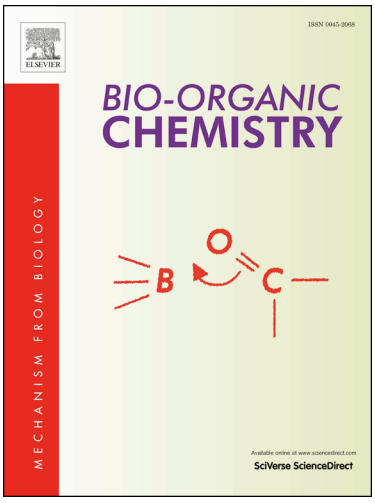

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Synthesis and anticholinesterase inhibitory activity of new 2-benzofuran carboxamide-benzylpyridinum salts

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This paper is dedicated to the memory of the late Professor Abbas Shafiee at Tehran University of Medical Sciences.

Abtract: A series of benzofuran-2-carboxamide-*N*-benzyl pyridinium derivatives (**6a-o**) are synthesized as new cholinesterase inhibitors. The synthetic pathway involves the reaction of salicylaldehyde derivatives and ethyl bromoacetate, followed by hydrolysis and amidation with 3- and 4-picolyl amine. Subsequently, *N*-((pyridin-4-yl) methyl) benzofuran-2-carboxamide and substituted *N*-((pyridin-3-yl) methyl) benzofuran-2-carboxamides reacts with benzyl halides to afford target compounds (**6a-o)**. The chemical structures of all derivatives are confirmed by spectroscopic methods. The studies revealed that some of the synthesized compounds exhibited potent butyrylcholinesterase inhibitory activities in the range of 0.054-2.7 μM. In addition, good inhibitory effects on Aβ self-aggregation are observed for **6h** and **6k** (33.1 and 46.4 % at 100 μM, respectively).

Keywords: Alzheimer's disease, Benzofuran-2-carboxamide, Benzylpyridinium, Cholinesterase inhibitor.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease, classified as the fourth leading cause of illness and death in people at old ages in developed countries. Scientists predict that the number of people, suffering from this disease will be nearly tripled by 2050 [1]. Alzheimer is a progressive disorder, characterized by loss of cognitive abilities, severe behavioral disorders and ultimately death. Low levels of acetylcholine (ACh) [2, 3], β-amyloid (Aβ) deposits [4], metal-ion imbalance [5, 6] and many other factors have been considered as important pathogenesis in AD [7-9].

The cognitive impairment in AD patients is due to the loss of cholinergic neurons and subsequently reduction in acetylcholine levels in the specific regions of the brain [10]. Therefore, according to cholinergic hypothesis, the reduced levels of acetylcholine in the brain have been introduced as the most important target among the treatment strategies [11].

The structures of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are very similar, expressing 65% identity in amino acid sequence [12]. The inhibition of acetylcholinesterase and butyrylcholinesterase enzymes led to the alleviation of the Alzheimer's symptoms, including cognitive level and short-term memory, by restoring ACh level. The increased levels of BuChE at the later stages of AD in the hippocampus and temporal cortex highlighted the importance of selective BuChE inhibitors in regulating the ACh level in cholinergic neurons. This fact was also proved by Mesulam's experiments with nullizygous and wild-type mice, exhibiting the constitutive function of BuChE in the hydrolysis of ACh in the normal brain [13]. Relied on multifactorial nature of AD, the synchronous inhibition, which is achieved by AChE and BChE inhibitors may be beneficial for AD treatment [14-16].

Another crucial hypothesis for AD suggested that the aggregation and accumulation of β-amyloid peptide in a brain could lead to toxic fibrils and consequently neuronal cell death [17-20]. Many strategies are currently being assessed for preventing the formation of amyloid and toxic oligomers. Several classes of small molecules have been reported in the literature, capable of preventing or reversing fibrillization [21-22], while none of them are successful to be moved to clinical phase.

Therefore, design and synthesis of new compounds with dual inhibitory activity may be promising in AD treatment [23]. Benzofurans are one of the most significant heterocyclic categories with wide range of bioactivities [24]. In addition, benzofuran-2-carboxamide derivatives are also known as antitumor [25-28], antimicrobial [29], anti-hyperlipidemic [30], AChE inhibitor [31], antibacterial [32] and suppressing agent in allergic rhinitis [33]. Recently, various benzofuran-based compounds were reported as potent acetylcholinesterase inhibitors. Considering the importance of heterocyclic compounds by our research team [34-40], herein we report the synthesis and anticholinesterase inhibitory activity of a series of derivatives bearing *N*-benzylpyridinium and benzofuran backbone which providing interactions with catalytic active site (CAS) and peripheral anionic site (PAS) of AChE, respectively (Figure 1) [41, 42]. It is expected that the intrinsic dual mechanism of action could result in efficient anticholinesterase agent discoveries.

