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New benzyl pyridinium derivatives bearing 2,4-dioxochroman moiety as potent agents for treatment of Alzheimer's disease: Design, synthesis, biological evaluation, and docking study

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ABSTRACT

A new series of benzyl pyridinium-2,4-dioxochroman derivatives **7a-o** was synthesized and evaluated as new anti-Alzheimer agents. Among the synthesized compounds, the compounds **7f** and **7i** exhibited the most potent anti-AChE and anti-BuChE activities, respectively. The kinetic study of the compound **7f** revealed that this compound inhibited AChE in a mixed-type inhibition mode. Furthermore, the docking study of the compounds **7f** and **7i** showed that these compounds bound to both the catalytic site (CS) and peripheral anionic site (PAS) of AChE and BuChE, respectively. The compound **7f** also exhibited a greater self-induced Aβ peptide aggregation inhibitory activity in compare to donepezil. Furthermore, the neuroprotective activity of this compound at 20 μM was comparable to that of the standard neuroprotective agent (quercetin).

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by decline in memory, cognition defects, and language deterioration [1]. It is the most common cause of dementia affecting elderly people [2]. At the molecular level, AD is associated with reduced synaptic levels of acetylcholine (ACh), abnormal deposits of β-amyloid peptide (Aβ), and presence of neurofibrillary tangles in the special areas of the brain such as the hippocampus and cortex (cholinergic hypothesis), which are related to memory and cognition [3].

One of the possible approaches for the symptomatic treatment of Alzheimer's is increasing the synaptic levels of acetylcholine (ACh) through the inhibition of the acetylcholinesterase enzyme (AChE) [4]. This enzyme is responsible for the degradation of ACh in the synaptic cleft [5]. Furthermore, several lines of recent evidence have suggested that in AD patients, AChE activity gradually decreases while BuChE levels significantly increase in the hippocampus and temporal cortex of the brain [6]. Accordingly, the concurrent inhibition of both AChE and BuChE may improve AD signs and symptoms [7,8]. On the other hand, recent studies have suggested that AChE plays a significant role in accelerating the formation of amyloid beta fibrils in the brain [9–12].

The structural analysis of the X-ray crystallographic structure of AChE revealed that this enzyme has two main substrate-binding sites: the peripheral anionic site (PAS), consisting of the residues Tyr70, ASP72, Tyr121, Trp279, and Tyr334, which is located at the entrance of the gorge and the catalytic site (CS) lying at the bottom of the gorge, including the following subunits: the catalytic triad with the residues Ser200-His440-Glu327 and the anionic site with the residues Trp84, Phe330, and Phe331 [13,14]. Several studies have suggested that AChE through PAS interacts with Aβ and accelerates the polymerization of these peptides [15,16]. Thus, dual binding site AChE inhibitors that interact with both CAS and PAS could be effective agents for the management of AD [17,18].

The docking study has uncovered further evidence suggesting that donepezil, the most popular drug for the palliative treatment of AD, interacts with the CS and PAS of AChE (Fig. 1**A**) [19]. In this regard, several series of donepezil analogs have been reported to show high

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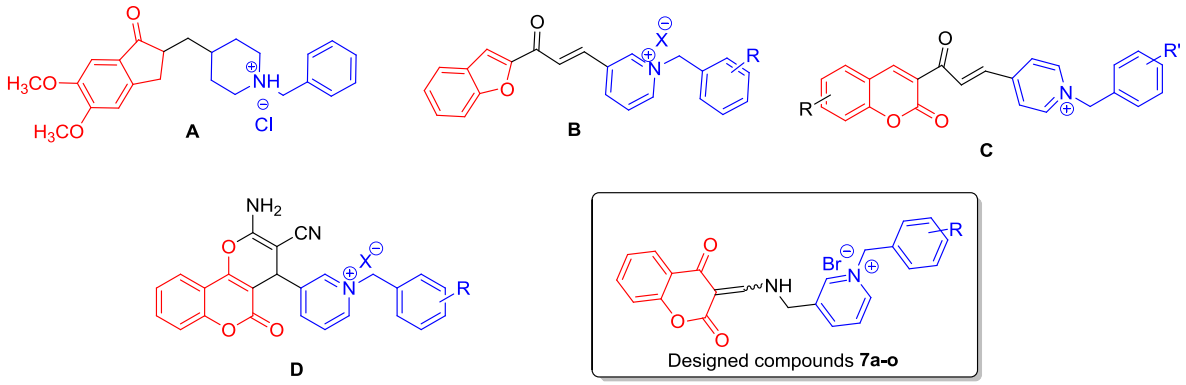


Fig. 1. Structures of donepezil (**A**), pyridinium containing AChE inhibitors (**B**, **C**, **D**), and designed compounds **7a-o** (**D**).

anti-AChE inhibitory effects and interact with both CS and PAS. For example, as can be observed in Fig. 1, in some of these new AChE inhibitors, the piperidine and indanone rings of donepezil were replaced with pyridinium ring and cores such as benzofuran (**B**) and coumarin (**C** and **D**), respectively [20–22] (see Fig. 1).

Therefore, prompted by the mentioned observations and in continuation to our interest in developing anti-Alzheimer's agents, herein, a new series of 1-benzyl-3-((((2,4-dioxochroman-3-ylidene)methyl) amino)methyl)pyridinium bromides **7a-o** was designed, synthesized, and screened for their anti-Alzheimer's activities [23–27].

2. Results and discussion

2.1. Chemistry

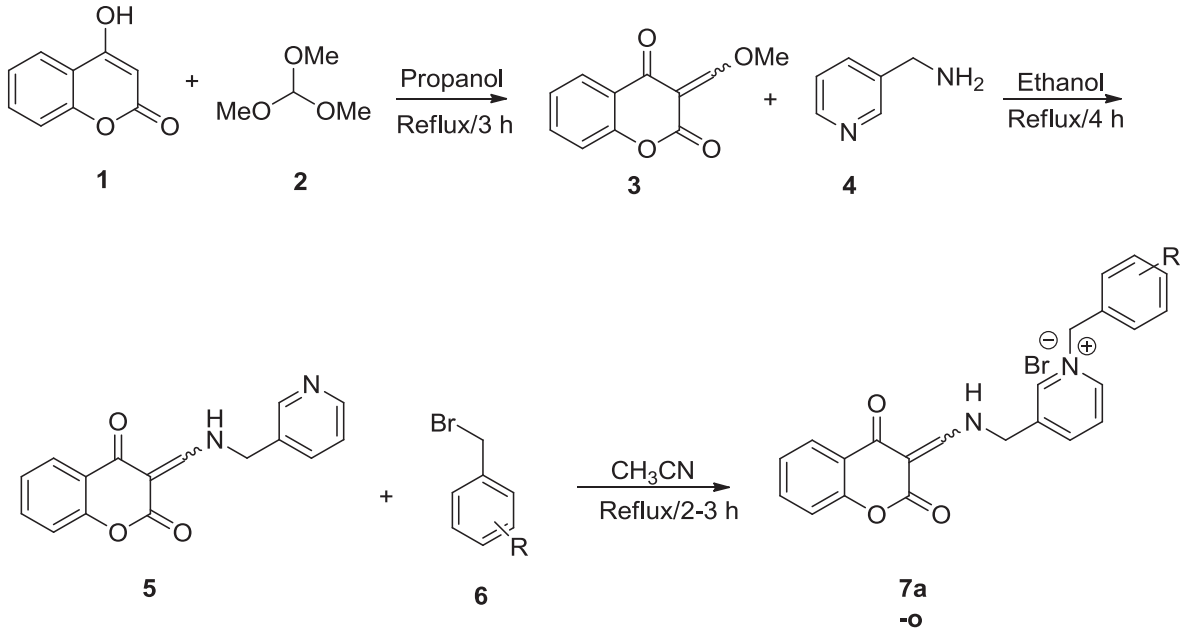
The synthetic procedure for the production of new 2,4-dioxochroman-pyridiniums **7a-o** is shown in Scheme 1. It was started from the reaction of 4-hydroxy-2H-chromen-2-one **1** and trimethoxymethane **2** in propanol at reflux for 3 h to give 3-(methoxymethylene)-3H-chromene-2,4-dione **3**. The letter compound easily reacted with pyridin-3-ylmethanamine **4** in ethanol at reflux for 4 h and afforded 3-((pyridin-3-ylmethylamino)methylene)-3H-chromene-2,4-dione **5**. Finally, the desired compound **7a-o** obtained from the reaction between the compound **5** and various benzyl bromides **6** in acetonitrile at reflux for 2–3 h.

2.2. Inhibitory activity against AChE and BuChE

The synthesized compounds **7a-o** were screened *in vitro* for their inhibitory activity against AChE and BuChE. These evaluations were performed using Ellman's method, and donepezil was used as the standard drug; the obtained results are shown in Table 1.

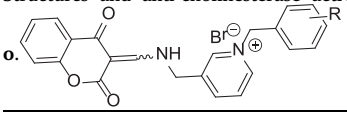
The IC_{50} values of the test compounds for AChE demonstrated that most of the synthesized compounds showed significant inhibition against this enzyme at concentrations less than 12.48 μM. The most active compounds were 2-chloro, 2-bromo, and 2-nitro derivatives (the compounds **7f**, **7j**, and **7m**, respectively) with IC_{50} values ≤ 1.41 ± 0.14 μM. Furthermore, the compounds **7a**, **7d**, **7g**, and **7h** exhibited good anti-AChE activity (IC_{50} values = 2.54 ± 0.081–3.00 ± 0.17 μM).

The un-substituted compound **7a** exhibited a good anti-AChE inhibitory activity (IC_{50} = 3.00 ± 0.17 μM). The introduction of a methyl group at the 2-position of the pendant phenyl ring in the compound **7a**, as in the compound **7b**, led to a significant decrease in the anti-AChE activity (IC_{50} = 9.48 ± 0.25 μM). Changing the position of the methyl substituent in the pendant phenyl ring from C-2 to C-4 in the



Scheme 1. Synthesis of benzyl pyridinium-2,4-dioxochromans **7a-o**.

