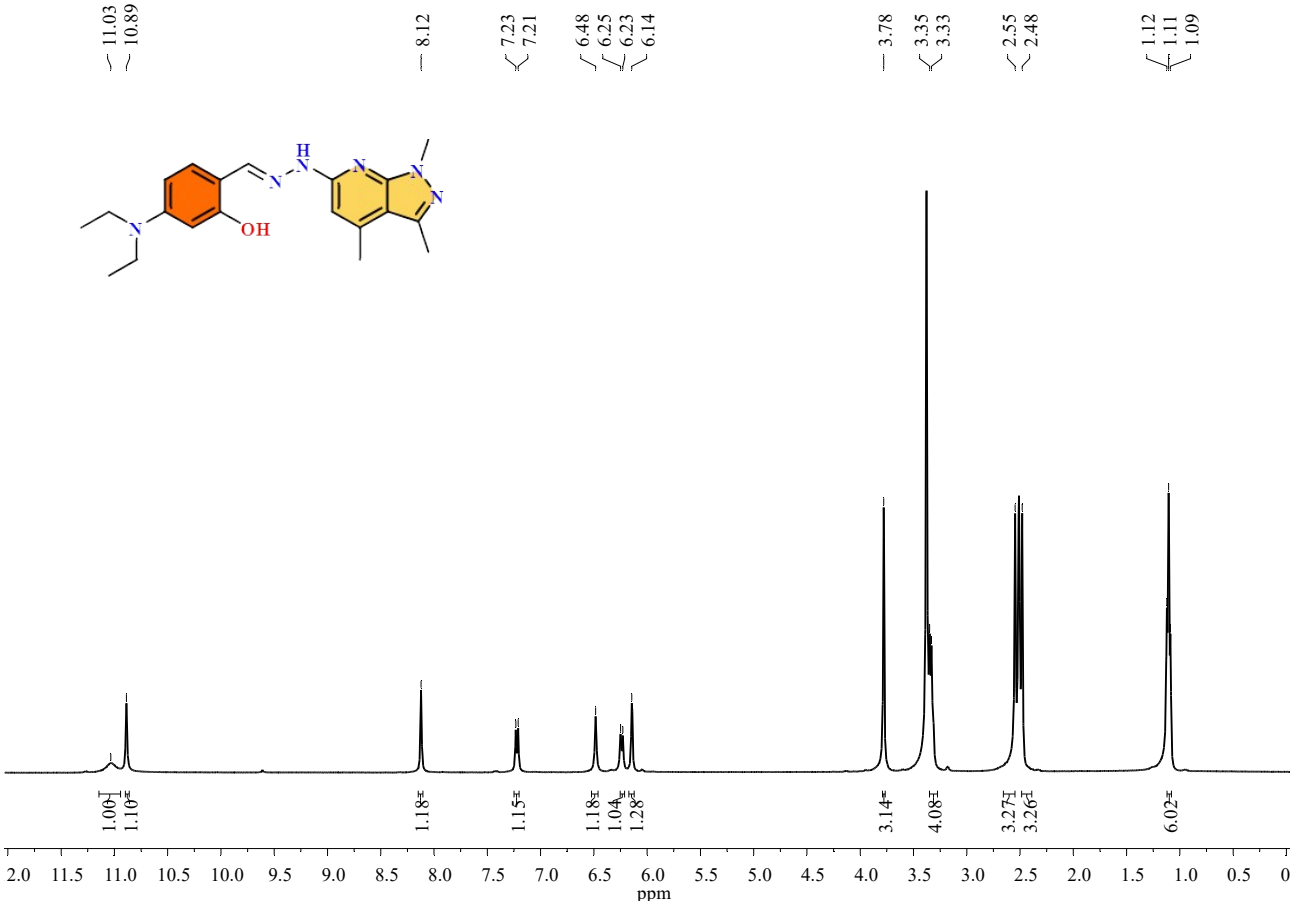


Figure 2: FTIR spectra of compounds **3a** and **3b**.