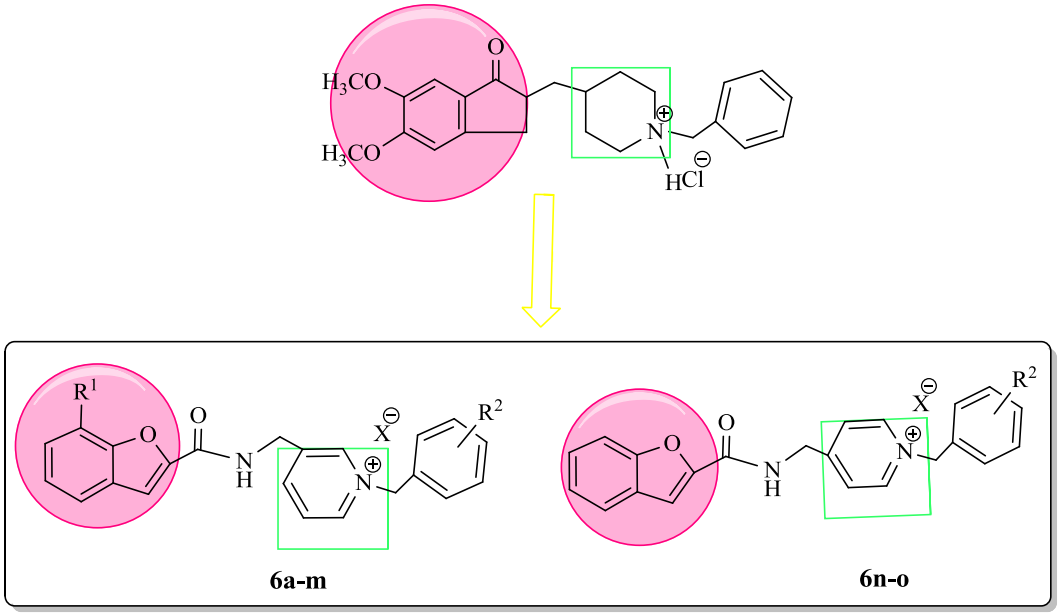


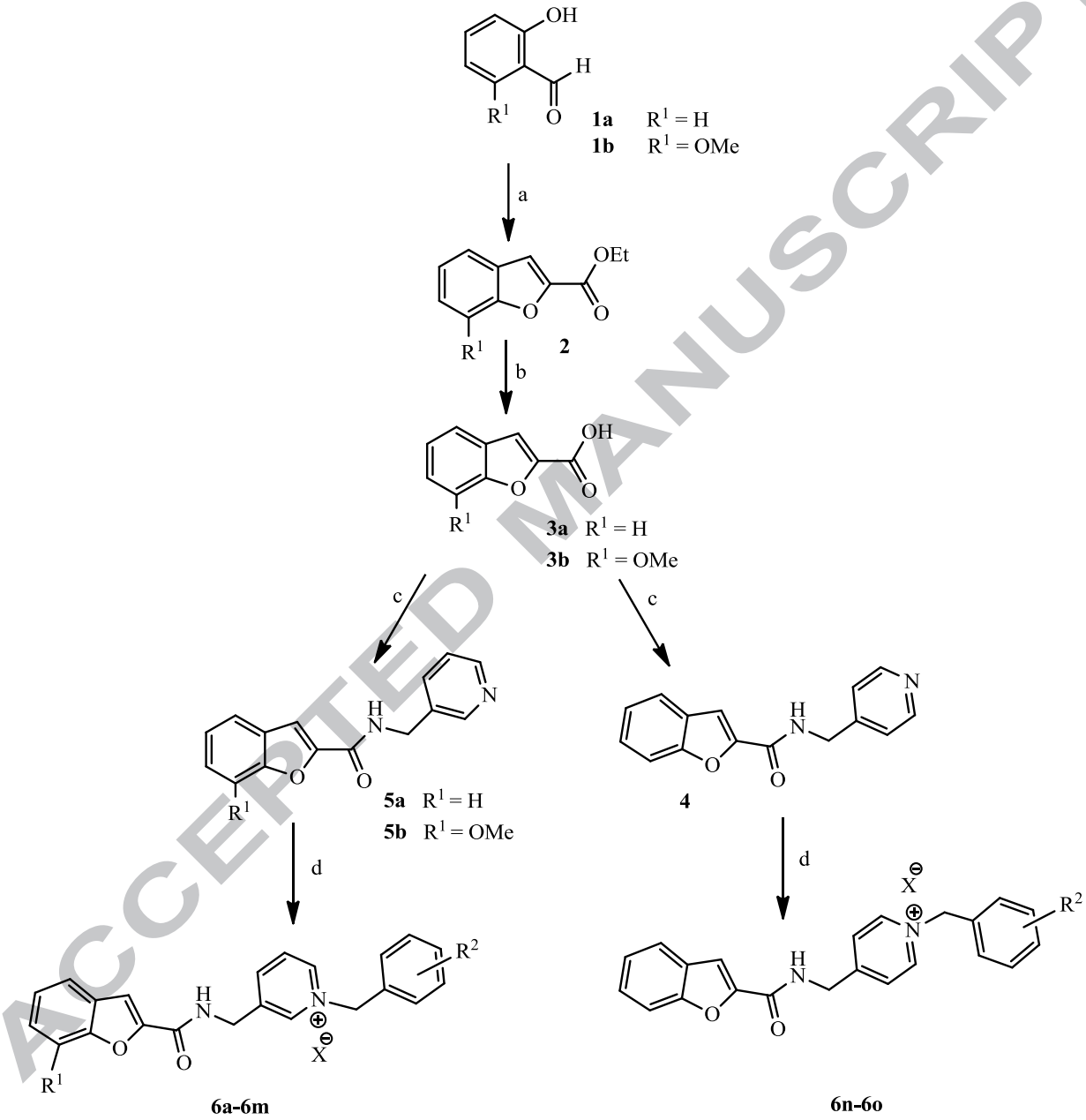
Figure 1. Structure of donepezil hydrochloride as a ChE inhibitor and designed compounds **6a-o** as selective ChE inhibitors

2. Results and discussion

2.1 Chemistry

The synthetic pathway toward benzofuran-2-carboxamide bearing pyridinium moiety is shown in scheme 1. The condensation reaction between salicylaldehyde derivatives **1** and ethyl bromoacetate in the presence of K_2CO_3 afforded ethyl benzofuran-2-carboxylates [43]. Subsequent hydrolysis in aqueous ethanol/KOH afforded **3a-b** [44]. The amidation reaction of **3a-b** with 3-(methylamino) pyridine or 4-(methylamino) pyridine in the presence of

hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDC) as coupling agents resulted in **4**, **5a-b** [45]. The *N*-benzylation of the latter compounds with appropriate benzyl halides in acetonitrile at reflux temperature resulted in benzyl pyridinium halide salts of desired products **6a-6o** in good yields [46].



Scheme 1. Synthesis of benzofuran-2-carboxamide **6a-o**: (a) Ethyl bromoacetate, K_2CO_3, Dry DMF, 90 ˚C, 4-6 h; (b) KOH, Ethanol: H_2O (2:1), reflux; (c) (Pyridin-3-yl)methanamine or (pyridin-4-yl)methanamine; HOBT, EDC, Dry CH_3CN, r.t., 24-48 h; (d) substituted benzyl halides, CH_3CN, reflux, 2-4 h.

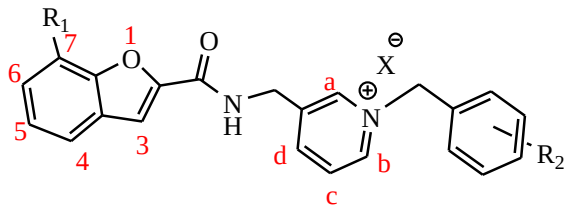
The presence of electron-donating groups including Me or OMe at the *ortho*, *meta* or *para* positions of benzyl moiety decreased the reaction yield. However, the difference in electronic

characters of substitutions, electron-donating and electron-withdrawing groups, had no distinguishing influence on the isolated yields.

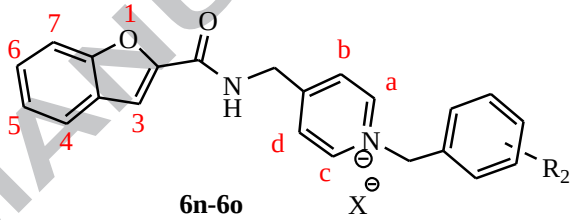
2.2 Cholinesterase activity evaluation

Inhibitory activities of **6a-o** series of benzofuran-carboxamide benzyl pyridinium derivetives towards AChE and BChE were measured by modified Ellman's protocol [47]. The results are summarized in Table 1 and are reported as IC_{50} and the percent of inhibition at 56 µM for target compounds (Table 1).

