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Research Article

Synthesis and Anticholinergic Activity of 4-hydroxycoumarin Derivatives Containing Substituted Benzyl-1,2,3-triazole Moiety

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A series of 4-hydroxycoumarin-derived compounds 8a-p containing *N*-benzyl-1,2,3-triazole motif were designed as AChE inhibitors. The title compounds were obtained conveniently using multicomponent click reaction. The in vitro anticholinesterase evaluation of synthesized compounds against AChE and BuChE showed that some of them are potent and selective inhibitors of AChE. Among them, 2-chlorobenzyl derivative 8k showed the most potent activity against AChE (IC_{50} = 0.18 μM). Its activity was also superior to that of standard drug tacrine. The kinetic study and molecular docking simulation of the most potent compound 8k were also described.

Key words: 1,2,3-Triazole, acetylcholinesterase, Alzheimer's disease, click chemistry, coumarin, docking study

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Alzheimer's disease (AD) is a prevalent neurodegenerative disorder in elderly adults which causes dementia and other cognitive dysfunctions (1). The number of patients suffering from AD is expected to increase to 25 million by 2025 (2). The increasing incidence of AD as well as the lack of effective strategies for its treatment makes this area as a very important research field in human health (3). The etiology of AD at the molecular level is not completely known, but several possible factors are proposed to be involved in the accession of AD. These include β-amyloid (Aβ) deposition, hyperphosphorylated τ-protein, oxidative stress, metal ion imbalance, inflammation, and low level of acetylcholine (ACh) in brain (4). Among different theories, cholinergic hypothesis has been considered more extensively. The ACh action can be terminated by rapid hydrolyzing with two types of cholinesterase enzymes, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Therefore, the inhibition of cholinesterase enzymes would be a useful approach for increasing the level of ACh in brain and management of AD (5). It has been indicated that AChE contains two distinct binding sites; the catalytic active site (CAS) at the bottom and the peripheral anionic site (PAS) near the entrance of the gorge (6). The previous studies demonstrated that the formation of stable AChE–Aβ complexes via interacting with the PAS of the enzyme accelerates β-Amyloid aggregation (7). Thus, the dual-binding AChE inhibitors that could interact with both the CAS and PAS of enzyme appear to be very promising therapeutic agents for management of AD (4).

Structurally, many aromatic and heteroaromatic structures have potential hydrophobic interactions and π–π stacking with the aromatic residues of the enzyme gorge in AChE. For example, several studies have shown that coumarin nucleus can bind primarily to the PAS of AChE (8–10). Accordingly, coumarin system has been used as a skeleton to discover new AChE inhibitors. Besides, basic side chain containing an amino group is an integral part of the structure of several reported AChE inhibitors such as donepezil, tacrine, and galantamine. It is known that this nitrogen atom plays an important role in enzyme–inhibitor interaction (11).

In light of these findings, recently we have chosen 4-hydroxycoumarin system as a scaffold and basic cyclic amino residues were incorporated to this scaffold with amidic linker (12). In this study, we replaced the basic side chain containing an cyclic amino group with benzyl-1,2,3-triazole moiety (Figure 1). The functionalized-1,2,3-triazole motif could be constructed by application of click chemistry. Several AChE inhibitors containing 1,2,3-triazole have

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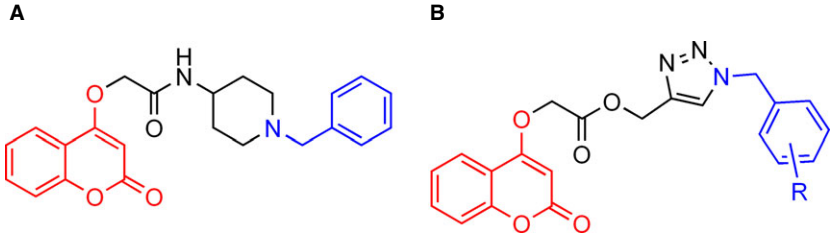


Figure 1: (A) Previously reported compound as AChE inhibitor (IC_{50} = 1.2 μM); (B) new designed compounds bearing *N*-benzyl triazole moiety.

been reported previously (13–15). Therefore, we report here, synthesis and biological evaluation of new 4-hydroxycoumarin derivatives containing substituted benzyl-1,2,3-triazole moiety as AChE inhibitors with potential usefulness in the field of AD therapy.