Table 1
Structures and anti-cholinesterase activities (IC_{50}s, μM) of compounds **7a-o**.



Compound	R	IC_{50} AChE μM	IC_{50} BuChE μM[a]
7a	H	3.00 ± 0.17	> 100
7b	2-CH_3	9.48 ± 0.25	6.43 ± 0.31
7c	4-CH_3	4.75 ± 0.18	> 100
7d	3-F	2.34 ± 0.13	15.83 ± 0.19
7e	4-F	4.70 ± 0.32	33.54 ± 0.24
7f	2-Cl	0.89 ± 0.011	> 100
7g	3-Cl	2.29 ± 0.23	> 100
7h	2,3-Cl	2.54 ± 0.081	6.18 ± 0.28
7i	3,4-Cl	> 100	5.22 ± 0.19
7j	2-Br	1.10 ± 0.069	> 100
7k	3-Br	8.23 ± 0.31	7.23 ± 0.11
7l	4-Br	> 100	> 100
7m	2-NO_2	1.41 ± 0.14	> 100
7n	4-NO_2	> 100	> 100
7o	4-CN	12.48 ± 0.21	> 100
Donepezil	–	0.028 ± 0.023	5.38 ± 0.34

[a] Mean ± S.E.; values are means of three independent experiments.

compound **7b**, producing **7c** (IC_{50} = 4.75 ± 0.18 μM) increased the inhibitory activity. The introduction of a 3-fluoro substituent on the pendant phenyl ring improved anti-AChE potency, as observed in the compound **7e** (IC_{50} = 2.34 ± 0.13 μM). Interestingly, the compound **7e** with an electron withdrawing substituent (fluoro) and the compound **7c** with an electron donating substituent (methyl) at the 4-position of the pendant phenyl ring showed approximately same inhibitory activity against AChE (Table 1).

The compound **7f**, bearing a 2-chloro substituent at the 2-position of the pendant phenyl group, was proved to be the most potent compound among all the synthesized compounds with an IC_{50} value of 0.89 ± 0.011 μM against AChE. Changing the position of the chloro substituent from C-2 to C-3 and or adding another chloro substituent to the 3-position of the pendant phenyl group led to a significant decrease in the inhibitory activity (the compound **7f** vs. the compounds **7g** and **7h**). On the other hand, adding another chloro substituent to the 4-position of the pendant phenyl group of the compound **7f**, producing the compound **7i**, led to the loss of activity against AChE. The second most potent compound was the 2-bromo derivative **7j** (IC_{50} = 1.10 ± 0.069 μM); movement of the bromo substituent from the 2-position into the 3-position (the compound **7k**) led to a decreased inhibitory activity while movement of this substituent from the 2-position into the 4-position (the compound **7k**) led to the loss of activity. The latter trend was also observed in the nitro derivatives **7m** (the third most potent compound) and **7n** (the inactive compound). It appears that the presence of an electron withdrawing substituent with appropriate size at the 2-position of the pendant phenyl ring can help to create an effective interaction with AChE. Finally, the compound **7o** with a 4-carbonitrile substituent on the pendant phenyl group showed a moderate inhibitory activity against AChE.

The inhibitory activity of the synthesized compounds against BuChE revealed that the compounds **7b**, **7d-e**, **7h-i**, and **7k** showed good to moderate enzyme inhibition while the remaining derivatives showed IC_{50} > 100 and thus considered as inactive compounds. Among the synthesized compounds, the 3,4-dichloro derivative **7i** with inhibitory activity similar to donepezil had the highest anti-BuChE activity. Furthermore, the 2,3-dichloro derivative **7h** was the second most potent compound against BuChE. By comparing the IC_{50} values of the 3,4-chloro derivative **7i** (IC_{50} = 5.22 ± 0.19 μM) with the 3-chloro derivative **7g** (IC_{50} > 100 μM) and or the 2,3-dichloro derivative **7h** (IC_{50} = 6.18 ± 0.28 μM) with the 2-chloro derivative **7f** (IC_{50} > 100 μM) and 3-chloro derivative **7g** (IC_{50} > 100 μM), it could be concluded that the presence of two chloro substituents on the pendant phenyl group increased the anti-BuChE activity. Moreover, the 2-methyl derivative **7b** (IC_{50} = 6.43 μM) and 3-bromo derivative **7k** (IC_{50} = 7.23 ± 0.11 μM), unlike their 4-substituented regioisomers (the inactive compounds **7c** and **7l**, respectively) showed good inhibitory activity against BuChE. In contrast, among the fluoro derivatives **7d** and **7e**, the 3-fluoro derivative **7d** showed more anti-BuChE activity in comparison with it 4-fluoro regioisomer **7e**.

The BBB penetration of the most potent compound **7f** was predicted by the admetSAR server [28], indicating that the compound **7f** was able to cross the BBB.

2.3. Kinetic study

To investigate the inhibition mechanism of the synthesized compounds against AChE, an enzyme kinetic study was performed on the most potent compound **7f**. Graphical analysis of the reciprocal Lineweaver–Burk plot revealed that both slopes (V_{max}) and intercepts (K_m) increased at higher inhibitor concentration (Fig . 2). This pattern

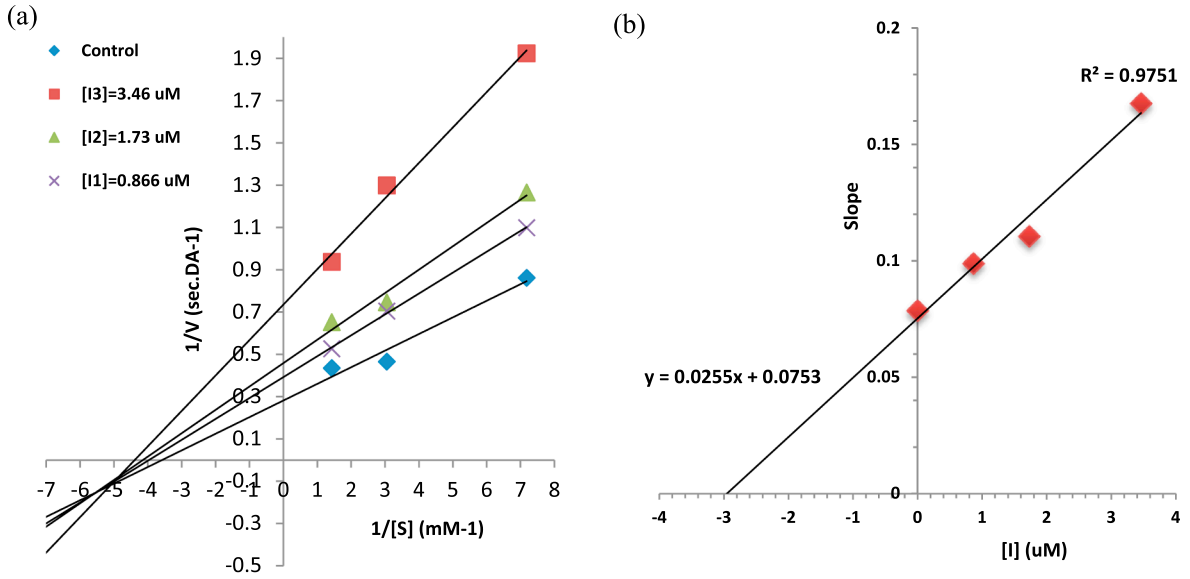


Fig. 2. Kinetic study of inhibitor **7f** (I) against AChE. Lineweaver-Burk plot (left) and double reciprocal Lineweaver-Burk plot (right) are shown.

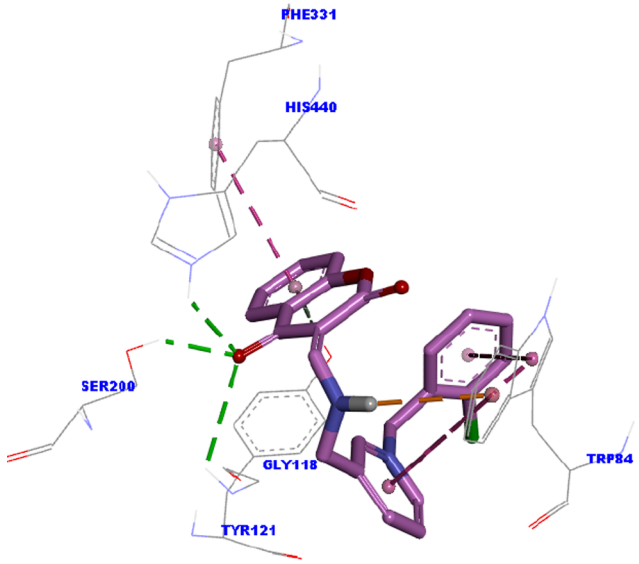


Fig. 3. Docking pose of the most potent compound **7f** in the active site of AChE.

could be attributed to the mixed-type inhibition, and it could be concluded that the compound **7f** might be able to bind to the CA and PAS of AChE. The K_i value of 2.95 μM was determined using plots of the slopes of the Lineweavere–Burk reciprocal plots versus concentrations of **7f** (Fig . 2).

2.4. Docking study

A docking study was performed to find a possible interaction mode of the synthesized compounds (the compounds **7f**, **7j**, and **7l**) with AChE (PDB code: 1EVE) using Auto Dock Tools (version 1.5.6) and Discovery Studio 4.0.