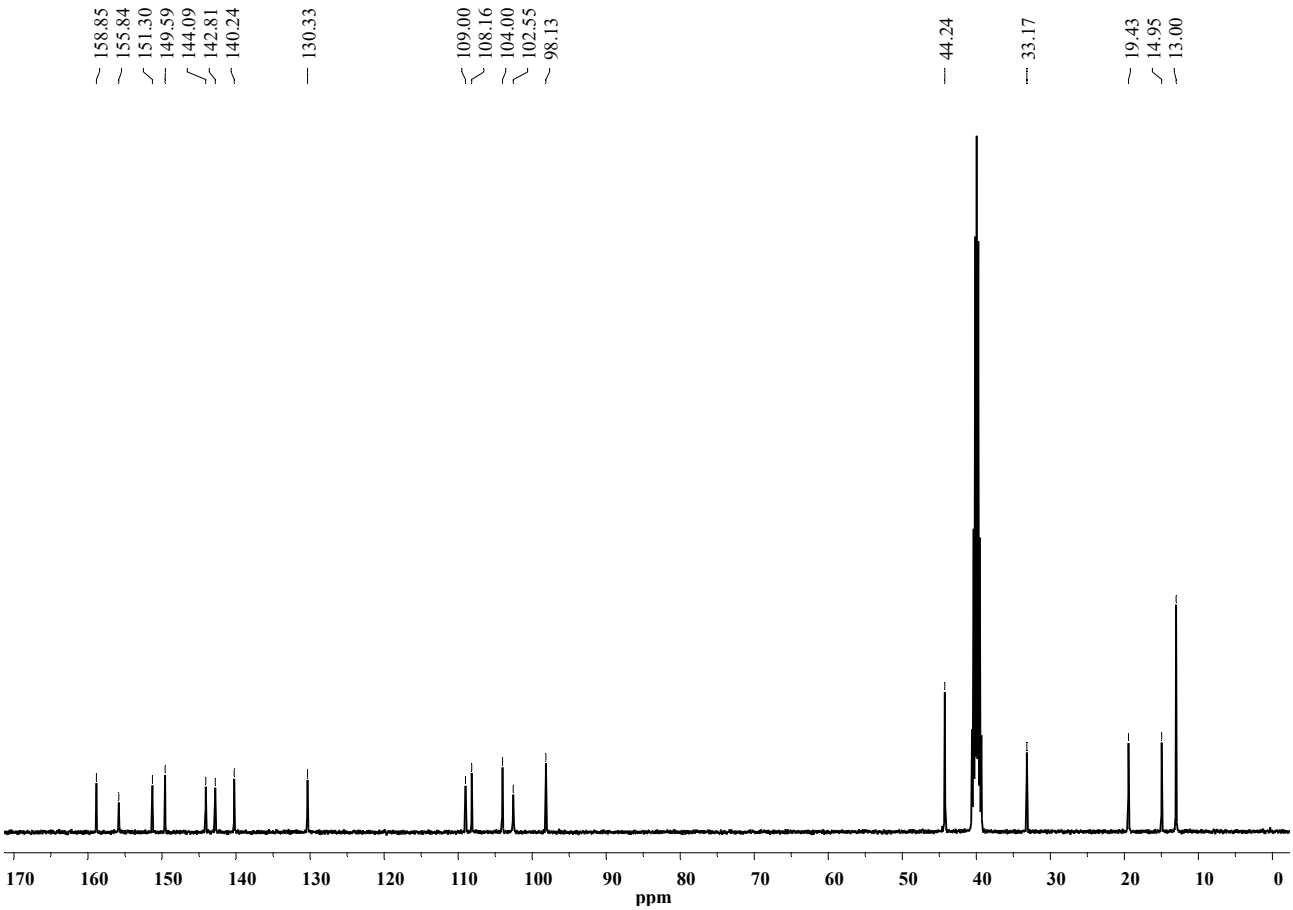
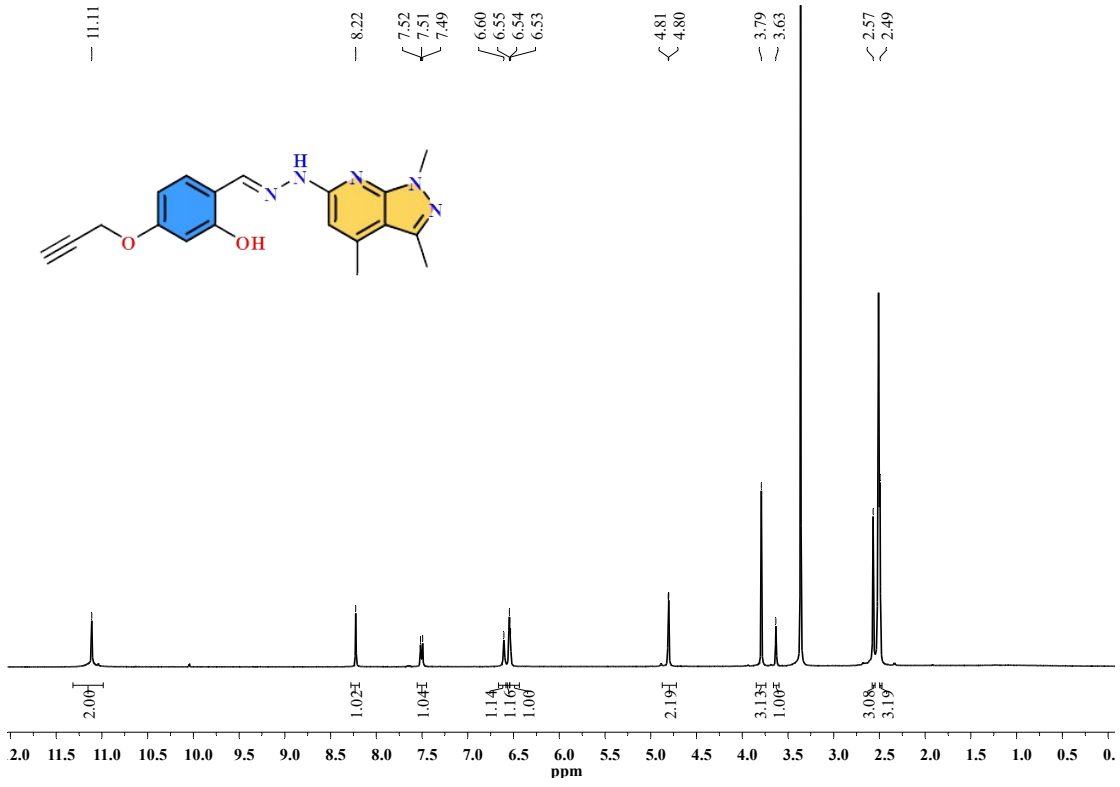


