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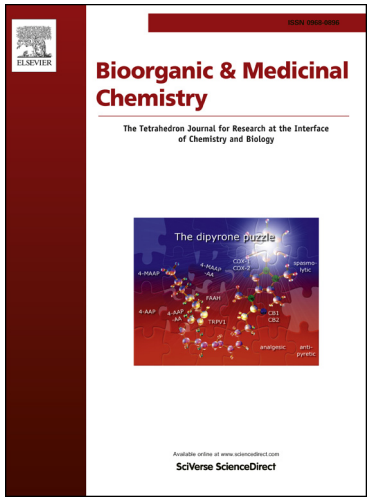

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Design, Synthesis and Evaluation of Novel 7-Aminoalkyl-Substituted Flavonoid Derivatives with Improved Cholinesterase Inhibitory Activities

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ABSTRACT

A novel series of 7-aminoalkyl-substituted flavonoid derivatives **5a**-**5r** were designed, synthesized and evaluated as potential cholinesterase inhibitors. The results showed that most of the synthesized compounds exhibited potent acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities at the micromolar range. Compound 2-(naphthalen-1-yl)-7-(8-(pyrrolidin-1-yl) octyloxy)-4H-chromen-4-one (**5q**) showed the best inhibitory activity (IC_{50}, 0.64 μM for AChE and 0.42 μM for BChE) which were better than our previously reported compounds and the commercially available cholinergic agent Rivastigmine. The results from a Lineweaver-Burk plot indicated a mixed-type inhibition for compound **5q** with AChE and BChE.

Furthermore, molecular modeling study showed that **5q** targeted both the catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE. Besides, these compounds (**5a-5r**) did not affect PC12 and HepG2 cell viability at the concentration of 10 μM. Consequently, these flavonoid derivatives should be further investigated as multipotent agents for the treatment of Alzheimer's disease.

KEYWORDS

Flavonoid derivatives

Cholinesterase

Anti-Alzheimer agent

Molecular modeling

Cytotoxicity

1. Introduction

Alzheimer's disease (AD) represents a progressive neurodegenerative brain disorder that is characterized by dementia, memory loss and other cognitive impediments, particularly among elderly individuals.[1] A variety of factors are considered key pathological hallmarks of AD, such as low levels of acetylcholine, β-amyloid (Aβ) deposits and oxidative damage.[2] These factors provide a basis for cholinergic, amyloid and oxidative stress hypotheses potentially explaining the origin of Alzheimer's disease.[3, 4]

Current treatment options for AD mainly focus on improving the cholinergic neurotransmission in the brain, which is mostly based on the 'cholinergic hypothesis'.[5] Various approaches have explored this hypothesis, however, cholinesterase inhibitors were the first and, to date, are the only agents that offer promising results in the treatment of AD.[6] Currently, four AChE inhibitors have been approved by the European and US regulatory authorities: Tacrine, Donepezil, Rivastigmine and Galantamine. These inhibitors exhibit beneficial effects on cognitive, functional and behavioural symptoms of AD. However, accumulative side effects or demerits such as hepatotoxicity, periphery side effect, short half-life, or gastrointestinal tract disorders diminish the overall clinical applicability.[8] Therefore, the development of efficient AChE inhibitors with low toxicity still continues to be a critical goal in AD therapy.

Two types of cholinesterase (ChE) species are found in the central nervous system, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), with both species able to hydrolyze acetylcholine. In the healthy brain, AChE predominates over BChE activity, however, during the early development of AD, AChE activity decreases in certain brain areas, whereas BChE levels remain unchanged or may even rise with disease progression.[9] Furthermore, BChE may also participate in the

aggregation of Aβ. As a consequence, a good balance in the AChE and BChE inhibition profiles may provide additional benefits in the treatment of AD.