As can be observed in Fig. 3, the most potent compound **7f** occupied the CS (anionic site and catalytic triad) and PAS of the AChE active site. The 2,4-dioxochroman ring of this compound formed a π-π interaction and a non-classical hydrogen bond with the residues Phe331(anionic site) and Tyr121 (PAS). The carbonyl unit at the 4-position of 2,4-dioxochroman moiety created the following hydrogen bonds: two hydrogen bonds with the residues His440 and Ser200 in the catalytic triad and a hydrogen bond with Gly118 in the oxyanione hole [14]. Furthermore, Trp84 of the anionic site established three interactions with the NH fragment (π-cation), pyridinium moiety (π-π), and pendant phenyl ring (π-π) of the compound **7f**.

Replacement of the 2-chloro substituent of the compound **7f** with a 2-bromo substituent led to a decrease in inhibitory activity (Table 1) and change in the orientation of the producing compound **7j** in comparison to the 2-chloro analog **7f** (Figs. 3 and 4a). In the 2-chloro derivative **7f**, the carbonyl unit at the 4-position of 2,4-dioxochroman moiety interacted with catalytic triad while in the 2-bromo derivative **7j**, the carbonyl unit at the 2-position of 2,4-dioxochroman moiety interacted with catalytic triad. Furthermore, the compound **7j** interacted with Phe330 in the anionic site and Tyr334 in the PAS while the compound **7f** interacted with Phe331 in the anionic site and Tyr121 in the PAS. Moreover, in the compound **7j**, only an interaction with Trp84 (the anionic site) was observed while in the compound **7f**, three interactions with Trp84 can be observed. The compound **7j** also formed a weak hydrophobic interaction with Gly117. Further modification, including changing the position of the bromo group in the pendant phenyl ring from C-2 to C-4, resulted in the loss of effect, as was observed in the compound **7l**. This effect appeared to be due to the removal of the interaction of the compound **7l** with catalytic triad (Fig. 3). In this regard, as can be observed in Fig. 4b, this compound interacted well with the anionic site (Phe330 and Trp84) and PAS (Asp72, Tyr121, and Tyr334).

Results of the study on the interaction mode of the compound **7i** as the most active compound against BuChE in the active site of this enzyme can be observed in Fig. 5. The compound **7i** interacted with the residues Gly121, Leu125, Trp82 (PAS), His438 (catalytic triad), Phe329 (anionic site), Gly117, Leu286, and Trp231 (Fig. 5) [14]. The 2,4-dioxochroman moiety of this compound established two hydrogen bonds with Gly117 and His438. Furthermore, the mentioned moiety formed two π-π interactions with the residues Phe329 and Trp231 and a weak hydrophobic interaction with Leu286. In this compound, pyridinium ring exhibited a π-π interaction and a π-cation interaction with the imidazole ring of His438. In addition, the pendant aryl group of the molecule involved in a π-π interaction with Trp82. The chloro substituents of this group also interacted with the residues Gly121 and Leu125.

2.5. Inhibition of self-induced Aβ aggregation

The self-induced Aβ aggregation inhibition of the compound **7f**, as the most potent compound against AChE, was evaluated by Thioflavin T(ThT)-binding assay [29]. As can be observed in Table 2, the compound **7f** at 10 μM concentration (20.38 ± 1.51%) showed a higher inhibitory activity than donepezil against self-induced Aβ peptide aggregation (14.70 ± 2.35%).

2.6. The protective effect of 7f against H_2O_2-Induced cell death

In addition to the AChE and BuChE inhibitions, protection against oxidative stress may be useful in the management of AD [30]. Thus, the neuroprotective activity of the most potent compound **7f** was evaluated against oxidative stress-induced cell death in the neuronal cell line PC12. For this purpose, PC12 cells were incubated with different concentrations (5, 10 and 20 μM) of the compound **7f** and quercetine (5 μM) as the reference compound for 3 h, before treatment with H_2O_2 (350 μM). For induction of apoptosis, H_2O_2 was added to the medium and occurrence of apoptosis was established after staining with DAPI. The cell viability was measured after 24 h by using the MTT assay. As shown in Fig. 6, the compound **7f** inhibited H_2O_2-induced cell death in PC12 cells with a dose-dependently mode. The neuroprotective activity of the compound **7f** at 20 μM was comparable to quercetin as a powerful natural antioxidant.

2.7. β-secretase inhibitory activity

The compounds with β-secretase (BACE-1) inhibitory activity can be useful in the AD symptomatic treatment [31]. Thus, the BACE-1 inhibitory activity of the compound **7f** as the most potent inhibitor among the synthesized compounds against AChE was evaluated via a fluorescence resonance emission transfer (FRET) method. The used kit consisted of BACE-1 enzyme and APP peptide based substrate (Rh-EVNL-DAEFK-quencher). Experiments were repeated for three times and compared with OM99-2 (IC_{50} = 0.014 μM) as the reference. Results showed that the compound **7f** possessed low inhibitory activity against β-secretase (Table 3).

3. Conclusion

A new series of benzyl pyridinium derivatives bearing 2,4-dioxochroman moiety was synthesized and evaluated as potent anti-Alzheimer agents. The two compounds 1-(2-chlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-ium bromide **7f** and 1-(3,4-dichlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-1-ium bromide **7i** were found as the most potent inhibitors against AChE and BuChE, respectively. The compound **7f** showed a mixed-type inhibition against AChE. The docking study of the most potent compounds **7f** and **7i** showed that these compounds bound to both the CS and PAS of AChE and BuChE, respectively.

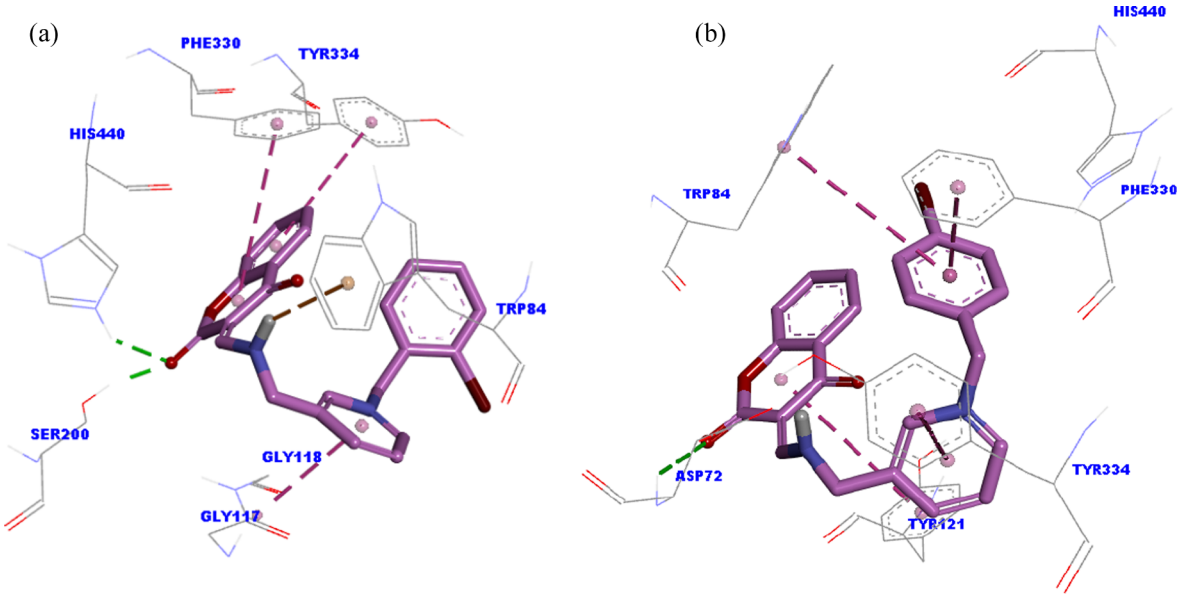


Fig. 4. Docking poses of the compounds **7j** (a) and **7l** (b) in the active site of AChE.

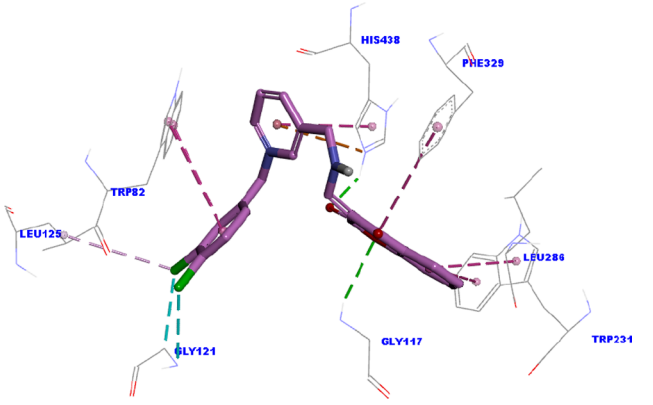


Fig. 5. Interaction mode of the most potent compound **7i** in the active site of BuChE.

Table 2
Inhibition of self-induced Aβ1–42 aggregation.

Compound	Inhibition at 10 μM[a] (%)
7f	20.38 ± 1.51
Donepezil	14.70 ± 2.35

[a] Values are expressed as means ± SEM of three experiments.

Furthermore, the biological results indicated that the most potent compound **7f** had neuroprotective activity against H_2O_2-induced damage in PC12 cells. This compound also exhibited a higher self-induced Aβ peptide aggregation inhibitory activity, as compared to donepezil.

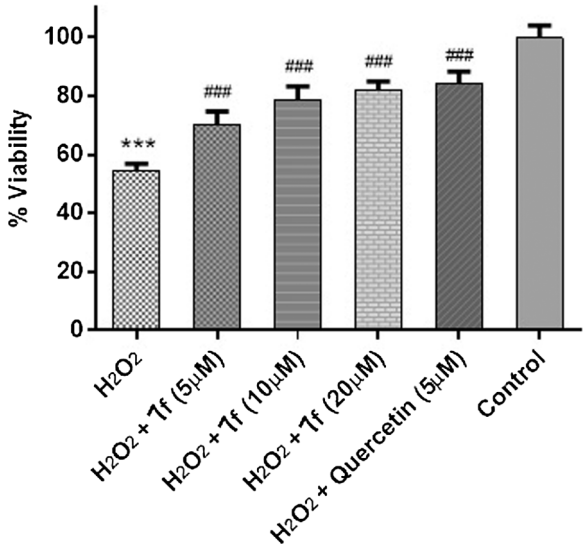


Fig. 6. Neuroprotective effect of compound **7f** against H_2O_2-induced cell death in PC12 cells. Data are expressed as Mean ± SD (n = 3). In each series labeled mean values are significantly different (at $p < 0.001$) according to H_2O_2 group. (*versus Control and #versus H_2O_2).