Experimental Section

Chemistry

The designed compounds **8a-p** were synthesized starting from 4-hydroxycoumarin (**1**) as outlined in Scheme 1. Compounds **4** and **6** were prepared according to the reported methods in literature (12,16).

General procedure for the synthesis of target compounds 8a-p

Compound **6** (1 mmol), NaN_3 (2 mmol), CuI (10 mol%), and different benzyl halides **7** (1 mmol) were added to a mixture of H_2O (6 ml) and PEG 300 (0.2 mmol). The mixture was allowed to stir at 70 °C for appropriate time. Progress of the reaction was monitored by TLC and after completion of the reaction; the mixture was extracted with ethyl acetate. The organic phase was evaporated, and the residue was recrystallized from EtOH.

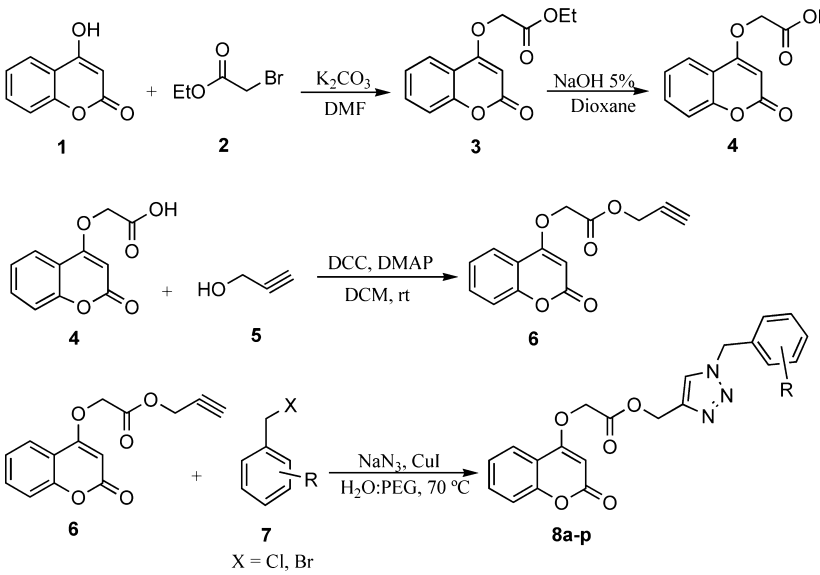
(1-(2-Bromobenzyl)-1H-1,2,3-triazol-4-yl)methyl 2-(2-oxo-2H-chromen-4-yloxy)acetate (8a)

White solid; yield 88%; mp 145–147 °C; IR (KBr, cm^{-1}) ν_{max}: 3143 (CH aromatic), 1747 and 1722 (C=O), 1620 (C=C), 1221 (C-N); ^{1}H NMR (400.0 MHz, $CDCl_3$) δ: 7.90 (dd, 1H, J = 8.0 and 1.6 Hz, H_5 coumarin), 7.69 (s, 1H, triazole), 7.63 (dd, 1H, J = 8.0 Hz, benzyl), 7.58 (dt, 1H, J = 8.6 and 1.6 Hz, H_7 coumarin), 7.36-7.30 (m, 3H, $H_{6,8}$ coumarin, and 1H benzyl), 7.26-7.24 (m, 2H, benzyl), 5.69 (s, 2H, OCH_2), 5.52 (s, 1H, H_3 coumarin), 5.39 (s, 2H, NCH_2), 4.80 (s, 2H, CH_2CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 166.3, 164.5, 162.2, 153.3, 141.8, 133.7, 133.3, 132.8, 130.7, 130.6, 128.3, 124.3, 124.1, 123.7, 123.2, 116.7, 115.1, 91.2, 65.1, 58.7, 54.1. EI-MS *m/z* (%) 471 (M^++2, 22), 469 (M^+, 21), 428 (39), 426 (38), 390 (9), 247 (13), 221 (15), 171 (100), 169 (99), 143 (31), 89 (52), 77 (11). Anal. Calcd for $C_{21}H_{16}BrN_3O_5$: C, 53.63; H, 3.43; N, 8.94. Found: C, 53.37; H, 3.72; N, 8.65.