Flavonoids represent a class of polyphenolic compounds found in plants, such as fruit, vegetables, tea and soybeans.[11] A wide range of physiological and biochemical functions are displayed by flavonoids, including UV-protection, anti-tumor, antioxidant, neuroprotection and anti-inflammatory characteristics.[12,13] In general, flavonoids feature low toxicity and have received much attention by a variety of research fields in medicinal chemistry.[14] However, not many articles have been reported focusing on new flavonoid derivatives as anti-AD agents. In recent years, only a few research groups have selected flavonoids as lead compounds in an effort to optimize the structures for the development of ChEs inhibitors as a treatment option for AD (Fig. 1, compounds A, B and C).[15-17]

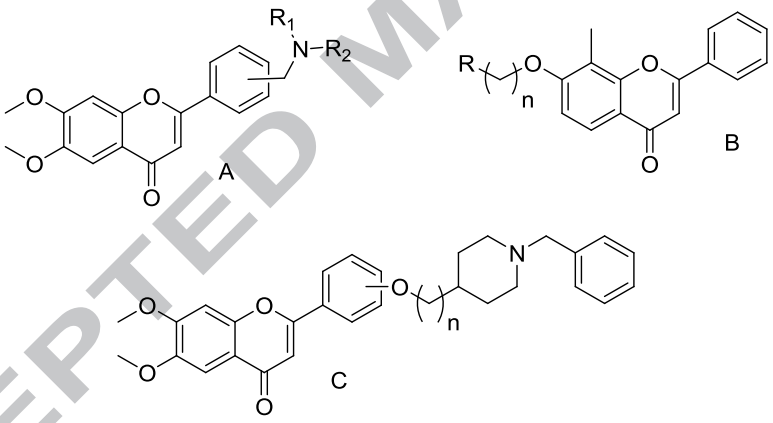


Figure 1. Flavonoids as new drugs or imaging agents in the treatment of AD

For many years, our group has focussed on the synthesis of flavonoids and the further development of ChE inhibitors.[18,19] We have previously studied a series of N, N-dimethylated flavonoid derivatives as multifunctional ChEs inhibitors and found that these derivatives feature a high inhibitory potency for both AChE and BChE (Fig. 2).[20] We now focus our interests on the development of novel flavonoid derivatives with high potency to find use as ChE inhibitors. In the present study, a series of new flavonoid derivatives with B ring modifications were designed, synthesized and evaluated for their biological activity, including cytotoxicity studies and investigations on the inhibition characteristics of

cholinesterase.