Table 3
BACE-1 inhibitory activity of the compound **7f**.

Compound	% inhibition at 10 μM	% inhibition at 50 μM	IC_{50} (μM)[a]
7f	28.0 ± 15.1	41.4 ± 22.4	ND
OM99-2	–	–	0.014 ± 0.003

[a] Mean ± S.E.; values are means of three independent experiments.

4. Experimental

All starting materials, solvents, and reagents were purchased from commercial suppliers and used without extra purification. The melting points of the compounds **7a-o** were measured by a Kofler hot stage apparatus and uncorrected. The IR spectra were obtained using a Nicolet FT-IR Magna 550 spectrometer (KBr disks). ^1H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz NMR instrument using TMS as an internal standard. The chemical shifts (δ) and coupling constants (J) were expressed in parts per million (ppm) and Hertz (Hz), respectively. Analytical HPLC was performed on Agilent 1290 Infinity II HPLC system. As column, a Zorbax 300SB-C18, 3.5 μm, 4.6 × 150 mm, Agilent, P/N: 863973-902 was used. HPLC purity was measured using the following binary solvent system: 0.1% trifluoroacetic acid (TFA) in HPLC grade water (90%) and 0.1% TFA in acetonitrile (10%) in 5 min, 0.1% TFA in HPLC grade water (50%) and 0.1% TFA in acetonitrile (50%) in 35 min, 0.1% TFA in HPLC grade water (50%) and 0.1% TFA

in acetonitrile (50%) in 38 min, 0.1% TFA in HPLC grade water (90%) and 0.1% TFA in acetonitrile (10%) in 39 min, 0.1% TFA in HPLC grade water (90%) and 0.1% TFA in acetonitrile (10%) in 45 min, flow rate 1 ml/min, λ 322 nm. The purity of the synthesized comppounds **7a-o** was determined to be > 95%.

4.1. Synthesis of 3-(methoxymethylene)-3H-chromene-2,4-dione *3*

A mixture of 4-hydroxy-2H-chromen-2-one **1** (1 mmol) and trimethoxymethane **2** (3 mmol) in propanol (5 ml) was stirred at reflux for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to stand overnight at room temperature for formation of pure 3-(methoxymethylene)-3H-chromene-2,4-dione precipitates **3**.

4.2. Synthesis of 3-((pyridin-3-ylmethylamino)methylene)-3H-chromene-2,4-dione *5*

A mixture of 3-(methoxymethylene)-3H-chromene-2,4-dione **3** (1 mmol) and pyridin-3-ylmethanamine **4** in ethanol (5 ml) was stirred at reflux for 4 h. Then, the reaction mixture was allowed to cool at room temperature and poured into crushed ices, and subsequently, the pure white precipitates **5** were filtered off.

4.3. General procedure for the preparation of 2,4-dioxochroman-pyridinium derivative *7a-o*

A solution of the 3-((pyridin-3-ylmethylamino)methylene)-3H-chromene-2,4-dione **5** (1 mmol) and benzyl bromide derivatives **6** (1.4 mmol) in dry acetonitrile (10 ml) was heated under reflux condition for 2–3 h. The reaction progress was monitored by TLC. The obtained precipitate was filtered off and washed with dry acetonitrile (2 ml) to give pure compounds **7a-o**. The target compounds were obtained as a mixture of Z and E isomers. The percentage of Z and E isomers was calculated by NMR.

4.3.1. 1-Benzyl-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl) pyridinium bromide (*7a*)

Yield 75% (Z isomer: 63% and E isomer: 37%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3386 (NH), 1699, 1642 (C=O). Purity 99.12% by HPLC. Anal. Calcd for $C_{23}H_{19}BrN_2O_3$: C, 61.21; H, 4.24; N, 6.21. Found: C, 61.39; H, 4.11; N, 61.09. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.27 (s, 1H, H_2 pyridine), 9.16 (d, 1H, H_6 pyridine, J = 6 Hz), 8.70 (d, 1H, =CH, J = 15 Hz), 8.66 (d, 1H, H_4 pyridine, J = 7.5 Hz), 8.20 (t, 1H, H_5 pyridine, J = 7.5 Hz), 7.91 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.52 (d, 2H, $H_{2,6}$ phenyl, J = 7.0 Hz), 7.47–7.41 (m, 3H, $H_{3,4,5}$ phenyl), 7.34–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.87 (s, 2H, CH_2N$^+$), 5.02 (s, 2H, CH_2N). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.26 (s, 1H, H_2 pyridine), 9.16 (d, 1H, H_6 pyridine, J = 6 Hz), 8.83 (d, 1H, =CH, J = 15 Hz), 8.64 (d, 1H, H_4 pyridine, J = 7.5 Hz), 8.20 (t, 1H, H_5 pyridine, J = 7.5 Hz), 7.96 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.52 (d, 2H, $H_{2,6}$ phenyl, J = 7.0 Hz), 7.47–7.41 (m, 3H, $H_{3,4,5}$ phenyl), 7.34–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.87 (s, 2H, CH_2N$^+$), 5.02 (s, 2H, CH_2N).

4.3.2. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(2-methylbenzyl)pyridinium bromide (*7b*)

Yield 90% (Z isomer: 66% and E isomer: 34%); white solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3405 (NH), 1686, 1642 (C=O). Purity 99.00% by HPLC. Anal. Calcd for $C_{24}H_{21}BrN_2O_3$: C, 61.95; H, 4.55; N, 6.02. Found: C, 61.82; H, 4.38; N, 6.16. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.84 (s, 1H, N*H*), 9.16 (s, 1H, H_2 pyridine), 9.09 (br.s, 1H, H_6 pyridine), 8.72 (d, 1H, =CH, J = 15.0 Hz), 8.73–8.71 (m, 1H, H_4 pyridine), 8.24 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.93 (d, 1H, H_5 coumarin, J = 6.0 Hz), 7.68–7.65 (m, 1H, H_7 coumarin), 7.32–7.20 (m, 7H, $H_{6,8}$ coumarin, phenyl), 5.99 (s, 2H, CH_2N$^+$), 5.08 (s, 2H, CH_2N), 2.33 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.3 (C=O), 162.6 (CH), 154.2 (C), 145.4 (CH), 144.4 (CH), 143.2 (CH), 138.3 (CH), 136.9 (C), 134.5 (CH), 131.9 (C), 130.9 (C), 129.4 (CH), 128.0 (CH), 126.7 (CH), 125.7 (CH), 125.2 (CH), 123.9 (CH), 120.1 (C), 116.7 (CH), 96.5 (C), 61.6 (CH_2), 50.1 (CH_2), 18.8 (CH_3). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.70 (s, 1H, N*H*), 9.16 (s, 1H, H_2 pyridine), 9.09 (br.s, 1H, H_6 pyridine), 8.84 (d, 1H, =CH, J = 15.0 Hz), 8.73–8.71 (m, 1H, H_4 pyridine), 8.24 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.96 (d, 1H, H_5 coumarin, J = 6.0 Hz), 7.68–7.65 (m, 1H, H_7 coumarin), 7.32–7.20 (m, 7H, $H_{6,8}$ coumarin, phenyl), 5.99 (s, 2H, CH_2N$^+$), 5.08 (s, 2H, CH_2N), 2.33 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.2 (C=O), 165.3 (C=O), 162.6 (CH), 154.2 (C), 146.5 (CH), 144.4 (CH), 144.1 (CH), 138.3 (CH), 136.9 (C), 134.5 (CH), 131.9 (C), 130.9 (C), 129.4 (CH), 128.0 (CH), 126.7 (CH), 125.7 (CH), 125.2 (CH), 123.9 (CH), 120.1 (C), 116.7 (CH), 96.5 (C), 61.6 (CH_2), 50.1 (CH_2), 18.8 (CH_3).

4.3.3. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(4-methylbenzyl)pyridinium bromide (*7c*)

Yield 95% (Z isomer: 66% and E isomer: 34%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3394 (NH), 1701, 1641 (C=O). Purity 95.56% by HPLC. Anal. Calcd for $C_{24}H_{21}BrN_2O_3$: C, 61.95; H, 4.55; N, 6.02. Found: C, 62.06; H, 4.41; N, 6.11. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.83 (br. s, 1H, N*H*), 9.30 (s, 1H, H_2 pyridine), 9.21 (br.s, 1H, H_6 pyridine), 8.71 (d, 1H, =CH, J = 15.0 Hz), 8.69–8.65 (m, 1H, H_4 pyridine), 8.19 (br.s, 1H, H_5 pyridine), 7.92 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.68–7.66 (m, 1H, H_7 coumarin), 7.46 (d, 2H, H_α phenyl, J = 6.5 Hz), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 7.25 (d, 2H, H_β phenyl, J = 6.5 Hz), 5.85 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N), 2.29 (s, 3H, Me). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.0 (C=O), 162.9 (C=O), 162.6 (CH), 154.2 (C), 145.2 (CH), 144.3 (CH), 143.6 (CH), 138.9 (C) 138.3 (CH), 134.5 (CH), 131.0 (C), 129.7 (C), 129.5 (CH), 128.9 (C), 128.7 (CH), 128.1 (CH), 125.3 (CH), 123.9 (CH), 120.1 (C), 116.8 (CH), 96.6 (C), 63.2 (CH_2), 50.2 (CH_2), 20.6 (CH_3). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.68 (br. s, 1H, N*H*), 9.28 (s, 1H, H_2 pyridine), 9.21 (br.s, 1H, H_6 pyridine), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.69–8.65 (m, 1H, H_4 pyridine), 8.19 (br.s, 1H, H_5 pyridine), 7.96 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.68–7.66 (m, 1H, H_7 coumarin), 7.46 (d, 2H, H_α phenyl, J = 6.5 Hz), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 7.25 (d, 2H, H_β phenyl, J = 6.5 Hz), 5.85 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N), 2.29 (s, 3H, Me). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.0 (C=O), 163.2 (C=O), 162.6 (CH), 154.2 (C), 147.0 (CH), 144.3 (CH), 144.0 (CH), 138.9 (C) 138.3 (CH), 134.5 (CH), 131.0 (C), 129.7 (C), 129.5 (CH), 128.9 (C), 128.7 (CH), 128.1 (CH), 125.3 (CH), 123.9 (CH), 120.1 (C), 116.8 (CH), 96.6 (C), 63.2 (CH_2), 50.2 (CH_2), 20.6 (CH_3).