Additional data for characterization of all target compounds **8a-p** may be found in Supporting Information.

Cholinesterase inhibition assay

Colorimetric Elman's method (17) was used for determination of inhibitory potency of target compounds against



Scheme 1: Synthesis of compounds **8a-p**.

AChE and BuChE enzymes. Acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from *electric eel,* 1000 unit), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), and butyrylthiocholine iodide (BTChI) were purchased from Sigma-Aldrich (St. Louis, MI, USA). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), potassium dihydrogen phosphate, di-potassium hydrogen phosphate, potassium hydroxide, sodium hydrogen carbonate, and acetyl thiocholine iodide (ATChI) were obtained from Fluka. The target compounds were dissolved in a mixture of 1 mL DMSO and 9 mL methanol and diluted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0) to obtain final concentrations. For each compound, five different concentrations were tested to obtain the range of 20–80% enzyme inhibition. The assay medium was composed of 3 mL of phosphate buffer (0.1 M, pH 8.0), 100 μL of DTNB (0.01 M), 100 μL of enzyme solution (AChE or BuChE, 2.5 unit/mL), and 50 μL of test compound. After incubation for 10 minutes in 25 °C, 10 μL of substrate (ATChI or BTChI, 0.15 M) was added and the rate of absorbance change was measured at 412 nm for 6 min. Finally, the IC_{50} values were determined graphically from inhibition curves (log inhibitor concentration vs. percent of inhibition). All spectrophotometric measurements were performed using UV Unico Double Beam Spectrophotometer (Nova Biotech, El Cajon, CA, USA).

Kinetic study

Kinetic study was carried out to obtain proposed inhibition mode and inhibition constant *K*i of the most potent compound. Similar to enzyme inhibition assay, the rate of enzymatic reaction was measured with different concentrations of inhibitor and without inhibitor at different concentrations of substrate (ATChI, 0.14–0.69 mM). Each experiment was started by adding substrate, and the progress curves were monitored for 2 min at 412 nm. Then, the double reciprocal plots of 1/v vs. 1/[s] were constructed using the slopes of progress curves. Afterward, the slopes of reciprocal plots were plotted vs. the concentrations of inhibitor to obtain *K*i value.

Docking studies

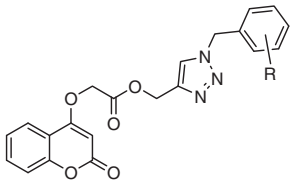
All docking calculations were performed by means of AUTODOCK VINA (ver. 1.1.1) (18). For docking purposes, the crystal structure of AChE (code ID: 1EVE) was taken from the Brookhaven protein database (www.rcsb.org) as a complex with inhibitor E2020 (donepezil). Then, the co-crystallized molecules (water, ligand, etc.) were removed from the protein structure. The 2D structures of ligands were sketched using MARVINSKETCH 5.8.3, 2012, Chemaxon (www.chemaxon.com) and then converted to 3D format by OPENBABEL (ver. 2.3.1) (19). Afterward, protein and ligands were converted to appropriate 3D pdbqt format using AUTODOCK TOOLS (ver. 1.5.4) (20) with default parameters. The center and dimensions of docking box was set as follows: center_x = 2.023, center_y = 63.29, center_z = 67.062; size_x = size_y = size_z = 20Å. The exhaustiveness value was set as 80, and other parameters were left as default. The best docked poses with lowest energy of each ligand were selected for analyzing the interactions between ligand and AChE. CHIMERA 1.6 (21) and POSEVIEWWEB 1.97.035 (PoseView. http://poseview.zbh.uni-hamburg.de/poseview/wizard, 2012) were used for representation of the ligand–protein complexes.