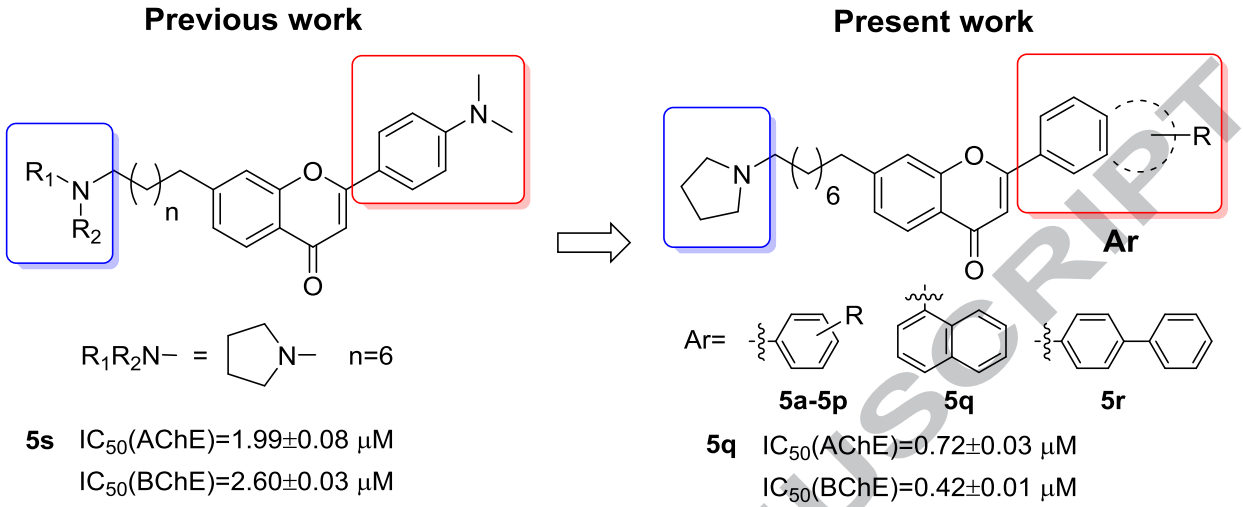
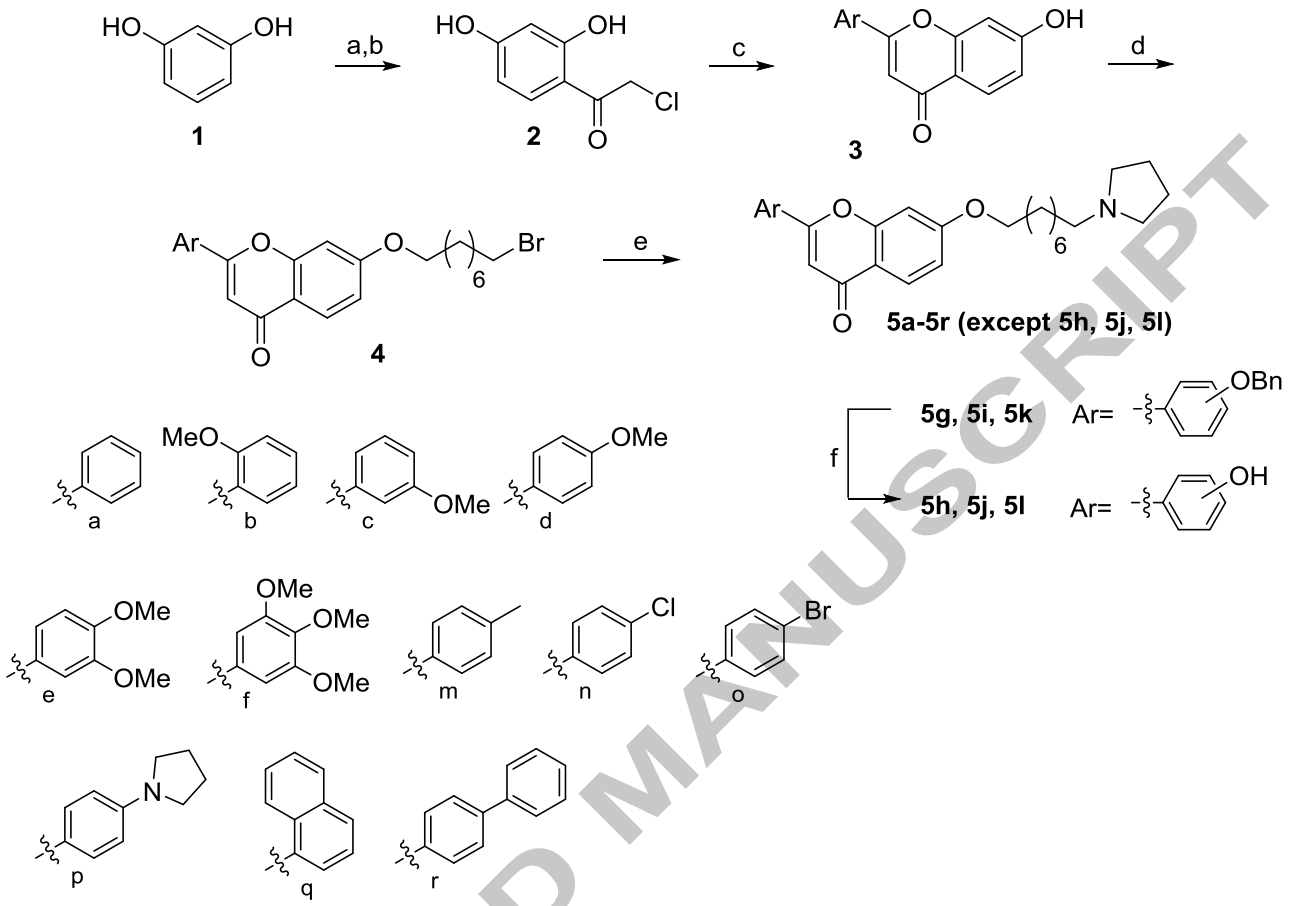