4.3.4. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(3-fluorobenzyl)pyridin bromide (*7d*)

Yield 80% (Z isomer: 66% and E isomer: 34%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3337 (NH), 1710, 1637 (C=O). Purity 98.83% by HPLC. Anal. Calcd for $C_{23}H_{19}Br_2FN_2O_3$: C, 50.21; H, 3.48; N, 5.09. Found: C, 50.32; H, 3.39; N, 5.14. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.70 (br. s, 1H, N*H*), 9.36 (s, 1H, H_2 pyridine), 9.27 (d, 1H, H_6 pyridine, J = 6 Hz), 8.73 (d, 1H, =CH, J = 15 Hz), 8.71–8.68 (m, 1H, H_4 pyridine), 8.24 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.93 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.68 (t, 1H, H_7 coumarin, J = 7.5 Hz), 7.53–7.27 (m, 6H, H phenyl, $H_{6,8}$ coumarin), 5.96 (s, 2H, CH_2N$^+$), 5.07 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.1 (C=O), 162.6 (C=O), 160.1 (CH), 155.3 (C), 151.3 (C), 145.5 (CH), 144.6 (CH), 143.8 (CH), 138.0 (CH), 136.0(CH), 133.6 (C), 132.0 (C), 127.5 (CH), 125.7 (CH), 123.5 (CH), 112.3 (CH), 112.2 (CH), 110.4 (CH), 101.1 (C), 55.9 (CH_2), 50.0 (CH_2). NMR data for the E isomer (34%). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.67 (br. s, 1H, N*H*), 9.34 (s, 1H, H_2 pyridine), 9.27 (d, 1H, H_6 pyridine, J = 6 Hz), 8.85 (d,

1H, =CH, J = 15 Hz), 8.71–8.68 (m, 1H, H_4 pyridine), 8.24 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.97 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.68 (t, 1H, H_7 coumarin, J = 7.5 Hz), 7.53–7.27 (m, 6H, H phenyl, $H_{6,8}$ coumarin), 5.96 (s, 2H, CH_2N$^+$), 5.07 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.1 (C=O), 164.0 (C=O), 160.1 (CH), 155.3 (C), 151.3 (C), 147.0 (CH), 144.6 (CH), 145.2 (CH), 138.0 (CH), 136.0 (CH), 133.6 (C), 132.0 (C), 127.5 (CH), 125.7 (CH), 123.5 (CH), 112.3 (CH), 112.2 (CH), 110.4 (CH), 101.1 (C), 55.9 (CH_2), 50.0 (CH_2).

4.3.5. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(4-fluorobenzyl)pyridinium bromide (**7e**)

Yield 86% (Z isomer: 66% and E isomer: 34%); white solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3457 (NH), 1698, 1644 (C=O). Purity 95.56% by HPLC. Anal. Calcd for $C_{23}H_{18}BrFN_2O_3$: C, 58.86; H, 3.87; N, 5.97. Found: C, 58.73; H, 3.76; N, 6.09. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.82 (br. s, 1H, N*H*), 9.33 (s, 1H, H_2 pyridine), 9.24 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.70 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.67 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.92 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.69–7.66 (m, 3H, H_7 coumarin, $H_α$ phenyl), 7.32–7.29 (m, 4H, $H_{6,8}$ coumarin, $H_β$ phenyl), 5.91 (s, 2H, CH_2N$^+$), 5.05 (s, 2H, CH_2N).). ^{13}CNMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.5 (C=O), 163.4 (C), 162.6 (CH), 154.2 (C), 145.2 (CH), 144.4 (CH), 144.1 (CH), 138.4 (CH), 134.5 (CH), 131.5 (C), 130.3 (C), 128.2 (CH), 128.1 (CH), 125.2 (CH), 124.1 (CH), 120.1 (C), 117.0 (CH), 116.8 (CH), 116.2 (CH), 96.6 (C), 62.4 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.68 (br. s, 1H, N*H*), 9.31 (s, 1H, H_2 pyridine), 9.24 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.67 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.97 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.69–7.66 (m, 3H, H_7 coumarin, $H_α$ phenyl), 7.32–7.29 (m, 4H, $H_{6,8}$ coumarin, $H_β$ phenyl), 5.91 (s, 2H, CH_2N$^+$), 5.05 (s, 2H, CH_2N). ^{13}CNMR:(500 MHz, DMSO-d_6) δ: 177.5 (C=O), 165.0 (C=O), 163.4 (C), 162.6 (CH), 154.2 (C), 146.0 (CH), 145.1 (CH), 144.4 (CH), 138.4 (CH), 134.5 (CH), 131.5 (C), 130.3(C), 128.2 (CH), 128.1 (CH), 125.2 (CH), 124.1 (CH), 120.1 (C), 117.0 (CH), 116.8 (CH), 116.2 (CH), 96.6 (C), 62.4 (CH_2), 50.2 (CH_2).

4.3.6. 1-(2-chlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-ium bromide (**7f**)

Yield 73% (Z isomer: 60% and E isomer: 40%); white solid; mp > 250 °C. IR (KBr, cm-1) vmax: 3346 (NH), 1706, 1637 (C=O). Purity 96.00% by HPLC. Anal. Calcd for $C_{23}H_{18}BrClN_2O_3$: C, 56.87; H, 3.73; N, 5.77. Found: C, 56.63; H, 3.91; N, 5.83. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.80 (br. s, 1H, N*H*), 9.17 (s, 1H, H_2 pyridine), 9.05 (d, 1H, H_6 pyridine, J = 6.0 Hz), 8.72 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.69 (m, 1H, H_4 pyridine), 8.22 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.91 (d, 1H, H_5 coumarin, J = 7.3 Hz), 7.76 (d, 1H, H_3 phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.51–7.29 (m, 5H, $H_{6,8}$ coumarin, $H_{4,5,6}$ phenyl), 5.97 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.3 (C=O), 162.7 (CH), 154.2 (C), 145.5 (CH), 144.6 (CH), 143.9 (CH), 138.3 (CH), 134.6 (CH), 132.9 (C), 131.6 (C), 131.2 (CH), 129.4 (CH), 128.9 (CH), 125.8 (CH), 125.3 (CH), 123.3 (CH), 120.1 (C), 117.0 (CH), 96.6 (C), 63.5 (CH_2), 50.2(CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.70 (br. s, 1H, N*H*), 9.16 (s, 1H, H_2 pyridine), 9.05 (d, 1H, H_6 pyridine, J = 6.0 Hz), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.69 (m, 1H, H_4 pyridine), 8.22 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.96 (d, 1H, H_5 coumarin, J = 7.3 Hz), 7.76 (d, 1H, H_3 phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.51–7.29 (m, 5H, $H_{6,8}$ coumarin, $H_{4,5,6}$ phenyl), 5.97 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.5 (C=O), 165.0 (C=O), 162.7 (CH), 154.2 (C), 147.0 (CH), 145.2 (CH), 144.6 (CH), 138.3 (CH), 134.6 (CH), 132.9 (C), 131.6 (C), 131.2 (CH), 129.4 (CH), 128.9 (CH), 125.8 (CH), 125.3 (CH), 123.3 (CH), 120.1 (C), 117.0 (CH), 96.6 (C), 63.5 (CH_2), 50.2(CH_2).

4.3.7. 1-(3-chlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-1-ium bromide (**7g**)

Yield 80% (Z isomer: 66% and E isomer: 34%); white solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3425 (NH), 1707, 1634 (C=O). Purity 99.19% by HPLC. Anal. Calcd for $C_{23}H_{18}BrClN_2O_3$: C, 56.87; H, 3.73; N, 5.77. Found: C, 56.68; H, 3.89; N, 5.83. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.81 (br. s, 1H, N*H*), 9.31 (s, 1H, H_2 pyridine), 9.26 (d, 1H, H_6 pyridine, J = 4.5 Hz), 8.73 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.68 (m, 1H, H_4 pyridine), 8.22 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.95 (s, 1H, H_2 phenyl), 7.92 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76–7.60 (m, 4H, H_7 coumarin, $H_{4,5,6}$ phenyl), 7.33–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.93 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.3 (C=O), 162.6 (CH), 154.2 (C), 145.5 (CH), 144.6 (CH), 143.9 (CH), 138.3 (CH), 134.6 (CH), 132.3 (C), 132.5 (C), 131.6 (C), 131.2 (CH), 129.4 (CH), 128.2 (CH), 125.8 (CH), 125.3 (CH), 124.1 (CH), 120.1 (C), 117.0 (CH), 96.6 (C), 61.7 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.67 (br. s, 1H, N*H*), 9.29 (s, 1H, H_2 pyridine), 9.25 (d, 1H, H_6 pyridine, J = 4.5 Hz), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.70–8.68 (m, 1H, H_4 pyridine), 8.22 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.95 (s, 1H, H_2 phenyl), 7.94 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76–7.60 (m, 4H, H_7 coumarin, $H_{4,5,6}$ phenyl), 7.33–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.93 (s, 2H, CH_2N$^+$), 5.04 (s, 2H, CH_2N). ^{13}C NMR: (500 MHz, DMSO-d_6) δ: 177.5 (C=O), 165.0 (C=O), 162.6 (CH), 154.2 (C), 146.6 (CH), 144.6 (CH), 144.3 (CH), 138.3 (CH), 134.6 (CH), 132.3 (C), 132.5 (C), 131.6 (C), 131.2 (CH), 129.4 (CH), 128.2 (CH), 125.8 (CH), 125.3 (CH), 124.1 (CH), 120.1 (C), 117.0 (CH), 96.6 (C), 61.7 (CH_2), 50.2 (CH_2).