Results and Discussion

Chemistry

The target compounds **8a-p** were synthesized via the route outlined in Scheme 1. At first, compound **3** was synthesized by *O*-alkylation of commercially available 4-hydroxycoumarin (**1**) with ethyl 2-bromoacetate (**2**) in the presence of K_2CO_3 in DMF. In the next step, the obtained ethyl ester **3** was hydrolyzed with aqueous solution of sodium hydroxide in dioxane to yield the corresponding acid **4**. The best condition for the esterification of acid **4** and propargyl alcohol **5** to obtain the key intermediate **6** was achieved by DCC/DMAP in dichloromethane. Finally, one-pot three-component reaction of sodium azide, alkyne **6,** and various benzyl halides **7** was used for the preparation of target compounds. Several conditions were screened to find the optimized condition, but the best result was achieved using mixture of water as solvent and CuI as catalyst (see Table S1). Copper-catalyzed azide–alkyne cycloaddition lead to obtain only pure 1,4-substituted regioisomer. All target compounds were isolated by

Table 1: Anticholinesterase activity of the synthesized compounds **8a-p**



Compound	R	AChE IC_{50} (μM)	BuChE IC_{50} (μM)
8a	2-Br	2.14	43
8b	3-Br	1.79	>50
8c	4-Br	87.9	>50
8d	3-OMe	7.0	>50
8e	4-OMe	104.0	>50
8f	2-Me	1.79	>50
8g	4-Me	90.0	>50
8h	2-F	5.70	>50
8i	3-F	1.64	>50
8j	4-F	2.54	>50
8k	2-Cl	0.18	>50
8l	3-Cl	0.34	>50
8m	4-Cl	13.0	>50
8n	2-NO_2	0.53	>50
8o	4-NO_2	137.0	>50
8p	H	15.77	>50
Tacrine	–	0.35	0.28

simple crystallization from ethanol without the need for chromatography.

Anticholinesterase activity

The *in vitro* anticholinesterase activity of synthesized compounds **8a-p** was evaluated against AChE and BuChE in comparison with standard drug tacrine. The obtained results were presented in Table 1 as IC_{50} values (μM). A survey on IC_{50} values of compounds **8a-p** against AChE revealed that some compounds had potent activity. Among them, 2-chlorobenzyl derivative **8k** with IC_{50} value of 0.18 μM was the most potent compound. It was twofold more potent than standard drug tacrine. Furthermore, the activity of compounds **8b**, **8f, 8i**, **8l,** and **8n** against AChE was remarkable (IC_{50} values = 0.34–1.79 μM). In the cases of bromo, chloro, nitro, and methyl derivatives, the displacement of substitution from *ortho*- to *para*-position dramatically diminished the anti-AChE activity (41–256 times). In contrast, in the case of fluorobenzyl analogs, the *para*-regioisomer was twofold more potent than *ortho* one and the order of activity was as follows: *meta* > *para* > *ortho*. Among the 2-halobenzyl derivatives, the order of activity was as follows: Cl > Br > F, while for 4-halobenzyl analogs, the order was F > Cl > Br. The comparison of *para*-substituted compounds such as **8c**, **8e**, and **8g** with unsubstituted derivative **8p** revealed that the insertion of *para*-substituent diminished the activity. Furthermore, the inhibitory activity of *para*-substituted compounds **8c**, **8e**, **8g**, **8m,** and **8o** was less than their related *ortho*- or *meta*-congeners. Based on these results, it seems that the steric effect plays an important role at this position. Interestingly, the small fluoro group was favorable for *para*-position as observed with compound **8j**. By comparing the IC_{50} values of 3-substituted benzyl derivatives with their related regioisomers, it revealed that substitution at