Figure 2. Our previous work and present work

2. Results and discussion

2.1 Chemistry

The synthetic route to the target compounds (**5a**–**5r**) starting from commercially available 1,3-benzenediol (**1**) was shown in Scheme 1. Condensation of 1,3-benzenediol (**1**) with chloroacetonitrile in the presence of $ZnCl_2$ in ether was followed by hydrolysis in water and produced ketone in 87% yield. The ketone **2** was treated with aryl aldehydes in the presence of excess NaOH in EtOH, was followed by acidification with aqueous HCl and resulted in the flavonoids **3** in satisfactory yield.[20] The alkylation of **3** with 1,8-dibromooctane in acetone provided **4** in good yields. Finally, the reaction of **4** with pyrrolidine produced the targets compounds **5a-5r** (except **5h**, **5j** and **5l**) in 42-65% yields. **5h**, **5j** and **5l** were obtained by deprotection from compounds **5g**, **5i** and **5k**. The structures of the target compounds were confirmed by ^{1}H NMR, ^{13}C NMR and MS.



Scheme 1. Synthesis of flavonoid derivatives. Reagents and conditions: (a) $ClCH_2CN$, $ZnCl_2$, EtOEt, HCl, 0 °C; (b) HCl, H_2O, reflux; (c) aryl aldehyde, 10% NaOH, rt, EtOH; (d) 1,8-dibromooctane, K_2CO_3, acetone, reflux; (e) pyrrolidine, K_2CO_3, CH_3CN, 60 °C, (f) HOAc/HCl, 80 °C.

2.2 In vitro inhibition studies on AChE and BChE

The synthesized compounds **5a**-**5r** were evaluated for their *in vitro* inhibitory activities towards AChE and BChE using the Ellman method.[21] The results were subsequently compared to the commercially available standard cholinergic agent Rivastigmine. The anti-cholinesterase activities and their selectivity indexes are summarized in Table 1.

The IC_{50} values obtained reveal that all of the tested compounds **5a-5r** exhibit very potent inhibitory activities (IC_{50} values = 0.64-4.80 μM), far superior to the standard drug Rivastigmine (IC_{50} values = 6.35 μM). Particularly the 2-methoxy derivative **5b** appears to be the most potent compound against

AChE, with an IC_{50} value of 0.64 µM. Concurrently, this compound proves to be about 10-fold more potent than Rivastigmine and 3-fold more potent than our previously reported compound (**5s**). Moreover, the 3-methoxy, 2,3-dimethoxy and the 1-naphthyl derivatives **5c**, **5e** and **5q** with IC_{50} values of 0.67, 0.83 and 0.72 µM, respectively, also feature high activities against AChE. Comparing the unsubstituted compound **5a** to the *ortho*-, *meta*- or *para*-substituted analogs **5b**, **5c** and **5d** shows that the introduction of a methoxy group in 2- or 3- but not in 4-position on the B ring in the flavonoid scaffold significantly improved the inhibitory activity against AChE. Furthermore, a bigger or smaller group such as a benzyloxy group (**5g** and **5i**) or a hydroxy group (**5h** and **5j**) in 2- or 3-position leads to a decrease in the AChE inhibitory activity. Comparing the the *para*-substituted analogs **5d**, **5k-5p**, **5r** and **5s**, revealed that the introduction of a larger group in 4-position (**5k**, **5o** and **5r**) does in fact not increase the AChE inhibitory activity.

The observed IC_{50} values of the target compounds against BChE indicate that all compounds with the exception of **5b**, **5h** and **5q** were determined to be less potent than the standard drug Rivastigmine. Most impressively, the anti-BChE activity of the most potent compound **5q** was determined to be 4 times higher than that of Rivastigmine and 6 times higher than our previously reported compound (**5s**). The 2-methoxy and 2-hydroxy derivatives **5b** and **5h** with IC_{50} values of 0.76 and 1.26 µM, respectively, also showed significant anti-BChE activities. However, most of the substituted benzyl derivatives were not less potent than the substituted analog **5a** against BChE. Taken in concert, these results demonstrate that an unsuitable substituent and/or functional group on the B ring results in a detrimental effect on the anti-BChE activity.

As calculated in Table 1, most of compounds (except **5h** and **5q**) showed a high selectivity for AChE (SI > 1) over BChE. The most active species (**5q**) against BChE features a similar selectivity index (SI = 0.58) as Rivastigmine (SI = 0.27).