4.3.8. 1-(2,3-dichlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-1-ium bromide (**7h**)

Yield 75% (Z isomer: 65% and E isomer: 35%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3330 (NH), 1688, 1628 (C=O). Purity 95.42% by HPLC. Anal. Calcd for $C_{23}H_{17}BrCl_2N_2O_3$: C, 53.10; H, 3.29; N, 5.39. Found: C, 53.01; H, 3.36; N, 5.47. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.80 (br. s, 1H, N*H*), 9.22 (s, 1H, H_2 pyridine), 9.16 (d, 1H, H_6 pyridine, J = 5.0 Hz), 8.74 (d, 1H, =CH, J = 15.0 Hz), 8.74–8.70 (m, 1H, H_4 pyridine), 8.25 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.92 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76 (d, 1H, H_6 phenyl, J = 7.5 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.50 (t, 1H, H_5 phenyl, J = 7.5 Hz), 7.44 (d, 1H, H_4 phenyl, J = 7.5 Hz), 7.35–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.11 (s, 2H, CH_2N$^+$), 5.06 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 162.6 (C=O), 162.0 (CH), 154.2 (C), 145.8 (CH), 144.7 (CH), 144.5 (CH), 138.3 (CH), 134.5 (CH), 133.8 (CH), 132.6 (C), 131.6 (CH), 130.0 (C), 129.0 (C), 128.1 (CH), 125.8 (CH), 125.2 (CH), 123.9 (CH), 120.1 (C), 117.0 (CH), 96.5 (C), 61.6 (CH_2), 50.1 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.70 (br. s, 1H, N*H*), 9.21 (s, 1H, H_2 pyridine), 9.16 (d, 1H, H_6 pyridine, J = 5.0 Hz), 8.84 (d, 1H, =CH, J = 15.0 Hz), 8.74–8.70 (m, 1H, H_4 pyridine), 8.25 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.96 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76 (d, 1H, H_6 phenyl, J = 7.5 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.50 (t, 1H, H_5 phenyl, J = 7.5 Hz), 7.35–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.11 (s, 2H, CH_2N$^+$), 5.06 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.3 (C=O), 163.4 (C=O), 162.0 (CH), 154.2 (C), 147.5 (CH), 145.0 (CH), 144.7 (CH), 138.3 (CH), 134.5 (CH), 133.8 (CH), 132.6 (C), 131.6 (CH), 130.0 (C), 129.0 (C), 128.1 (CH), 125.8 (CH), 125.2 (CH), 123.9 (CH), 120.1 (C), 117.0 (CH), 96.5 (C), 61.6 (CH_2), 50.1 (CH_2).

4.3.9. 1-(3,4-dichlorobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-1-ium bromide (**7i**)

Yield 79% (Z isomer: 65% and E isomer: 35%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3337 (NH), 1714, 1632 (C=O). Purity 96.71% by HPLC. Anal. Calcd for $C_{23}H_{17}BrCl_2N_2O_3$: C, 53.10; H, 3.29; N, 5.39. Found: C, 53.17; H, 3.21; N, 5.45. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.84 (br. s, 1H, N*H*), 9.34 (s,

1H, H_2 pyridine), 9.26 (d, 1H, H_6 pyridine, J = 3.5 Hz), 8.72 (d, 1H, =CH, J = 15.0 Hz), 8.71–8.69 (m, 1H, H_4 pyridine), 8.24 (br. S, 1H, H_5 pyridine), 7.92 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.73–7.51 (m, 4H, H_7 coumarin, phenyl), 7.33–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.94 (s, 2H, CH_2N$^+$), 5.07 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.4 (C=O), 162.6 (CH), 154.2 (C), 145.4 (CH), 144.6 (CH), 143.8 (CH), 138.3 (CH), 136.2 (CH), 134.5 (C), 133.6 (C), 131.1 (C), 129.4 (C), 128.8 (CH), 127.5 (CH), 125.7 (CH), 125.2 (CH), 123.9 (CH), 120.3 (C), 116.8 (CH), 96.4 (C), 62.4 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.70 (br. s, 1H, N*H*), 9.31 (s, 1H, H_2 pyridine), 9.26 (d, 1H, H_6 pyridine, J = 3.5 Hz), 8.85 (d, 1H, =CH, J = 15.0 Hz), 8.71–8.69 (m, 1H, H_4 pyridine), 8.24 (br. S, 1H, H_5 pyridine), 7.97 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.73–7.51 (m, 4H, H_7 coumarin, phenyl), 7.33–7.30 (m, 2H, $H_{6,8}$ coumarin), 5.94 (s, 2H, CH_2N$^+$), 5.07 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.3 (C=O), 164.2 (C=O), 162.6 (CH), 154.2 (C), 146.0 (CH), 145.0 (CH), 144.6 (CH), 138.3(CH), 136.2 (CH), 134.5 (C), 133.6 (C), 131.1 (C), 129.4 (C), 128.8 (CH), 127.5 (CH), 125.7 (CH), 125.2 (CH), 123.9 (CH), 120.3 (C), 116.8 (CH), 96.4 (C), 62.4 (CH_2), 50.2 (CH_2).

*4.3.10. 1-(2-bromobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl) amino)methyl)pyridinium bromide (**7j**)*

Yield 73% (Z isomer: 65% and E isomer: 35%); yellow solid; mp > 250 °C; IR (KBr, cm^{-1}) ν_{max}: 3406 (NH), 1711, 1628 (C=O). Purity 96.28% by HPLC. Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3$: C, 52.10; H, 3.42; N, 5.28. Found: C, 52.23; H, 3.35; N, 5.41. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.86 (br. s, 1H, N*H*), 9.16 (s, 1H, H_2 pyridine), 9.04 (d, 1H, H_6 pyridine, J = 6.0 Hz), 8.72 (d, 1H, =CH, J = 15.0 Hz), 8.69–8.68 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.90 (d, 1H, H_5 coumarin, J = 7.3 Hz), 7.76 (d, 1H, H_3 phenyl, J = 8.0 Hz), 7.69–7.65 (m, 1H, H_7 coumarin), 7.50–7.28 (m, 5H, $H_{6,8}$ coumarin, $H_{4,5,6}$ phenyl), 5.99 (s, 2H, CH_2N$^+$), 5.16 (s, 2H, CH_2N). ^{13}CNMR: (500 MHz, DMSO-d_6) δ: 179.6 (C=O), 161.7 (C=O), 161.0 (CH), 154.2 (C), 147.0 (CH), 145.5 (CH), 142.5 (CH), 138.0 (CH), 136.0 (CH), 134.5 (C), 133.1 (C), 132.0 (C), 129.5 (CH), 129.0 (CH), 128.0 (CH), 126.3 (CH), 126.2 (CH),126.0 (CH), 123.5 (CH), 120.1 (C), 118.0 (CH), 96.8 (C), 62.7 (CH_2), 52.3 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.73 (br. s, 1H, N*H*), 9.15 (s, 1H, H_2 pyridine), 9.04 (d, 1H, H_6 pyridine, J = 6.0 Hz), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.69–8.68 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.95 (d, 1H, H_5 coumarin, J = 7.3 Hz), 7.76 (d, 1H, H_3 phenyl, J = 8.0 Hz), 7.69–7.65 (m, 1H, H_7 coumarin), 7.50–7.28 (m, 5H, $H_{6,8}$ coumarin, $H_{4,5,6}$ phenyl), 5.99 (s, 2H, CH_2N$^+$), 5.16 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.0 (C=O), 162.7 (C=O), 161.0 (CH), 154.2 (C), 147.0 (CH), 146.2 (CH), 138.0 (CH), 136.0 (CH), 134.5 (C), 133.1 (C), 132.0 (C), 129.5 (CH), 129.0 (CH), 128.0 (CH), 126.3 (CH), 126.2 (CH),126.0 (CH), 123.5 (CH), 120.1 (C), 118.0 (CH), 96.8 (C), 62.7 (CH_2), 52.3 (CH_2).