Table 1. Cholinesterase inhibitory activity of the synthesized compounds (**6a-6o**)



6a-6m



6n-6o

Compound	R_1	R_2	X	AChE inhibition[a]	BChE inhibition
6a	H	H	Br	5.5±0.3	0.29±0.01
6b	H	3-Me	Br	13.8±0.5	0.11±0.01
6c	H	4-Me	Br	19.8±0.8	0.65±0.04
6d	H	2-NO_2	Br	3.5±0.3	0.15±0.01
6e	H	4-NO_2	Br	(40.0±4.0)	0.76±0.05
6f	H	4-F	Br	12.3±0.3	0.37±0.01
6g	H	2,4-Cl_2	Cl	4.4±0.3	0.37±0.01
6h	H	2-F-6-NO_2	Br	33.8±1.2	0.054±0.002
6i	OMe	H	Cl	4.7±0.4	0.87±0.07
6j	OMe	3-OMe	Cl	(2.0±0.8)	9.6±0.3
6k	OMe	2-Me	Br	2.1±0.1	0.45±0.05
6l	OMe	2,4-Cl_2	Cl	14.5±1.0	0.18±0.01
6m	OMe	4-Br	Br	29.3±2.8	2.45±0.30
6n	H	2-Br	Br	61.0±1.6	2.7±0.2
6o	H	4-Br	Br	(44.0±5.0)	4.3±0.4
Donepezil	-	-	-	0.031±0.005	5.4±0.1

[a] IC_{50} (µM) or inhibition % at 56.3 µM concentration (in parentheses). Values were the means of three replicates ± standard deviation (SD)

None of the compounds exhibited higher AChE inhibition compared to donepezil, but all of them except **6j** are better BChE inhibitors. Among the target compounds, **6k** showed the best

inhibitory activity against AChE and **6h** was more potent than donepezil against BChE, exhibiting IC_{50} value of 0.054 µM, which is 100 times stronger than positive control.

As depicted in Table 1, in both series, the *ortho* and *meta* substituted derivatives exhibit better inhibition towards AChE and BChE than the *para* substituted ones. The presence of strong electron withdrawing groups at *para* position in **6e** decreases the inhibitory activity against AChE in 3-pyridinium series. As shown in Table 1, the presence of methoxy group at 7-position of benzofuran leads to the reduced butyrylcholinesterase inhibitory activity (**6a** vs. **6i**). The 4-pyridinium series display the weakest inhibitory activity against both AChE and BChE compared to 3-pyridinium series.

To predict BBB penetration and intestinal absorption the admetSAR server was used [48]. Calculated logP and tPSA were retrieved from chembiodraw ultra 14.0 (PerkinElmer). Based on the obtained data from the server all compounds were predicted as CNS active with satisfactory probability (Table 2).

Table 2. BBB and HIA prediction. All prediction was performed by admetSAR server.

Compound	ClogP*	tPSA(Å^2)[a]	Blood-Brain Barrier probability	Human Intestinal Absorption probability
6a	0.226	41.34	0.9915	0.9791
6b	0.725	41.34	0.9887	0.9828
6c	0.725	41.34	0.9870	0.9877
6d	-0.111	93.15	0.9564	0.9837
6e	-0.031	93.15	0.9648	0.9850
6f	0.369	41.34	0.9920	0.9824
6g	1.65	41.34	0.9824	0.9810
6h	0.031	93.15	0.9569	0.9866
6i	0.356	50.57	0.9865	0.9879
6j	0.275	59.8	0.9656	0.9483
6k	0.805	50.57	0.9781	0.9844
6l	1.78	50.57	0.9744	0.9892
6m	1.21	50.57	0.9806	0.9857
6n	1.09	41.34	0.9904	0.9549
6o	1.09	41.34	0.9934	0.9584
Donepezil	4.59	38.77	0.9953	0.9966

[a] ClogP and tPSA were calculated by chembiodraw ultra (14.0)

2.3 Docking studies

Docking studies were carried out to provide insight into the binding mode of the synthesized compounds **6h** and **6k**. In order to validate the docking reliability, root-mean-square distance (RMSD) values of 0.774 Å and 0.615 Å were obtained between bounded ligands and the re-docked ligands tacrine and donepezil, which shows the high reliability of the GOLD method to reproduce the known binding mode.

According to the docking results, the best docked pose of molecule **6h** shows a hydrogen bond between oxygen atom of benzofuran and Ser198. The positively charged nitrogen induces the π-cation interactions with aromatic residues Phe329 and Trp82. Moreover, the benzyl pyridinium section of **6h** involves in the π-π interaction with Tyr332 (Figure 2).

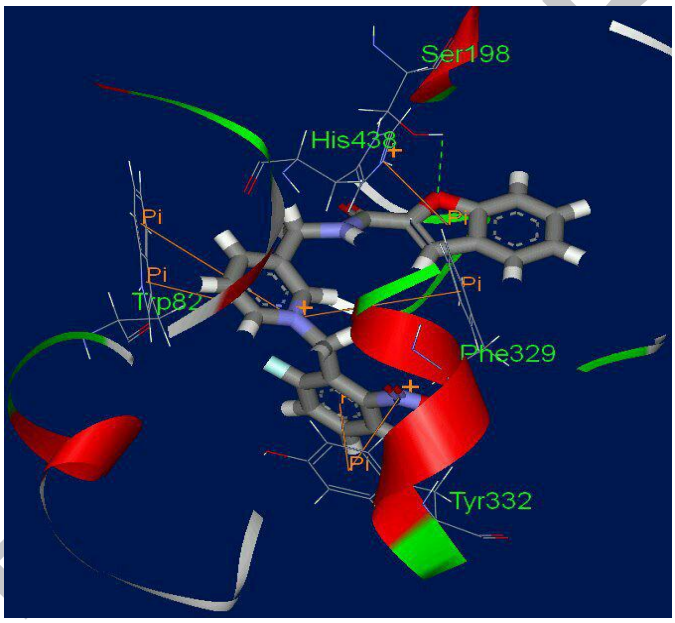


Figure 2. The interacting mode of the most active compound **6h** with the active site of BChE.

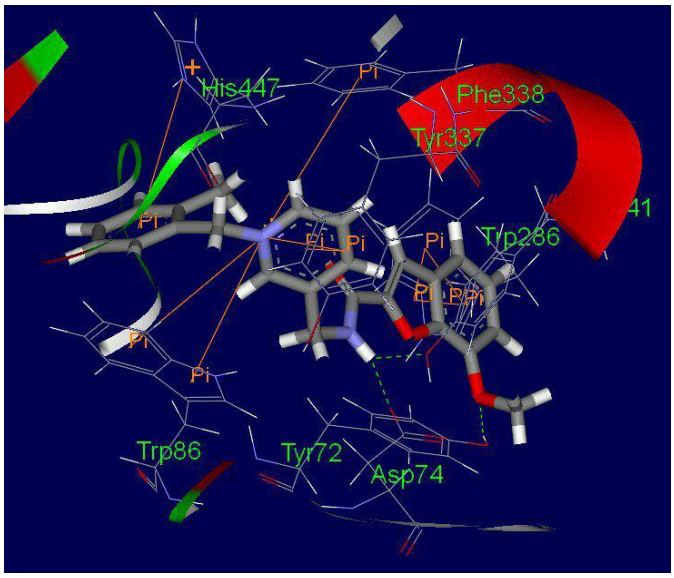


Figure 3. The interacting mode of the most active compound **6k** with the active site of AChE.

Amongst the synthetic compounds, **6k** (IC_{50} = 2.1 µM) with methoxy substitution as R_1 and methyl as R_2 showed the most potent compounds toward AChE comparing to other congeners of the series. As depicted in Figure 3, four π-cation interactions between quaternary nitrogen of pyridine ring with Trp86 and Tyr337 of AChE lead to the stabilization of the ligand in the active site of enzyme. In addition, benzofuran part of the molecule involves in a π-π interaction with Trp286 and Tyr341.