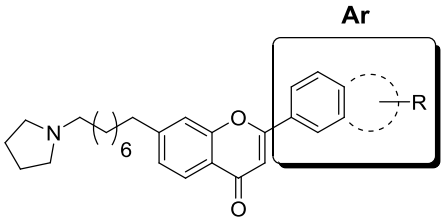
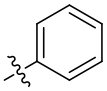
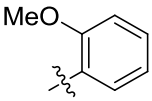
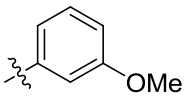
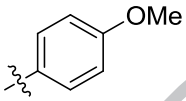
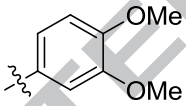
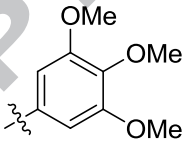
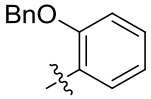
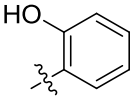
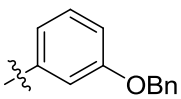
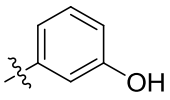
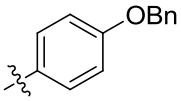
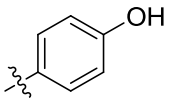
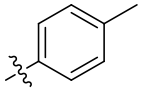
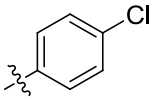
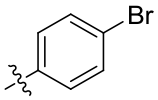
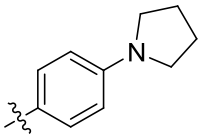
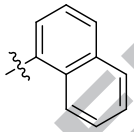
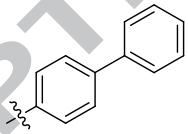
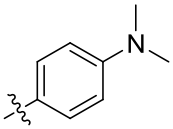


Table 1. Inhibition of ChE activity and selectivity index.

Compds	Ar	IC_{50} [a] for AChE (μM)	IC_{50} [b] for BChE (μM)	SI[c]
5a		1.09±0.03	2.24±0.01	2.05
5b		0.64±0.04	0.76±0.02	1.19
5c		0.67±0.05	2.29±0.04	3.42
5d		1.70±0.05	5.42±0.13	3.19
5e		0.83±0.01	3.43±0.22	4.13
5f		1.08±0.02	2.20±0.06	2.04
5g		2.01±0.03	5.26±0.21	2.62
5h		3.07±0.21	1.26±0.01	0.41
5i		1.58±0.12	>25	>15.8
5j		1.12±0.05	4.92±0.67	4.39

5k		1.90±0.13	>25	13.16
5l		1.21±0.08	4.58±0.08	3.79
5m		1.25±0.01	1.92±0.003	1.54
5n		1.12±0.02	3.46±0.23	3.09
5o		2.15±0.12	10.25±0.27	4.77
5p		1.38±0.11	4.10±0.43	2.97
5q		0.72±0.03	0.42±0.01	0.58
5r		4.80±0.21	>25	5.21
5s [d]		1.99±0.08	2.60±0.03	1.31
Rivastigmine	-	6.35±1.50	1.71±0.09	0.27

[a] AChE from *electric eel*; IC_{50}, 50% inhibitor concentration (means ± SEM of three experiments).

[b] BChE from *equine serum*; IC_{50}, 50% inhibitor concentration (means ± SEM of three experiments).

[c] SI: Selectivity Index =IC_{50} (BChE)/ IC_{50} (AChE)

[d] Data of ChEs inhibition were taken from ref. [20].

2.3 Kinetic characterization of ChE inhibition

The compound that appeared to be the most successful at inhibiting ChEs was **5q**. We further

investigated **5q** using graphical analysis of steady state inhibition data (Fig. 2, A). The Lineweaver–Burk plots showed both increasing slope (decreased V_{max}) and increasing intercept (higher K_m) for higher inhibitor concentrations, indicating a mixed-type inhibition. Replots of the slope versus concentration of compound **5q** gave an extimate of the inhibition constant, K_i of 0.206 μM. This suggested that compound **5q** was able to bind both CAS and PAS of AChE and supported the results of the molecular modeling study. The same inhibition type between **5q** and BChE was found in graphical analysis (Fig. 2, B).