*4.3.11. 1-(3-bromobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl) amino)methyl)pyridinium bromide (**7k**)*

Yield 75% (Z isomer: 66% and E isomer: 34%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) ν_{max}: 3458 (NH), 1706, 1633 (C=O). Purity 96.63% by HPLC. Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3$: C, 52.10; H, 3.42; N, 5.28. Found: C, 52.01; H, 3.31; N, 5.17. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.83–11.82 (m, 1H, N*H*), 9.32 (s, 1H, H_2 pyridine), 9.27 (d, 1H, H_6 pyridine, J = 4.5 Hz), 8.72 (d, 1H, =CH, J = 15.0 Hz), 8.71–8.69 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.94 (s, 1H, H_2 phenyl), 7.92 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76–7.60 (m, 4H, H_7 coumarin, $H_{4,5,6}$ phenyl), 7.33–7.31 (m, 2H, $H_{6,8}$ coumarin), 5.89 (s, 2H, CH_2N$^+$), 5.06 (s, 2H, CH_2N). ^{13}CNMR:(500 MHz, DMSO-d_6) δ: 179.0 (C=O), 165.0 (C), 162.1 (C=O), 157.7 (CH), 154.2 (C), 148.7 (CH), 145.2 (CH), 144.4 (CH), 142.8 (CH), 138.4 (CH), 136.2 (CH), 134.5 (C), 133.6 (C), 130.0 (CH), 126.5 (CH), 126.0 (CH), 125.1 (CH), 124.9 (CH), 120.1 (C), 116.2 (CH), 115.7 (CH), 115.6 (CH), 97.3 (C), 62.0 (CH_2), 52.3 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.66–10.66 (m, 1H, N*H*), 9.31 (s, 1H, H_2 pyridine), 9.26 (d, 1H, H_6 pyridine, J = 4.5 Hz), 8.85 (d, 1H, =CH, J = 15.0 Hz), 8.71–8.69 (m, 1H, H_4 pyridine), 8.21 (t, 1H, H_5 pyridine, J = 7.0 Hz), 7.94 (s, 1H, H_2 phenyl), 7.92 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.76–7.60 (m, 4H, H_7 coumarin, $H_{4,5,6}$ phenyl), 7.33–7.31 (m, 2H, $H_{6,8}$ coumarin), 5.89 (s, 2H, CH_2N$^+$), 5.06 (s, 2H, CH_2N). ^{13}C NMR: (500 MHz, DMSO-d_6) δ: 177.0 (C=O), 165.0 (C), 163.3 (C=O), 157.7 (CH), 154.2 (C), 148.7 (CH), 146.0 (CH), 145.2 (CH), 144.2 (CH), 138.4 (CH), 136.2 (CH), 134.5 (C), 133.6 (C), 130.0 (CH), 126.5 (CH), 126.0 (CH), 125.1 (CH), 124.9 (CH), 120.1 (C), 116.2 (CH), 115.7 (CH), 115.6 (CH), 97.3 (C), 62.0 (CH_2), 52.3 (CH_2).

*4.3.12. 1-(4-bromobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl) amino)methyl)pyridin-1-ium bromide (**7l**)*

Yield 85% (Z isomer: 66% and E isomer: 34%); white solid; mp > 250 °C. IR (KBr, cm^{-1}) ν_{max}: 3393 (NH), 1698, 1637 (C=O). Purity 97.11% by HPLC. Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3$: C, 52.10; H, 3.42; N, 5.28. Found: C, 52.19; H, 3.56; N, 5.39. NMR data for the Z isomer. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.81 (br. s, 1H, N*H*), 9.26 (s, 1H, H_2 pyridine), 9.18 (d, 1H, H_6 pyridine, J = 5.0 Hz), 8.69 (d, 1H, =CH, J = 15.0 Hz), 8.68–8.66 (m, 1H, H_4 pyridine), 8.20 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.92 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.67 (d, 2H, H_α phenyl, J = 7.5 Hz), 7.68–7.66 (m, 1H, H_7 coumarin), 7.52 (d, 2H, H_β phenyl, J = 7.5 Hz), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 5.88 (s, 2H, CH_2N$^+$), 5.03 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 163.3 (C=O), 161.9 (CH), 154.2 (C), 145.4 (CH), 144.5 (CH), 143.8 (CH), 138.4 (CH), 134.5 (CH), 133.3 (C), 132.0 (CH), 131.2 (CH), 131.0 (C), 128.2 (CH), 125.2 (C), 124.1 (C), 122.8 (CH), 117.0 (CH), 96.6 (C), 62.5 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.68 (br. s, 1H, N*H*), 9.24 (s, 1H, H_2 pyridine), 9.18 (d, 1H, H_6 pyridine, J = 5.0 Hz), 8.83 (d, 1H, =CH, J = 15.0 Hz), 8.68–8.66 (m, 1H, H_4 pyridine), 8.20 (t, 1H, H_5 pyridine, J = 6.5 Hz), 7.97 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.67 (d, 2H, H_α phenyl, J = 7.5 Hz), 7.68–7.66 (m, 1H, H_7 coumarin), 7.52 (d, 2H, H_β phenyl, J = 7.5 Hz), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 5.88 (s, 2H, CH_2N$^+$), 5.03 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 178.2 (C=O), 164.0 (C=O), 161.9 (CH), 154.2 (C), 146.0 (CH), 144.5 (CH), 144.2 (CH), 138.4 (CH), 134.5 (CH), 133.3 (C), 132.0 (CH), 131.2 (CH), 131.0 (C), 128.2 (CH), 125.2 (C), 124.1 (C), 122.8 (CH), 117.0 (CH), 96.6 (C), 62.5 (CH_2), 50.2 (CH_2).

*4.3.13. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(2-nitrobenzyl)pyridin-1-ium bromide (**7m**)*

Yield 75% (Z isomer: 66% and E isomer: 34%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) ν_{max}: 3310 (NH), 1708, 1640 (C=O), 1525, 1352 (NO_2). Purity 96.64% by HPLC. Anal. Calcd for $C_{23}H_{18}BrN_3O_5$: C, 55.66; H, 3.66; N, 8.47. Found: C, 55.54; H, 3.74; N, 8.38. NMR data for the Z isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.80 (br. s, 1H, N*H*), 9.18 (s, 1H, H_2 pyridine), 9.11 (d, 1H, H_6 pyridine, J = 4.0 Hz), 8.76 (d, 1H, =CH, J = 15.0 Hz), 8.76–8.70 (m, 1H, H_4 pyridine), 8.28 (d, 1H, H_6 phenyl, J = 7.0 Hz), 8.28 (overlap, 1H, H_5 pyridine), 7.92 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.85 (t, 1H, H_4 phenyl, J = 7.0 Hz), 7.77 (dd, 1H, H_3 phenyl, J = 7.0, 7.5 Hz), 7.70–7.67 (m, 1H, H_7 coumarin), 7.35–7.27 (m, 3H, $H_{6,8}$ coumarin, H_5 phenyl), 6.29 (s, 2H, CH_2N$^+$), 5.08 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C=O), 162.9 (C=O), 161.9 (CH), 162.6 (CH), 154.2 (C), 147.5 (CH), 145.7 (CH), 144.7 (CH), 138.4 (CH), 138.2 (CH), 134.9 (CH), 134.5 (CH), 130.6 (C), 129.9 (CH), 128.1 (CH), 125.5 (CH), 125.3 (CH), 123.9 (CH), 120.1 (C), 116.8 (CH), 96.6 (C), 60.6 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.70 (br. s, 1H, N*H*), 9.17 (s, 1H, H_2 pyridine), 9.11 (d, 1H, H_6 pyridine, J = 4.0 Hz), 8.84 (d, 1H, =CH, J = 15.0 Hz), 8.76–8.70 (m, 1H, H_4 pyridine), 8.28 (d, 1H, H_6 phenyl, J = 7.0 Hz), 8.28 (overlap, 1H, H_5 pyridine), 7.96 (d, 1H, H_5 coumarin, J = 7.0 Hz), 7.85 (t, 1H, H_4 phenyl, J = 7.0 Hz), 7.77 (dd, 1H, H_3 phenyl, J = 7.0, 7.5 Hz),

7.70–7.67 (m, 1H, H_7 coumarin), 7.35–7.27 (m, 3H, $H_{6,8}$ coumarin, H_5 phenyl), 6.29 (s, 2H, CH_2N^+), 5.08 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.2 (C═O), 163.2 (C═O), 161.9 (CH), 162.6 (CH), 154.2 (C), 148.5 (CH), 146.3 (CH), 144.7 (CH), 138.4 (CH), 138.2 (CH), 134.9 (CH), 134.5 (CH), 130.6 (C), 129.9 (CH), 128.1 (CH), 125.5 (CH), 125.3 (CH), 123.9 (CH), 120.1 (C), 116.8 (CH), 96.6 (C), 60.6 (CH_2), 50.2 (CH_2).

4.3.14. 3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)-1-(4-nitrobenzyl)pyridin-1-ium bromide (7n)

Yield 85% (Z isomer: 63% and E isomer: 37%); yellow solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3450 (NH), 1693, 1628 (C═O), 1463, 1347 (NO_2). Purity 96.32% by HPLC. Anal. Calcd for $C_{23}H_{18}BrN_3O_5$: C, 55.66; H, 3.66; N, 8.47. Found: C, 55.51; H, 3.54; N, 8.32. NMR data for the Z isomer: ^1H NMR (500 MHz, DMSO-d_6) δ: 11.80 (br. s, 1H, N*H*), 9.34 (s, 1H, H_2 pyridine), 9.27 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.71 (d, 1H, ═CH, J = 15.0 Hz), 8.72–8.69 (m, 1H, H_4 pyridine), 8.32 (d, 2H, H_α phenyl, J = 8.0 Hz), 8.25 (t, 1H, , H_5 pyridine, J = 7.0 Hz), 7.91 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.81 (d, 2H, H_β phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.09 (s, 2H, CH_2N^+), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C═O), 162.6 (C═O), 162.1 (CH), 154.2 (C), 147.9 (CH), 145.7 (CH), 144.5 (CH), 141.0 (C), 138.4 (CH), 134.6 (CH), 131.0 (C), 130.1 (CH), 128.3 (CH), 125.3 (CH), 124.0 (CH), 120.2 (C), 117.0 (CH), 96.6 (C), 62.2 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^1H NMR (500 MHz, DMSO-d_6) δ: 10.65 (br. s, 1H, N*H*), 9.32 (s, 1H, H_2 pyridine), 9.27 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.82 (d, 1H, ═CH, J = 15.0 Hz), 8.72–8.69 (m, 1H, H_4 pyridine), 8.32 (d, 2H, H_α phenyl, J = 8.0 Hz), 8.25 (t, 1H, , H_5 pyridine, J = 7.0 Hz), 7.96 (d, 1H, H_5 coumarin, J = 7.5 Hz), 7.81 (d, 2H, H_β phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.09 (s, 2H, CH_2N^+), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 177.4 (C═O), 163.4 (C═O), 162.1 (CH), 154.2 (C), 149.8 (CH), 145.7 (CH), 145.6 (CH), 141.0 (C), 138.4 (CH), 134.6 (CH), 131.0 (C), 130.1 (CH), 128.3 (CH), 125.3 (CH), 124.0 (CH), 120.2 (C), 117.0 (CH), 96.6 (C), 62.2 (CH_2), 50.2 (CH_2).