There are two hydrogen bonding interactions of nitrogen atom of amide group and methoxy moiety of the benzofuran with Asp74**.** The same binding mode and interactions were also observed for the reference drug (Donepezil).

2.4 Inhibitory Effects on the Amyloid-β self-aggregation

The potential of compounds **6h** and **6k** to inhibit amyloid-beta aggregation was evaluated using thioflavin T (ThT) assay. Results indicated that compounds **6h** and **6k** emerged as potent inhibitors of Aβ fibrillization (33.1 and 46.4 % inhibition, respectively) in comparison with donepezil (22 %) and rifampicin (27.5 %) (Table 2). Compound **6k** is two-fold more potent than reference drugs in inhibitory activity against Aβ aggregation. The superiority of the **6k** inhibitory activity on **6h**, confirmed the role of methoxy group in the benzofuran framework for Aβ binding**.**

Table 2. Inhibitory effects of compounds **6h** and **6k** on Aβ self-aggregation**.**

compound	Inhibition of Aβ self-aggregation (%)[a]
Donepezil	22.0±5.4
Rifampicin	27.5±4.3
6h	33.1±11.2
6k	46.4±2.2

[a]Inhibition of self-induced Aβ(1-42) aggregation (10 µM) produced by the tested compound at 100 µM concentration. Values are expressed as means ± SEM of three experiments.

3. Conclusion

Regarding the pivotal role of BuCh in the treatment of AD, herein, we presented the novel benzofuran carboxamide-based derivatives with significant anti butyrylcholinesterase activity. It was revealed that all the synthesized compounds are better BChE inhibitors rather than AChE. The 2-fluoro-6-nitro containing derivative, compound **6h**, along with compound

6k was subjected to docking studies and Aβ fibrillization inhibitory activity. On the other side, based on ClogP and tPSA calculation, all compounds were predicted as CNS active with satisfactory probability. Therefore, compound **6h** with such multifunctional properties could introduce as a candidate for further modification *in the* development of anti-AD drugs.

4. Materials and Methods

4.1 Chemistry

All the chemical compounds were purchased from Merck, Aldrich and Acros chemical and used without further purification. Melting points were determined with a Kofler hot stage apparatus and are uncorrected. The IR spectra were taken using Nicolet FT-IR Magna 550 spectrometer (KBr disks). Mass spectra were recorded on an Agilent Technologies (HP) 5973 mass spectrometer operating at an ionization potential of 70 eV. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) were recorded in DMSO-d_6 and/or $CDCl_3$ on a Bruker FT-500 MHz spectrometer with tetramethyl silane (TMS) as the internal standard. Coupling constants were reported in Hertz (Hz) and chemical shifts are given as δ value (ppm) relative to TMS as internal standard. To express spin multiplicities s (singlet), d (doublet), t (triplet), dd (doublet of doublet) and m (multiplet) were used. The atom numbering of products used for NMR interpretation is shown in Figure 4.

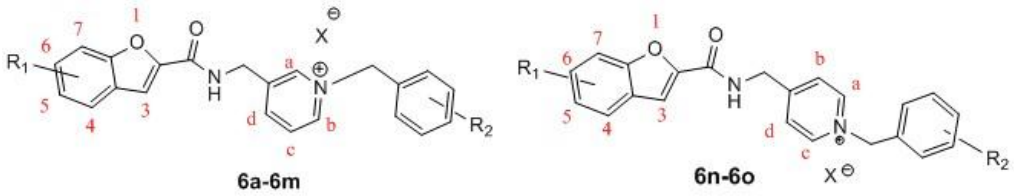


Figure 4. Atom numbering of compounds **6a-o**, used for NMR spectra interpretation

4.1.1 General Procedure for the Synthesis of Ethyl benzofuran-2-carboxylate (**2a, 2b**)

A mixture of 2-hydroxybenzaldehyde **1a** or **1b** (0.05 mol), ethyl bromoacetate (0.05 mol), anhydrous potassium carbonate (0.075 mol) and dried DMF (70 mL) were heated at 90 °C for 4 hours [28]. The solution was decanted into ice water. The precipitate was filtered off and washed with cold water.

4.1.1.1. Ethyl benzofuran-2-carboxylate (**2a**)

Pale yellow powder; yield: 75 %; mp 32 °C [mp lit. 30 °C][49]; ^{1}H NMR ($CDCl_3$, 500 MHz): 1.43 (m, 3H, -CH_3), 4.45 (q, 2H, *J* = 7 Hz, -CH_2), 7.30 (t, *J* = 7.5 Hz, 1H, H_5-benzofuran), 7.44 (t, *J* = 7.5 Hz, 1H, H_6-benzofuran), 7.53 (s, 1H, H_3-benzofuran), 7.59 (d, *J* = 8.5 Hz, 1H, H_7-benzofuran), 7.67 (d, *J* = 8 Hz, H_4-benzofuran).

4.1.1.2. Ethyl 7-methoxybenzofuran-2-carboxylate (**2b**)

Pale yellow powder; yield: 78 %; mp: 73 °C [mp lit. 73-75°C][49]; ^{1}H NMR ($CDCl_3$, 500 MHz): 1.41(m, 3H, -CH_3), 4.00 (s, 3H, -OCH_3), 4.42 (q, 2H, *J* = 7 Hz, -CH_2), 6.90 (d, *J* = 7 Hz, 1H, H_6-benzofuran), 7.20-7.24 (m, 2H, H_4-benzofuran, H_5-benzofuran), 7.50 (s, 1H, H_3-benzofuran).

4.1.2 General Procedure for the Synthesis of Benzofuran-2-carboxylic acid (**3a, 3b**)

To ethyl-1-benzofuran-2-carboxylate (**2)** (1 mmol) containing ethanol was added water (2:1, 30 mL) and potassium hydroxide (2 mmol). The mixture refluxed for 2 hrs. The reaction was monitored by thin layer chromatography. After completion of the reaction, the reaction mixture was decanted into ice water and extracted the ethyl acetate layer to give target compounds **3a** and **3b** [42].

4.1.2.1.Benzofuran-2-carboxylic acid (**3a**)

White crystal; yield: 85 %; mp 190 °C [mp lit. 191-192 °C][50]; ^{1}H NMR (500 MHz, DMSO-d_6): 7.35 (t, *J* = 7.5 Hz, 1H, H_5-benzofuran), 7.50 (t, *J* = 7.5 Hz, 1H, H_6-benzofuran), 7.66 (s, 1H, H_3-benzofuran), 7.69 (d, *J* = 8.5 Hz, 1H, H_7-benzofuran), 7.79 (d, *J* = 7.5 Hz, H_4-benzofuran), 13.5 (bs, 1H, -OH).

4.1.2.2. 7-MethoxyBenzofuran-2-carboxylic acid (**3b**)

Pale yellow crystal; yield: 85 %; mp 224 °C [lit. mp 220-222 °C][50]; ^{1}H NMR (500 MHz, DMSO-d_6): 3.94 (s, 1H, -CH_3), 7.06 (d, *J* = 7.5 Hz, 1H, H_6-benzofuran), 7.24 (t, 1H, H_5-benzofuran), 7.30 (d, *J* = 7.5 Hz, 1H, H_4-benzofuran), 7.63 (s, 1H, H_3-benzofuran) 13.5 (bs, 1H, OH).