Figure 3: $^{1}H(^{13}C)$ NMR spectra of compound **3a**.



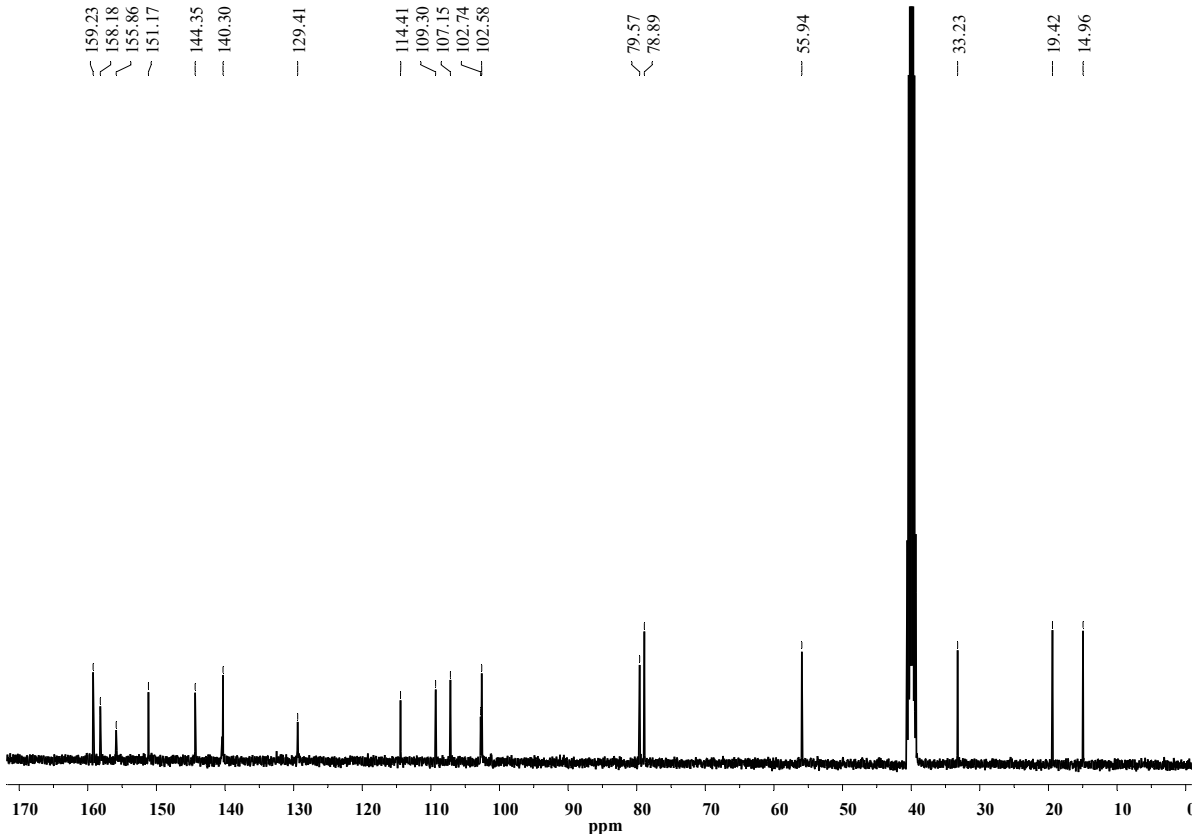


Figure 4: $^{1}H(^{13}C)$ NMR spectra of compound **3b**.