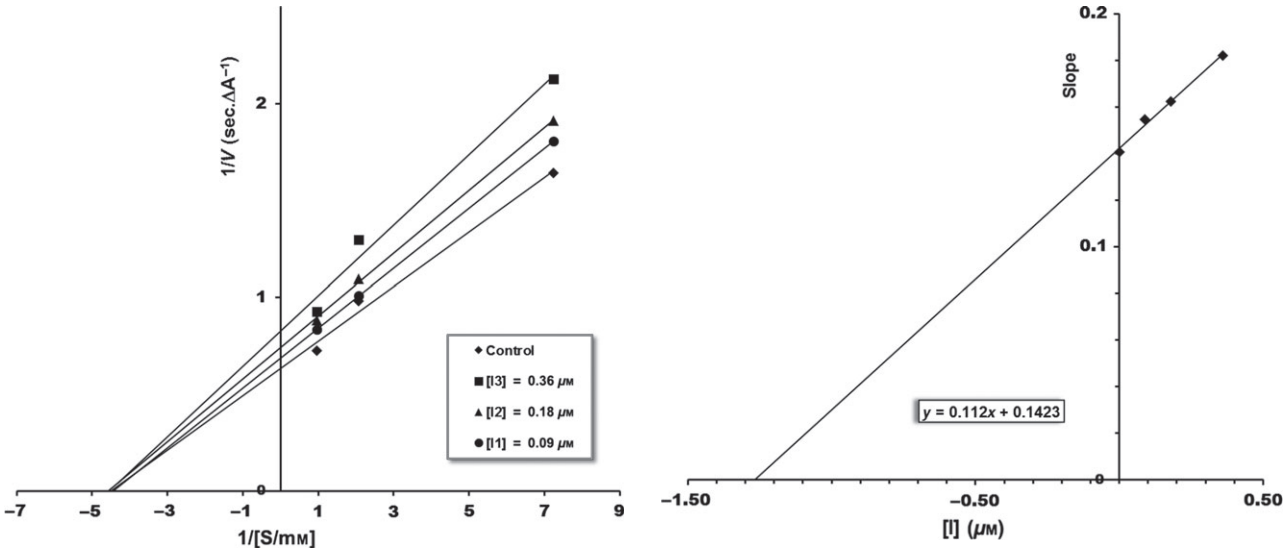


Figure 2: (Left) Lineweaver–Burk plot for the inhibition of AChE by compound **8k** at different concentrations of acetylthiocholine (ATCh). Right) Secondary plot for calculation of steady-state inhibition constant (*Ki*) of compound **8k**.

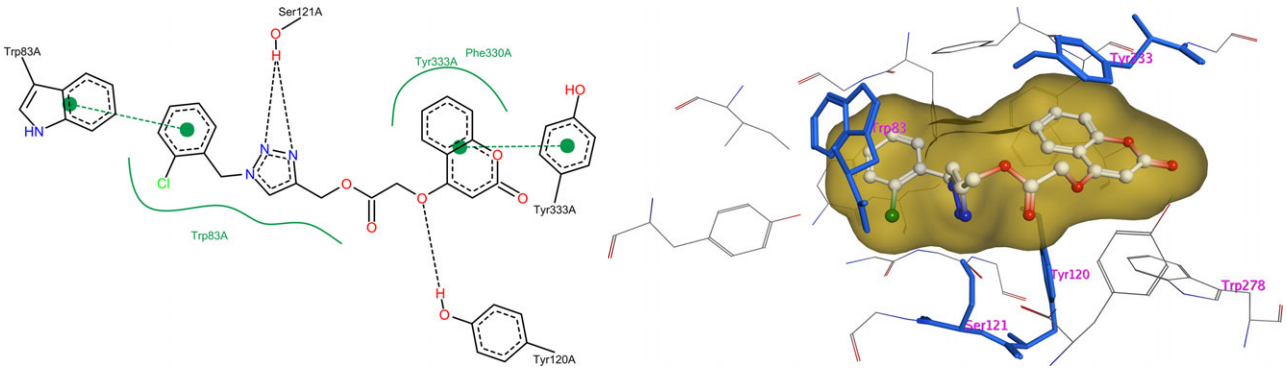


Figure 3: 2D (left) and 3D (right) representation of proposed binding mode and notable interactions of compound **8k** in the active site of AChE.

position 3 was better tolerated than position 4. While compounds **8b** and **8i** bearing a bromine or fluorine substituent at the *meta*-position were more active than their *ortho*-regioisomers (**8a** and **8h**, respectively), the *m*-chloro derivative **8l** was less active than *o*-chloro analog **8k**. In general, the obtained results for *ortho*- or *meta*-substituted derivatives in comparison with unsubstituted benzyl analog **8p** revealed that the substitution of benzyl group at *ortho*- or *meta*-position improves the anti-AChE activity.

The obtained IC_{50} values against BuChE demonstrated that all compounds with exception of 2-bromo derivative **8a** showed no significant activity (IC_{50} values >50 μM). The IC_{50} of compound **8a** was 43 μM. These results revealed that in contrast to the standard drug tacrine, the designed compounds are selective for AChE.