4.1.3 General Procedure for the Synthesis of N-(pyridine-N-ylmethyl) Benzofuran-2-carboxamide **(4, 5a-b)**

A mixture of benzofuran-2-carboxylic acid ethyl ester (**3a** or **3b**) (1.5 mmol), 1-hydroxy-benzotriazole (1.7 mmol) and *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (1.7 mmol) in dried acetonitrile (10 mL) were stirred for 30 minutes at ambient temperature. The 3 or 4-(methylamino) pyridine was added and the mixture was stirred for

another 24-48 hours. After completion, the crude product was extracted with chloroform, solution of sodium hydrogen carbonate (10%) and citric acid (10%). The organic layer was separated, dried and evaporated under vacuum to give **4, 5a** or **5b** [51].

4.1.4 General Procedure for the Synthesis of pyridinium halide salts derivatives (**6a-o**)

4a or **5a-b** (1 mmol) was dissolved in dried acetonitrile (7 mL) and heated under reflux. Then, appropriate benzyl halide (1.8 mmol) was added dropwise to the mixture. Upon completion (checked by TLC), the mixture was cooled down to room temperature. The precipitated solid was separated by filtration, washed with diethyl ether and dried to afford the corresponding compounds **6a-o**[46].

4.1.4.1 *3-((Benzofuran-2-carboxamido)methyl)-1-benzylpyridin-1-ium bromide* (**6a**)

Cream powder; 0.180 g, yield: 42 %; mp 178–180 ˚C; IR (KBr): 3027 (N-H), 1657 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) 4.69 (d, J = 6 Hz, 2H, CH_2-NH), 5.88 (s, 2H, -CH_2N), 7.36 (t, J = 7 Hz, 1H, H_5-benzofuran), 7.41-7.53 (m, 6H, H_6-benzofuran, H aromatic), 7.62 (s, 1H, H_3-benzofuran), 7.66 (d, J = 8.5 Hz, 1H, H_7-benzofuran), 7.80 (d, J = 7.5 Hz, 1H, H_4-benzofuran), 8.15 (t, J = 7 Hz, 1H, H_c-pyridine), 8.58 (d, J = 8 Hz, 1H, H_d-pyridine), 9.12 (d, J = 6 Hz, 1H, H_b-pyridine), 9.24 (s, 1H, H_a-pyridine), 9.48 (t, J = 6 Hz, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 63.19, 110.18, 110.25, 111.78, 122.82, 123.73, 127.02, 128.02, 128.22, 128.66, 129.23, 134.22, 140.51, 143.23, 143.44, 144.63, 148.42, 154.26, 158.61.

4.1.4.2 *3-((Benzofuran-2-carboxamido)methyl)-1-(3-methylbenzyl)pyridin-1-ium bromide* (**6b**)

White powder; 0.182 g, yield: 41 %; mp 178–180 ˚C; IR (KBr): 3277 (N-H), 2943, 1655 (C=O), 1504, 1288 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) 2.28 (s, 3H, -CH_3), 4.68 (d, J = 6 Hz, 2H, CH_2-NH), 5.81 (s, 2H, -CH_2N), 7.21 (d, J=6.5 Hz, 1H, H aromatic), 7.29-7.37 (m, 4H, H_5-benzofuran, H aromatic), 7.49 (t, J = 8 Hz, 1H, H_6-benzofuran), 7.62 (s, 1H, H_3-benzofuran), 7.66 (d, J = 8 Hz, 1H, H_7-benzofuran), 7.79 (d, J =8 Hz, 1H, H_4-benzofuran), 8.14 (t, J = 6 Hz, 1H, H_c-pyridine), 8.57 (d, J = 7.5 Hz, 1H, H_d-pyridine), 9.10 (d, J = 6 Hz, 1H, H_b-pyridine), 9.21 (s, 1H, H_a-pyridine), 9.51 (1H, bs, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 20.83, 63.26, 110.23, 111.74, 122.84, 123.72, 125.79, 127.97, 128.20, 129.92, 134.08, 138.52, 140.49, 143.41, 143.43, 144.60, 148.42, 154.26, 158.59; MS *m/z* (%): 357 (M^+, <1),

252.2 (49.5), 235.2 (22.5), 189.2 (9.3), 145.1 (43.9), 127.0 (8.6), 105.2 (100), 89.1 (34.5), 63.1 (13.8)

4.1.4.3 *3-((Benzofuran-2-carboxamido)methyl)-1-(4-methylbenzyl)pyridin-1-ium bromide* (**6c**)

White powder; 0.174 g, yield: 40 %; mp 180-182 ˚C; IR (KBr): 3176 (N-H), 3089, 3024, 1653 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz): 2.28 (s, 3H, -CH_3), 4.68 (d, J = 5.5 Hz, 2H, CH_2-NH), 5.81 (s, 2H, -CH_2N), 7.23 (d, J = 7 Hz, 2H, H aromatic), 7.36 (dd, J = 6, 7.5 Hz, 1H, H_5-benzofuran), 7.42 (d, J = 7 Hz, 2H, H aromatic), 7.49 (dd, J = 7.5, 8 Hz, 1H, H_6-benzofuran), 7.64 (s, 1H, H_3-benzofuran), 7.67 (d, J = 8 Hz, 1H, H_7-benzofuran), 7.80 (d, J = 7.5 Hz, 1H, H_4-benzofuran), 8.13 (dd, J = 6.5, 7 Hz, 1H, H_c-pyridine), 8.57 (d, J = 7.5 Hz, 1H, H_d-pyridine), 9.10 (d, J = 6 Hz, 1H, H_b-pyridine), 9.21 (s, 1H, H_a-pyridine), 9.56 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 20.66, 63.05, 110.20, 111.75, 122.80, 123.72, 127.03, 127.94, 128.12, 128.77, 128.89, 129.60, 131.26, 135.24, 138.92, 140.52, 143.33, 144.50, 148.87, 154.26, 158.23, 158.59.

4.1.4.4 *3-((Benzofuran-2-carboxamido)methyl)-1-(2-nitrobenzyl)pyridin-1-ium bromide* (**6d**)

White powder; 0.25 g; yield: 53%; mp 226-228 ˚C ; IR (KBr): 3239, 3027 (N-H), 1657 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.71 (d, J = 6 Hz, 2H, CH_2-NH), 6.24 (s, 2H, -CH_2N), 7.14 (d, J = 8 Hz, 1H, H_7-benzofuran), 7.36 (t, J = 7.5 Hz, 1H, H_5-benzofuran), 7.49 (t, J = 7.5 Hz, 1H, H_6-benzofuran), 7.60 (s, 1H, H_3-benzofuran), 7.66 (d, J = 8.5 Hz, 1H, H_4-benzofuran), 7.71-7.82 (m, 3H, H aromatic), 8.21 (dd, J = 6.5, 7 Hz, 1H, H_c-pyridine), 8.26 (d, J = 8 Hz, 1H, H aromatic), 8.66 (d, J = 8 Hz, 1H, H_d-pyridine), 9.01 (d, J = 5.5 Hz, 1H, H_b-pyridine), 9.07 (s, 1H, H_a-pyridine), 9.49 (1H, bs, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 60.61, 110.14, 111.79, 122.88, 123.74, 125.44, 127.01, 127.94, 128.18, 128.93, 134.78, 140.46, 143.83, 144.12, 145.19, 147.51, 148.41,154.25, 158.60.