3.3. Inhibitory Activity against AChE

The *in vitro* activities of compounds **3a** and **3b** were investigated against acetylcholinesterase (AchE), one of the main enzymes of Alzheimer's disease. Galantamine was used as a positive control. The inhibitory effects of compounds **3a** and **3b** on AChE (IC_{50} values of 282.37 μM and 104.4 μM, respectively) were evaluated and found to be the most active compound **3b**. Active compound **3b** also showed stronger inhibition than control galantamine (IC_{50} value 139.42 μM). *N,N*-diethyl ($N(C_2H_3)_2$) and propargyl ($OCH_2C{\equiv}CH$) groups were effective in determining the strength of the inhibitory effect of compounds **3a** and **3b** against AChE. The propargyl group end proton of the compound **3b** the hydrophilic interaction with the residues in the active site of enzyme may have been effective increase the inhibitory property. Molecular docking was performed to identify possible modes of binding between AChE and inhibitors.

3.4. Molecular Docking Studies of Compounds 3a and 3b

AChE inhibition effects of compounds based on 6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-b]pyridine (**3a** and **3b**) were investigated by *in vitro* method. Molecular docking study was performed to determine the possible binding poses, interactions, affinities and complex structures of these compounds with AChE. Autodock 4.2 software program was used to determine the active site of AChE. First, the GNT compound co-crystallized with the enzyme was redocking to the active site of the enzyme by the docking procedure. In consequence of placing on the AChE active site, the best binding affinity of GNT was determined as -9.39 kcal/mol.

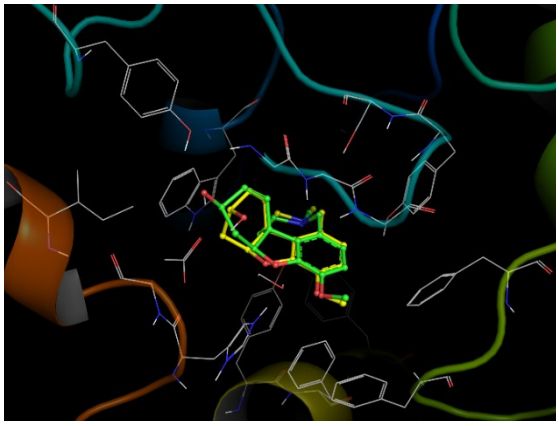


Figure 5: Docking verification. The carbon atoms of the co-crystallized ligand are represented by the ball and stick modeling in yellow, while the carbon atoms of the inserted ligand are represented by the ball and stick modeling in green.

Molecular docking studies of compounds against AChE have been performed. According to the docking results, compound **3b** showed the strongest inhibitory property with -10.28 kcal/mol the lowest binding affinity. Compound **3b** hydrogen bonded with residues Arg296 (2.2 Å), Phe295 (2.1 Å), Tyr337 (2.0 Å and 2.5 Å), Tyr124 (1.9 Å, 2.6 Å and 2.9 Å) and Ser125 (2.7 Å and 2.9 Å) in the active site of the enzyme. Compound **3b** made $\pi-\pi$ interactions with amino acids Tyr341 (4.33 Å), Phe297 (5.91 Å), and Trp86 (4.98 Å, 5.53 Å and 5.89 Å). In addition, compound **3b** formed numerous hydrophobic interactions with residues in the active site of AChE. Residues Tyr124 and Tyr341 in the peripheral anionic region (PAS), Phe295 and Phe297 in the acyl binding pocket and Trp86 is located in the active site. Compound **3b** interacts with Trp286 π-alkyl interaction and Asp74 attractive charge. Compound **3a** formed the most stable complex with a binding affinity of -10.05 kcal/mol as a result of docking at the active site of AChE. Compound **3a** interacted with similar residues as compound **3b** in the active site. Compound **3b** involved in more hydrogen bonds and $\pi-\pi$ interactions than that of compound **3a**.