Recently, we have described a series of 4-hydroxycoumarin derivatives bearing an amine functional group on alkyl side chain in which the presence of lipophilic moiety and often a tertiary amino group represents the key requirement for a good AChE inhibition. Among the coumarin-derived compounds tested toward AChE and BuChE, *N*-(1-benzylpiperidin-4-yl)acetamide derivative [Figure 1, structure (A)] displayed the highest anti-AChE activity (IC_{50} = 1.2 μM) with good selectivity (selectivity index = 37) (12). In this study, we introduced new 4-hydroxycoumarin derivatives bearing *N*-benzyl-1,2,3-triazole functionality instead of *N*-benzyl piperidine moiety resulted in improving anti-AChE activity as exemplified by compound **8k** (IC_{50} = 0.18 μM) with higher selectivity.

Kinetic characterization of AChE inhibition

For kinetic study, the rate of enzymatic reaction was measured for three different concentrations of compound **8k** (0.09, 0.18, and 0.36 μM) in the presence of different concentration of substrate (acetylthiocholine iodide). In each case, the reciprocal of initial velocity (1/v) was plotted against reciprocal of substrate concentration (1/[s]). The constructed Lineweaver–Burk plots showed different V_{max} and constant K_m values for different concentrations of substrate (Figure 2, Left). This pattern indicates non-competitive inhibition mode of AChE by compound **8k**. The calculated inhibitory constant *K*i value based on the secondary plot was 1.27 μM (Figure 2, right).

Protein–ligand docking study

To obtain better insight into the binding mode and effects of structural modifications on the inhibitory activity of compounds, docking studies were performed on 1EVE as AChE structure. To test the validity of docking protocol, the co-crystallized ligand (donepezil) was redocked on the target enzyme. The validity of used docking method was confirmed by RMSD value of 0.75 Å. Then, all target compounds were docked into the active site of enzyme and visual inspection of best docked poses in terms of free-binding energy showed similar orientation of compounds in the active site. Therefore, the interaction of the most potent inhibitor **8k** was further analyzed. As shown in Figure 3, the compound **8k** was spanning along the bottom of active site to mid-gorge in which the chlorophenyl moiety anchored the compound through a π–π interaction with Trp83. Furthermore, the π–π stacking between coumarin phenyl ring and Tyr333 could stabilize the ligand. The aforementioned interactions donate a specific conformation to the compound in such a way that makes feasible formation of two hydrogen bonds via linker heteroatoms as follows: a hydrogen bond between Ser121 and nitrogen's of triazole ring and the other between Tyr120 and oxygen of linker. The results suggest that these compounds could be easily accommodated in the active site of AChE and stabilized through multiple interactions with amino acids of catalytic site (CS) and central anionic site (CAS) of enzyme.

Conclusions

We designed a series of selective AChE inhibitors by introducing benzyltriazole moiety instead of usual benzylamine derivative on the 4-hydroxycoumarin skeleton. The title compounds were obtained conveniently using multicomponent click reaction. The *in vitro* anticholinesterase evaluation of synthesized compounds against AChE and BuChE showed that some of them are potent and selective inhibitors of AChE. Among them, 2-chlorobenzyl derivative **8k** showed the most potent activity against AChE (IC_{50} = 0.18 μM). Its activity was also superior to that of standard drug tacrine.

Acknowledgments

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Conflict of Interest

Authors declare no conflict of interest.

References

1. Blennow K., de Leon M.J., Zetterberg H. (2006) Alzheimer's disease. Lancet;368:387–403.
2. Abbott A. (2011) Dementia: a problem for our age. Nature;475:S2–S4.
3. Selkoe D.J. (2011) Resolving controversies on the path to Alzheimer's therapeutics. Nat Med;17:1060–1065.
4. Liu J., Qiu J., Wang M., Wang L., Su L., Gao J., Gu Q., Xu J., Huang S.-L., Gu L.-Q., Huang Z.-S., Li D. (2014) Synthesis and characterization of 1*H*-phenan-