4.1.4.5 *3-((Benzofuran-2-carboxamido)methyl)-1-(4-nitrobenzyl)pyridin-1-ium bromide* (**6e**)

White crystal; 0.21 g, yield: 44 %; mp 195-198 ˚C; IR (KBr): 3493 (N-H), 1646 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.70 (d, 2H, J = 6 Hz, CH_2-NH), 6.06 (s, 2H, -CH_2N), 7.35 (t, J =7.5 Hz, 1H, H_5-benzofuran), 7.5 (dd, 1H, J = 7.5 , 8 Hz, H_6-benzofuran), 7.64-7.67 (m, 2H, H_3- benzofuran, H_7-benzofuran), 7.77-7.80 (m, 3H, H aromatic, H_4-benzofuran), 8.20 (t, J = 7 Hz, 1H, H_c-pyridine), 8.28 (d, J = 8.5 Hz, 2H, H aromatic), 8.62

(d, *J* = 7.5 Hz, 1H, H_d-pyridine), 9.18 (d, *J* = 6 Hz, H_b-pyridine), 9.27 (s, 1H, H_a-pyridine), 9.55 (t, *J* = 5.5 Hz, 1H, N-H); MS *m/z* (%): 388 (M^+, <1), 252 (51.3), 145.1 (57.4), 136.1 (68.4), 118 (29.6), 108.1 (3.8), 89.1(100), 63.1(39.5), 51.1(15.7),

4.1.4.6 *3-((Benzofuran-2-carboxamido)methyl)-1-(4-fluorobenzyl)pyridin-1-ium bromide* (**6f**)

Cream powder; 0.28 g, yield: 62 %; mp 149-151 ˚C; IR (KBr): 3423 (N-H), 1662 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.69 (d, *J* = 6 Hz, 2H, CH_2-NH), 5.89 (s, 2H, -CH_2N), 7.28 (t, *J* = 8 Hz, 2H, H aromatic), 7.35 (t, *J* = 7.5 Hz, 1H, H_5-benzofuran), 7.49 (t, *J* =7.5 Hz, 1H, H_6-benzofuran), 7.63-7.68 (m, 4H, H_3- benzofuran, H_2-benzofuran and H aromatic), 7.80 (d, *J* = 7.5 Hz, 1H, H_4-benzofuran), 8.15 (t, 1H, *J* = 6 Hz, H_c-pyridine), 8.58 (d, *J* = 7.5 Hz, 1H, H_d-pyridine), 9.15 (d, *J* = 6 Hz, 1H, H_b-pyridine), 9.27 (s, 1H, H_a-pyridine), 9.51 (t, *J* = 6 Hz, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz) 62.26, 110.28, 111.77, 115.96 (d, $J_{C\text{-}F}$ = 19 Hz), 122.85, 123.84, 127.01, 128.01, 128.23, 130.34, 131.48, 140.48, 143.25, 143.37, 144.4, 148.4, 154.27, 158.62, 162.56 (d, $J_{C\text{-}F}$ = 245 Hz).

4.1.4.7 *3-((Benzofuran-2-carboxamido)methyl)-1-(2,4-dichlorobenzyl)pyridin-1-ium chloride* **(6g)**

Cream powder; 0.322 g, yield: 66 % ; mp 210-212 ˚C; IR (KBr): 3418(N-H), 1654 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.69 (d, *J* = 6 Hz, 2H, CH_2-NH), 6.02 (s, 2H, -CH_2N), 7.35 (dd, *J* = 6, *J* = 7.5 Hz, 1H, H_5-benzofuran), 7.48 (dd, *J* = 6.5, 8.5 Hz, 1H, H_6-benzofuran), 7.60-7.64 (m, 2H, H aromatic), 7.66 (d, *J* = 8.5 Hz, 1H, H_7-benzofuran), 7.69 (s, 1H, H_3-benzofuran), 7.76-7.80 (m, 2H, H_4-benzofuran, H aromatic), 8.16 (t, *J* = 6 Hz, 1H, H_c-pyridine), 8.64 (d, *J* = 7.5 Hz, 1H, H_d-pyridine), 9.02 (d, *J* = 6 Hz, 1H, H_b-pyridine), 9.14 (s, 1H, H_a-pyridine), 9.71 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz) 60.48, 110.20, 110.91, 111.76, 122.82, 123.69, 127.00, 127.86, 128.09, 129.52, 130.44, 133.04, 134.43, 135.19, 140.45, 143.77, 145.15, 148.42, 154.26, 158.59; MS *m/z* (%): 411 (M^+, <1), 252.2 (83.4), 235.2 (37.3), 196 (19), 159.1 (100), 145 (64), 107.1 (44.4), 89.1 (62.4), 63.1 (26.8), 51.1 (8.9).

4.1.4.8 *3-((Benzofuran-2-carboxamido)methyl)-1-(2-fluoro-6-nitrobenzyl)pyridin-1-ium bromide* **(6h)**

White powder; 0.20 g; yield: 41%; mp 230 ˚C (dec.); IR (KBr): 3239 (N-H), 1657 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.68 (d, *J* = 6 Hz, 2H, CH_2-NH), 6.16 (s, 2H, -

CH_2N), 7.36 (t, *J* = 6 Hz, 1H, H_5-benzofuran), 7.50 (t, *J* = 7.6 Hz, 1H, H_6-benzofuran), 7.59 (s, 1H, H_3-benzofuran), 7.66 (d, *J* = 8.5 Hz, 1H, H_7-benzofuran), 7.80 (d, *J* = 7.5 Hz, 1H, H_4-benzofuran), 7.85-7.88 (m, 2H, H aromatic), 8.12-8.15 (m, 2H, H_c-pyridine, H aromatic), 8.60 (d, *J* = 8 Hz, 1H, H_d-pyridine), 8.99 (d, *J* = 6 Hz, 1H, H_b-pyridine), 9.01 (s, 1H, H_a-pyridine), 9.46 (t, *J* = 6 Hz, 1H, N-H); ^{13}C NMR (DMSO-d_6,125 MHz) 53.86, 110.27, 111.76, 115.00, 122.03, 123.86, 127.01, 127.75, 127.85(d, $^2J_{C\text{-}F}$ = 25), 133.17, 140.22, 143.13, 143.46, 144.94, 148.39, 149.49, 154.26, 158.58, 161.45(d, $^1J_{C\text{-}F}$ = 250).

4.1.4.9 *1-Benzyl-3-((7-methoxybenzofuran-2-carboxamido)methyl)pyridin-1-ium chloride* **(6i)**

White powder; 0.140 g; yield: 31 %; mp 174-176 ˚C; IR (KBr): 3591 (N-H), 1652 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz): 3.96 (s, 3H, -OCH_3), 4.68 (d, *J* = 6 Hz, 2H, CH_2-NH), 5.93 (s, 2H, -CH_2N), 7.10 (t, *J* = 8 Hz, 2H, H_5-benzofuran, H aromatic), 7.23-7.33 (m, 6H, H_4- benzofuran, H_6-benzofuran and H aromatic), 7.58 (s, 1H, H_3-benzofuran), 8.16 (t, *J* = 7 Hz, 1H, H_c-pyridine), 8.61 (d, 1H, *J* = 8 Hz, H_d-pyridine), 8.94 (d, *J* = 6 Hz, 1H, H_b-pyridine), 9.04 (s, 1H, H_a-pyridine), 9.42 (t, *J* = 6 Hz, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz) 16.74, 55.78, 61.55, 108.92, 110.6, 114.48, 124.45, 126.66, 128.02, 128.21, 128.58, 128.93, 129.32, 130.88, 132.17, 136.89, 140.51, 143.58, 143.70, 144.83, 145.23, 148.46, 158.48.