4. CONCLUSION

Two different compounds based on 6-hydrazinyl-1,3,4-trimethyl-1H-pyrazolo[3,4-b]pyridine (**3a** and **3b**) designed and synthesized and their structures were determined by FTIR and $^{1}H/^{13}C$ NMR spectroscopic methods. *In vitro* inhibitory effects of compounds **3a** and **3b** against AChE were evaluated. Compound **3b** exhibited stronger inhibitory activity than both the reference drug galantamine and compound **3a**. Docking studies were performed to identify possible binding modes of the compounds. In consequence of docking, Compound **3b** was found to have the highest activity with the lowest binding affinity. The pyrazole, pyridine, and hydrazine units were found to make hydrophobic and hydrophilic interactions with the residues in the active site of the enzyme. In addition, the propargyl group was thought to be responsible in the strong inhibitory activity of compound **3b**.

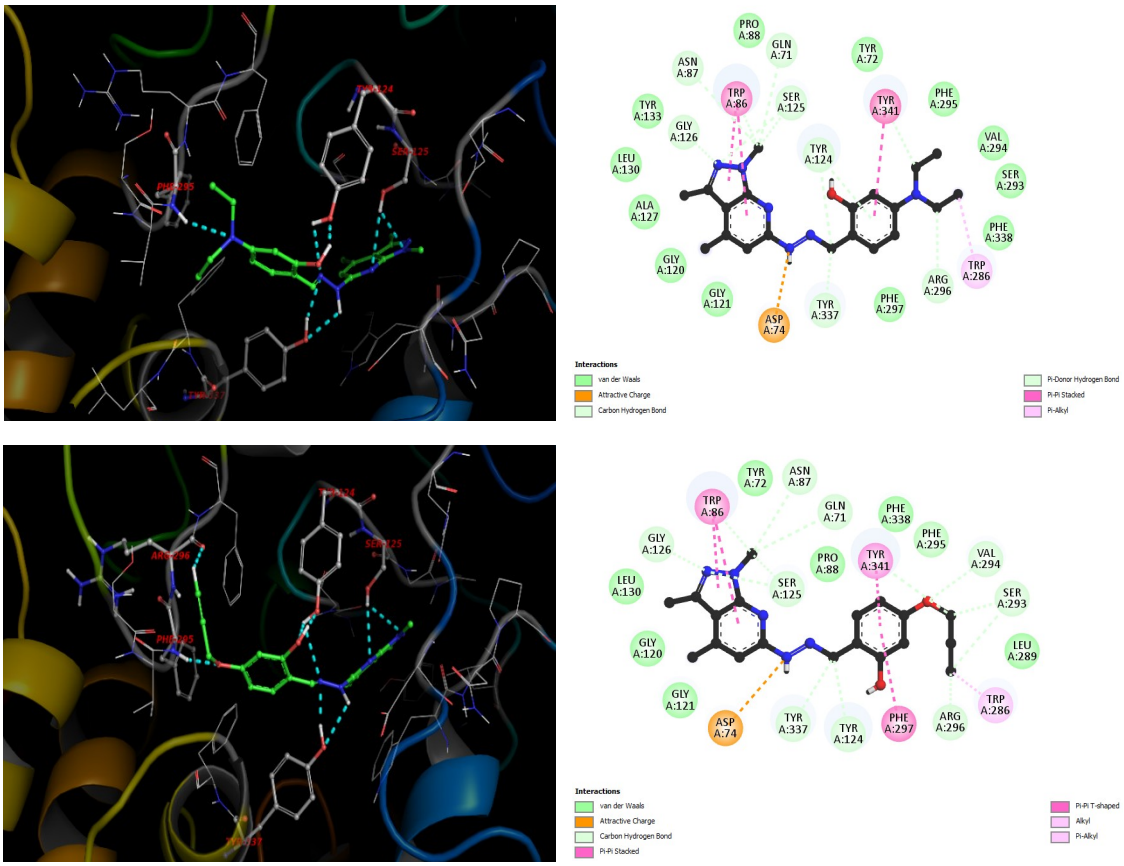


Figure 6: 2D Hydrophobic interaction profile with AChE of compounds **3a** (upper right) and **3b** (lower right). 3D bonding mode of the lowest energy conformation with AChE of compounds **3a** (upper left) and **3b** (lower left). The carbon atoms green color of compounds **3a** and **3b** and the carbon atoms gray color of amino acids are shown as ball and stick model. The hydrogen bond interaction between ligands and residues is shown by turquoise dashed lines.

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