4.3.15. 1-(4-cyanobenzyl)-3-((((2,4-dioxochroman-3-ylidene)methyl)amino)methyl)pyridin-1-ium bromide (7o)

Yield 86% (Z isomer: 63% and E isomer: 37%); white solid; mp > 250 °C. IR (KBr, cm^{-1}) v_{max}: 3417 (NH), 2200 (CN), 1661, 1604 (C═O). Purity 99.19% by HPLC. Anal. Calcd for $C_{24}H_{18}BrN_3O_3$: C, 60.52; H, 3.81; N, 8.82. Found: C, 60.63; H, 3.72; N, 8.95. NMR data for the Z isomer: ^1H NMR (500 MHz, DMSO-d_6) δ: 11.83 (br. s, 1H, N*H*), 9.30 (s, 1H, H_2 pyridine), 9.21 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.72–8.70 (m, 2H, H_4 pyridine, ═CH), 8.23 (t, 1H, , H_5 pyridine, J = 6.5 Hz), 7.95 (d, 2H, H_α phenyl, J = 8.0 Hz), 7.92 (d, 1H, H_5 coumarin, J = 8.5 Hz), 7.74 (d, 2H, H_β phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.35–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.02 (s, 2H, CH_2N^+), 5.04 (s, 2H, CH_2N). ^{13}C NMR:(500 MHz, DMSO-d_6) δ: 179.4 (C═O), 162.6 (C═O), 162.0 (CH), 154.2 (C), 145.5 (CH), 144.8 (CH), 144.4 (CH), 144.1 (CH), 139.1 (CH), 138.6 (CH), 134.5 (C), 133.0 (CH), 129.6 (CH), 128.3 (CH), 128.2 (C), 125.3 (CH), 123.9 (CH), 120.1 (C), 118.2 (CH), 117.0 (CN), 112.0 (CH), 96.7 (C), 62.5 (CH_2), 50.2 (CH_2). NMR data for the E isomer: ^1H NMR (500 MHz, DMSO-d_6) δ: 10.68 (br. s, 1H, N*H*), 9.28 (s, 1H, H_2 pyridine), 9.21 (d, 1H, H_6 pyridine, J = 5.5 Hz), 8.83 (d, 1H, ═CH, J = 15.0 Hz), 8.72–8.70 (m, 1H, H_4 pyridine), 8.23 (t, 1H, , H_5 pyridine, J = 6.5 Hz), 7.95 (d, 2H, H_α phenyl, J = 8.0 Hz), 7.95 (d, 1H, H_5 coumarin, J = 8.0 Hz), 7.74 (d, 2H, H_β phenyl, J = 8.0 Hz), 7.70–7.66 (m, 1H, H_7 coumarin), 7.33–7.29 (m, 2H, $H_{6,8}$ coumarin), 6.02 (s, 2H, CH_2N^+), 5.04 (s, 2H, CH_2N). ^{13}CNMR: (500 MHz, DMSO-d_6) δ: 177.5 (C═O), 163.4 (C═O), 162.0 (CH), 154.2 (C), 147.0 (CH), 146.0 (CH), 144.4 (CH), 144.1 (CH), 139.1 (CH), 138.6 (CH), 134.5 (C), 133.0 (CH), 129.6 (CH), 128.3 (CH), 128.2 (C), 125.3 (CH), 123.9 (CH), 120.1 (C), 118.2 (CH), 117.0 (CN), 112.0 (CH), 96.7 (C), 62.5 (CH_2), 50.2 (CH_2).

4.4. AChE and BChE inhibition assay

For evaluation of the anti-cholinesterase activity, all the synthesized compounds **7a–o** were subjected to AChE and BuChE inhibition assays using Ellman's method [32]. For this purpose, AChE (E.C. 3.1.1.7, type V-S, lyophilized powder, from electric eel, 1000 unit), BChE (E.C. 3.1.1.8, from equine serum), acetylthiocholine iodide (ATCI), and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. Phosphate buffer components (potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium hydroxide, and sodium hydrogen carbonate) were purchased from Fluka. The compounds **7a–o** were dissolved in a mixture of 5 ml DMSO and 5 ml methanol and diluted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0). Then, 50 μL potassium phosphate buffer (KH_2PO_4/K_2HPO_4, 0.1 M, pH 8), 25 μL test compound (prepared sample as described above), and 25 μL enzyme (AChE or BuChE) with a final concentration of 0.22 U/mL in the buffer were added to each well of a 96-well plate and were pre-incubated for 15 min at room temperature. Then, 125 μL of DTNB (3 mM in the buffer) was added to each well and the change of absorbance was measured at 412 nm for 15 min. The IC_{50} values were obtained graphically from the inhibition curves (log inhibitor concentration vs. percent of inhibition). For all the synthesized compounds, four different concentrations in triplicate were tested to obtain the range of 20–80% enzyme inhibition for AChE and BuChE.

4.5. Kinetic study of AChE inhibition

The kinetic study carried out according to the protocol was used for the inhibition assay [32]. By using three different concentrations ([I1] = 0.866, [I2] = 1.73 and [I3] = 3.46 μM) of the compound **7f**, the Lineweaver–Burk reciprocal plot was constructed by plotting 1/velocity against 1/[substrate] at variable concentrations of the substrate acetylthiocholine (0.23, 0.58, 1.17 mM). The inhibition constant K_i was achieved by the plot of the slopes versus the corresponding concentrations of the compound **7f**.

4.6. Molecular docking study

A docking study was performed using Auto Dock Tools (version 1.5.6) and the pdb structures of 1EVE (AChE) and 1P0I (BuChE) were taken from the Brookhaven protein database (http://www.rcsb.org). The 3D structures of the selected compounds were created by MarvineSketch 5.8.3, 2012, ChemAxon (http://www.chemaxon.com) and by Auto Dock Tools converted to pdbqt coordinates. Moreover, the pdbqt coordinates of the enzymes were prepared using the Auto Dock Tools. Before preparation of the pdbqt form of the enzymes, the water molecules and inhibitors were removed. Then, by using Auto Dock Tools, polar hydrogen atoms were added, Koullman charges were assigned, and the obtained protein structures were used as input files for the AUTOGRID program. In AUTOGRID for each atom type in the inhibitors, maps were calculated with 0.375 Å spacing between grid points, and the center of the grid box was placed at x = 2.023, y = 63.295, and z = 67.062 for AChE and x = 137.985, y = 122.725, and z = 38.78 for BuChE. The dimensions of the active site box were set at 40 × 40 × 40 Å for AChE and 55 × 55 × 55 Å for BuChE. Flexible ligand dockings were accomplished for the selected compounds. Each docked system was carried out by 50 runs of the AUTODOCK search by the Lamarckian genetic algorithm (LGA). The best position of each compound was selected for analyzing the interactions between AChE or BuChE and the inhibitor, and its results were visualized using Discovery Studio 4.0 Client.

4.7. In vitro assay of self-induced Aβ1–42 aggregation inhibitory activity

The inhibition of the self-induced Aβ1–42 aggregation was measured using a Thioflavin T(ThT)-binding assay [29]. HFIP pretreated

Aβ1–42 samples (Anaspec Inc) were resolubilized with a 50 mM phosphate buffer (pH 7.4) to give a 20 μM solution. Each tested compound was firstly prepared in dimethyl sulfoxide (DMSO) in order to have a stable stock solution. A mixture of the peptide (10 μL, 10 μM, final concentration) with or without the tested compound (10 μL, 10 and 50 μM, final concentration) was incubated at 30 °C for 48 h. Blanks containing 50 mM phosphate buffer (pH 7.4) instead of Aβ with or without inhibitors were also carried out. After incubation, the samples were diluted to a final volume of 200 μM with 50 Mm glycine–NaOH buffer (pH 8.0) containing thioflavin-T (5 μM). Each measurement was run in triplicate. Fluorescence was measured on a Synergy HTX Multi-Mode reader from BioTek Instruments with excitation and emission wavelengths at 440 nm and 485 nm, respectively. The percent inhibition of the aggregation was calculated by the expression $(1 - IF_i/IF_O) \times 100\%$, in which IF_i and IF_O are the fluorescence intensities obtained for Aβ in the presence and absence of the inhibitors, respectively.

4.8. Neuroprotection assay

The neuroprotection assay was conducted by using the rat differentiated neuronal cell line PC12. The differentiated PC12 cells were pretreated with the compound **7f** (5, 10, and 20 μM) and quercetin (5 μM) for 3 h before treatment with H_2O2. Then, to initiate apoptosis, H_2O_2 (350 μM) was added to the cells and they were incubated for 24 h. Induction of apoptosis was recognized by DAPI staining. After 24 h of incubation, the medium was removed. The cells were washed with PBS, and 10 μL of the MTT solution (5 mg/ml, Sigma) was added to each well. After 3.5 h of incubation, the medium was removed and 150 μL DMSO was added to dissolve the formazan precipitates. Finally, optical density (OD) was measured at 560 nm using a microplate reader (BioTek synergy HT). The results were adjusted considering the OD measured in the blank [29].

4.9. BACE1 enzymatic assay

A FRET-based BACE1 enzyme assay kit to evaluate the enzyme inhibition was purchased from Invitrogen (former Pan Vera Corporation, Madison, WI), and the assay was performed according to the manufacturer's instructions (Invitrogen. http://tools.invitrogen.com/content/sfs/manuals/L0724.pdf) [31].

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