4.1.4.10 *3-((7-Methoxybenzofuran-2-carboxamido)methyl)-1-(3-methoxybenzyl) pyridin-1-ium chloride* **(6j)**

White crystal; 0.183 g; yield: 42 %; mp>230 ˚C; IR (KBr): 3424 (N-H), 1622 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 2.28 (s, 3H, -OCH_3), 3.95 (s, 3H, -OCH_3 benzofuran), 4.68 (d, *J* = 5.5 Hz, 2H, CH_2-NH), 5.96 (s, 2H, -CH_2N), 7.09-7.12 (2H, bs, H_4, H_6-benzofuran), 7.25-7.31 (5H, m, H_5-benzofuran, H aromatic), 7.61 (s, 1H, H_3-benzofuran), 8.17 (bs, 1H, H_c-pyridine), 8.61 (d, *J* = 5.5 Hz, 1H, H_d-pyridine), 8.99 (bs, 1H, H_b-pyridine), 9.08 (s, 1H, H_a-pyridine), 9.49 (bs, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 18.83, 55.80, 61.49, 108.89, 110.67, 114.50, 114.80, 124.65, 126.66, 128.13, 128.57, 128.91, 128.94, 129.31, 130.88, 132.30, 136.91, 140.48, 143.49, 143.63, 144.80, 145.22, 148.44, 158.50.

4.1.4.11 *3-((7-Methoxybenzofuran-2-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide* **(6k)**

White powder; 0.200 g; yield: 43 % ; mp 174-176 ˚C; IR (KBr): 3592 (N-H), 1653 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) 2.29 (s, 3H, -CH_3), 3.96 (s, 3H, -OCH_3), 4.69 (d, J = 5.5 Hz, 2H, CH_2-NH), 5.97 (s, 2H, -CH_2N), 7.08-7.13 (m, 2H, H_4, H_6-benzofuran), 7.27-7.33 (m, 5H, H_5-benzofuran, H aromatic) ,7.62 (s, 1H, H_3-benzofuran), 8.18 (t, J = 7 Hz, 1H, H_c-pyridine), 8.62 (d, J = 8 Hz, 1H, H_d-pyridine), 8.99 (d, J = 6 Hz, 1H, H_b-pyridine), 9.09 (s, 1H, H_a-pyridine), 9.49 (bs, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 18.77, 55.80, 61.50, 108.91, 110.57, 110.66, 114.48, 124.59, 126.63, 128.0, 128.20, 128.57, 128.95, 129.30, 130.86, 132.19, 136.90, 140.47, 143.58, 143.70, 144.83, 145.22, 148.44, 158.48.

4.1.4.12 *1-(2,4-Dichlorobenzyl)-3-((7-methoxybenzofuran-2-carboxamido)methyl) pyridin-1-ium chloride* **(6l)**

White crystal; 0.190 g; yield: 40 %; mp>230 ˚C; IR (KBr): 3277 (N-H), 1654 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) 3.74 (3H, s, -OCH_3), 4.69 (d, J = 5.5 Hz, 2H, CH_2-NH), 5.83 (2H, s, -CH_2N), 6.97 (d, J =7.8 Hz, 1H, H_6-benzofuran), 7.09 (d, J = 7.4 Hz, 1H, H_4-benzofuran), 7.16 (s, 1H, H_3-benzofuran), 7.49 (1H, t, J = 8 Hz, H_5-benzofuran), 7.61 (s, 1H, H aromatic), 7.68 (d, J = 8 Hz, 1H, H aromatic), 7.80 (d, J = 8 Hz, 1H), 8.16 (t, J = 6 Hz, 1H, H_c-pyridine), 8.58 (d, J = 7.9 Hz, 1H, H_d-pyridine), 9.14 (d, J = 5.7 Hz, 1H, H_b-pyridine), 9.24 (s, 1H, H_a-pyridine), 9.50 (1H, t, NH); ^{13}C NMR (DMSO-d_6, 125 MHz) 55.25, 63.21, 110.27, 111.81, 114.55, 114.77, 120.74, 122.93, 123.87, 127.07, 127.17, 128.16, 130.43, 130.53, 135.57, 140.52, 143.25, 143.41, 144.63, 148.46, 154.28, 158.64, 159.62.

4.1.4.13 *3-((7-Methoxybenzofuran-2-carboxamido) methyl) 1-(4-bromobenzyl)-pyridin-1-ium bromide* (**6m**)

Yellow crystal; 0.238 g; yield: 44 %; 127-130 ˚C; IR (KBr): 3609 (N-H), 1656 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz): 3.96 (s, 3H, -OCH_3), 4.67 (d, J = 5 Hz, 2H, CH_2-NH), 5.86 (s, 2H, -CH_2N), 7.10 (d, J = 7.5 Hz, 1H, H_4-benzofuran), 7.26 (m, 1H, H_5-benzofuran), 7.32 (d, J = 7.5 Hz, 1H, H_6-benzofuran), 7.50 (d, J = 8 Hz, 2H, H aromatic), 7.58 (s, 1H, H_3-benzofuran), 7.65 (d, J = 8 Hz, 2H, H aromatic), 8.15 (t, J = 6 Hz, 1H, H_c-pyridine), 8.58 (d, J = 8 Hz, 1H, H_d-pyridine), 9.11 (d, J = 6.5 Hz, 1H, H_b-pyridine), 9.20 (s, 1H, H_a-pyridine), 9.39 (t, J = 6.5 Hz, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 15.11, 55.78, 62.47, 64.84, 108.93, 110.57, 110.68, 114.54, 122.86, 124.66, 128.06, 128.26, 128.57, 130.98, 131.12, 132.09, 133.49, 140.58, 143.45, 143.69, 144.76, 145.52, 148.47, 158.48; MS: *m/z* (%) 43.1 (100), 53.1 (75.3), 63.1 (74.1), 69.1 (30.2), 77.1 (37.2), 83.1 (20.6), 89.1 (81.3),

97.1 (14.6), 107.1 (43.8), 119.1 (19.1), 127 (18.4), 169 (86.9), 175.1 (25.6), 192 (3.9), 282 (21.9).