thro[9,10-*d*]imidazole derivatives as multifunctional agents for treatment of Alzheimer's disease. Biochim Biophys Acta;1840:2886–2903.
5. Čolović M.B., Krstić D.Z., Lazarević-Pašti T.D., Bondžić A.M., Vasić V.M. (2013) Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol;11:315–335.
6. Harel M., Sonoda L.K., Silman I., Sussman J.L., Rosenberry T.L. (2008) Crystal structure of thioflavin T bound to the peripheral site of *Torpedo californica* acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site. J Am Chem Soc;130:7856–7861.
7. Carvajal F.J., Inestrosa N.C. (2011) Interactions of AChE with Aβ aggregates in Alzheimer's brain: therapeutic relevance of IDN 5706. Front Mol Neurosci;4:19.
8. Alipour M., Khoobi M., Moradi M., Nadri M., Homayouni Moghadam F., Emami S., Hasanpour Z., Foroumadi A., Shafiee A. (2014) Synthesis and anticholinesterase activity of new 7-hydroxycoumarin derivatives. Eur J Med Chem;82:536–544.
9. Khoobi M., Alipour M., Moradi A., Sakhteman A., Nadri H., Razavi S.F., Ghandi M., Foroumadi A., Shafiee A. (2013) Design, synthesis, docking study and biological evaluation of some novel tetrahydrochromeno [3′,4′:5,6]pyrano[2,3-*b*]quinolin-6(7*H*)-one derivatives against acetyl- and butyrylcholinesterase. Eur J Med Chem;68:291–300.
10. Khoobi M., Alipour M., Sakhteman A., Nadri H., Moradi A., Ghandi M., Emami S., Foroumadi A., Shafiee A. (2013) Design, synthesis, biological evaluation and docking study of 5-oxo-4,5-dihydropyrano[3,2-c] chromene derivatives as acetylcholinesterase and butyrylcholinesterase inhibitors. Eur J Med Chem;68:260–269.
11. Refolo L.M., Fillit H.M. (2004) Drug discovery for Alzheimer's disease: the end of the beginning. J Mol Neurosci;24:1–8.
12. Razavi S.F., Khoobi M., Nadri H., Sakhteman A., Moradi A., Emami S., Foroumadi A., Shafiee A. (2013) Synthesis and evaluation of 4-substituted coumarins as novel acetylcholinesterase inhibitors. Eur J Med Chem;64:252–259.
13. Bourne Y., Kolb H.C., Radić Z., Sharpless K.B., Taylor P., Marchot P. (2004) Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation. Proc Natl Acad Sci USA;101:1449–1454.
14. Krasiński A., Radić Z., Manetsch R., Raushel J., Taylor P., Sharpless K.B., Kolb H.C. (2005) In situ selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. J Am Chem;127:6686–6692.
15. Shi A., Huang L., Lu C., He F., Li X. (2011) Synthesis, biological evaluation and molecular modeling of novel triazole-containing berberine derivatives as acetylcholinesterase and β-amyloid aggregation inhibitors. Bioorg Med Chem;19:2298–2305.
16. Pramitha P., Bahulayan D. (2012) Stereoselective synthesis of bio-hybrid amphiphiles of coumarin derivatives by Ugi-Mannich triazole randomization using copper catalyzed alkyne azide click chemistry. Bioorg Med Chem;22:2598–2603.
17. Ellman G.L., Courtney K.D., Andres V. Jr, Featherstone R.M. (1961) A new and rapidcolorimetric determination of acetylcholinesterase activity. Biochem Pharmacol;7:88–95.
18. Trott O., Olson A.J. (2010) AutoDockVina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem;31:455–461.
19. O'Boyle N.M., Banck M., James C.A., Morley C., Vandermeersch T., Hutchison G.R. (2011) Open Babel: an open chemical toolbox. J Cheminform;3:33.
20. Sanner M.F. (1999) A programming language for software integration and development. J Mol Graphics Model;17:57–61.
21. Pettersen E.F., Goddard T.D., Huang C.C., Couch G.S., Greenblatt D.M., Meng E.C., Ferrin T.E. (2004) UCSF Chimera - a visualization system for exploratory research and analysis. J Comput Chem;25:1605–1612.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. General chemistry information.

Table S1. Optimization of reaction conditions for the synthesis of compound **8a**

Appendix S2. Physicochemical and spectroscopic data of compounds **8a-p**.

Appendix S3. ^{1}H and ^{13}C NMR spectra for synthesized compounds **8a-p**.