4.1.4.14 *4-((Benzofuran-2-carboxamido) methyl)-1-(2-bromobenzyl)pyridin-1-ium bromide* **(6n)**

White powder; 0.198 g; yield: 47%; mp 175-178 ˚C ; IR (KBr): 3372 (N-H), 1650 (C=O), 1599 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.76 (d, 2H, J = 5 Hz, CH_2-NH), 5.82 (2H, s, -CH_2N), 7.36 (dd, J = 6, 7.5 Hz, 1H, H_5-benzofuran), 7.53-7.49 (m, 3H, H_7-benzofuran, H aromatic), 7.62-7.71 (m, 4H, H_3-benzofuran, H_6-benzofuran and H aromatic), 7.81 (d, J = 8 Hz, 1H, H_4-benzofuran), 8.09 (d, J = 6 Hz, 2H, H_b-pyridine, H_d-pyridine), 9.08 (d, J = 6 Hz, 2H, H_a-pyridine, H_c-pyridine), 9.80-9.83 (m, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 41.78, 61.82, 110.57, 111.79, 122.82, 123.90, 126.02, 126.08, 126.22, 127.01, 127.10, 130.99, 131.11, 132.12, 132.23, 133.58, 144.43, 144.51, 148.30, 154.30, 158.69, 159.68.

4.1.4.15 *4-((Benzofuran-2-carboxamido)methyl)-1-(4-bromobenzyl)pyridin-1-ium bromide* **(6o)**

Cream powder; 0.246 g; yield: 49 %; mp 165-167 ˚C; IR (KBr): 3375 (N-H), 1657 (C=O), 1600 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6, 500 MHz) 4.75 (d, J = 5.5 Hz, 2H, CH_2-NH), 5.84 (s, 2H, -CH_2N), 7.35 (t, J = 8 Hz, 1H, H_5-benzofuran), 7.47-7.52 (m, 3H, H_7-benzofuran, H aromatic), 7.64-7.69 (m, 4H, H_3-benzofuran, H_6-benzofuran and H aromatic), 7.80 (d, J = 7.5 Hz, 1H, H_4-benzofuran), 8.09 (d, J = 6 Hz, 2H, H_b-pyridine, H_d-pyridine), 9.14 (d, J = 7.5 Hz, 2H, H_a-pyridine, H_c-pyridine), 9.62-9.64 (m, 1H, N-H); ^{13}C NMR (DMSO-d_6, 125 MHz) 41.76, 61.74, 110.38, 111.79, 122.81, 123.73, 123.76, 126.02, 126.21, 127.00, 127.12, 131.15, 131.18, 132.09, 133.58, 144.44, 145.28, 148.28, 148.31, 154.29, 158.69, 159.66.

4.2 Molecular modeling

The crystal structures of BChE enzyme complexed with tacrine (PDB Code: 4BDS) and AChE enzyme complexed with donepezil (PDB Code: 4EY70) were taken from RCSB protein databank (http://www.pdb.org). Since BChE and AChE in PDB are not complexed with any of the understudy molecules, in docking step their original ligands BChE and AChE were removed and then compounds in this study data set were docked in the active sites of BChE and AChE one by one. Ligands were sketched and minimized in SYBYL 7.3 molecular modeling package (Tripos Inc., St. Louis, USA) running on a Red Hat Linux

workstation 4.7. The resultant structures were imported into Discovery Studio 4.1 (Accelrys Inc, San Diego, CA, USA), CHARMm force field was applied and Momany-Rone option was used to calculate partial charges [52]. The 1000 steps of steepest descent minimization with a RMS gradient tolerance of 3, followed by conjugate gradient minimization was performed. To prepare the structure of BChE and AChE, protein preparation protocol was used. In this step, CHARMm force field was applied, hydrogen atoms were added, all water molecules were removed and pH of protein was adjusted to near neutral 7.4. All molecules were again minimized *in-situ* with Smart Minimizer option [53]. The binding sites of proteins were defined as a sphere with a radius of 10Å around the bounded ligand which confirms the movement of the ligand atoms and the side-chains of the residues of the protein within 10 Å from the center of the active site. Then bounded ligands BChE and AChE were removed from the active site. Other parameters were set by default protocol settings. GOLD program was used to dock the compounds into receptors [54].

4.3 Anticholinesterase assay

According to the spectrophotometric method of Ellman [47], the target compounds (**6a-6o**) revealed anticholinesterase activity against butyrylcholinesterase from equin serum and acetylcholinestrase from *electrophorus electricus* (AChE, *eel*). Each compound was tested in five different concentrations against the enzyme to achieve a range of inhibition between 20-80 %. After five minutes incubation of a mixture containing phosphate buffer (0.1 M, pH=8.0, 2 mL), acetylcholinesterase or butyrylcholinesterase (20 µL), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB, 60 µL) and compounds solution (30 µL), acetylthiocholine iodide or butyrylthiocholine iodide (20 µL) was added as substrate and the change of absorbance was recorded at 412 nm for two minutes using a Synergy Biothech® multiplate reader. The IC_{50} values were obtained from Log concentration vs. percent of inhibition curves. To guarantee the optimum result, each experiment was accomplished in triplicate.

4.4 Determination of the inhibitory potency on $A\beta_{1-42}$ self-aggregation

Inhibitory properties of compounds on self-aggregation of amyloid β protein 1-42 was determined using a thioflavin T (ThT)-based fluorescence assay [55]. Amyloid β protein 1-42 (Sigma A9810) was dissolved in Phosphate Buffer Saline pH 7.4 (PBS, HyClone Thermo Scientific) containing 1% ammonium hydroxide. 50 µM Amyloid β protein 1-42 was incubated for 72 hours at 37 °C for prefibrillation .

For the assay, Aβ1-42 (10 µl) were added to 0.05 M KP buffer pH 7.4 and incubated at 37˚C for 48 h in the absence and presence of compounds (100 µM). Incubated mixture (100 µL) was mixed with 50 µL of thioflavin T (ThT, 200 µM) in 50 mM glycine-NaOH buffer (pH 8.5). The ThT excitation/emission was measured at 448 nm/490 nm at 48 hours using a SpectraMax® Microplate Reader. Rifampicin (100 µM, Sigma R-3501) and Donepezil (100 µM, Sigma D-6821) were also tested as reference agents .

Self-aggregation due to the presence of the tested compounds (**6h** and **6k**) were determined by the following calculation: [(IFi/IFo) × 100] where IFi and IFo are the fluorescence intensities obtained for Aβ in the presence and in the absence of inhibitors.

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Graphical Abstract

Synthesis and anticholinesterase inhibitory activity of new 2-benzofuran carboxamide-benzylpyridinum salts

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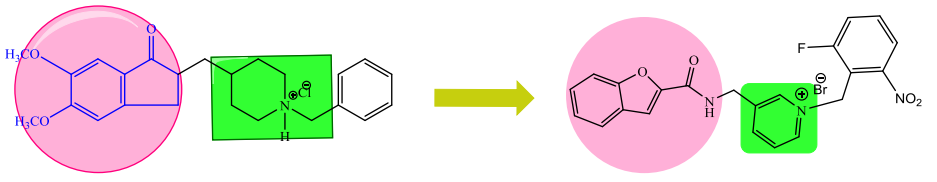

donepezil hydrochloride
BChE IC_{50}=54 μM
Inhibition of Aβ self-aggregation (%)=22.0±5.4

target compound(**6h**)
BChE IC_{50}=0.054 μM
Inhibition of Aβ self-aggregation (%)=33.1±11.2

Highlights

- Benzofuran-2-carboxamide-pyridinium salts were evaluated as anticholinesterase agents.
- Compound **6h** showed good BChE and Aβ-self aggregation inhibitory activity.
- Docking studies indicate the interactions of **6h** resulted in stabilization of it.