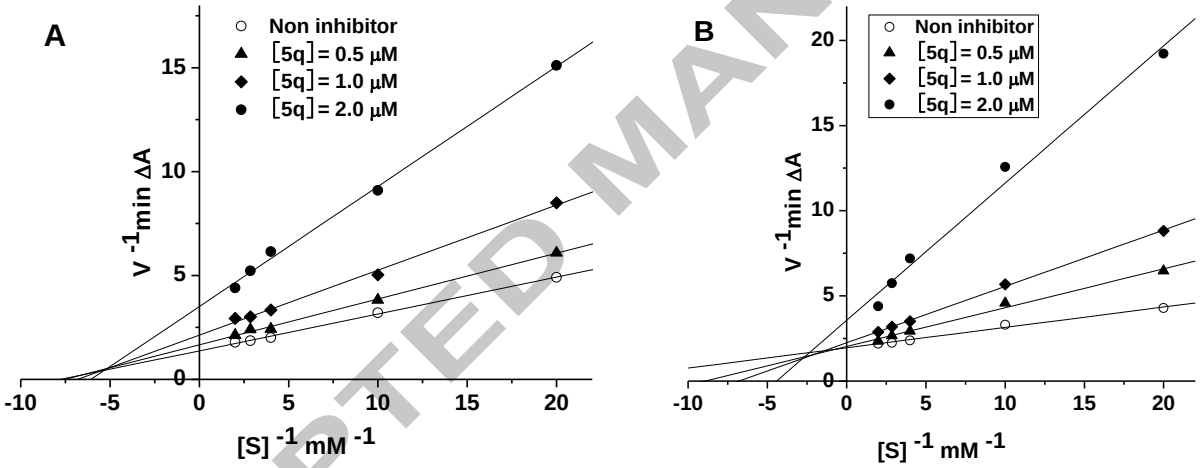


Figure 2. Lineweaver−Burk plot for the inhibition of AChE (A) and BChE (B) by compound **5q**.

2.4 Molecular modeling study

In order to obtain functional and structural insight into the binding interactions between compound **5q** and *Tc*AChE (PDB code: 1ACJ), molecular docking simulation was performed using Autodock 4.0 [22] and PyMOL software as shown in Figure 3. Regarding the docking studies, compound **5q** occupied the enzymatic CAS, mid-gorge and PAS, the charged nitrogen of pyrrolidine showed a cation-π interaction with Trp84 and Phe330, and a hydrogen bond (3.6 Å) was found with His440 at the CAS. At the PAS, the naphthalene ring of flavonoid stacked against the Trp279 through π-π stacking with a distance of 3.8

Å. The docking studies showed that compound **5q** exhibited a mixed type of inhibition, consistent with our kinetic analysis result. Since the crystal structure of BChE from equine serum has not been reported, so we did not perform the docking study for compound **5q** with BChE.

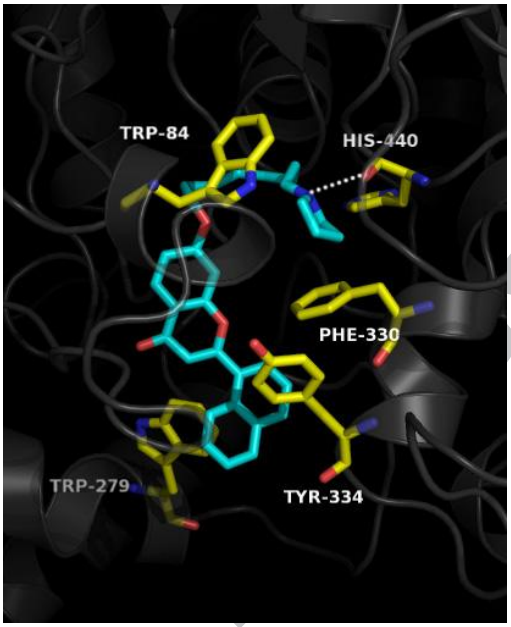


Figure 3. Docking model of **5q**-*Tc*AChE complex.

2.5. MTT assay of PC12 and HepG2 cells viability

To investigate the effect of the new compounds on cell viability, a MTT assay was conducted using PC12 and HepG2 cell lines. The cells were incubated for 48 hours with varying target compound concentrations (1 and 10 μM). At a concentration of 1 μM, all of the compounds were shown to be nontoxic and had no effect on the cell viability of PC12 and HepG2 cells. At a concentration of 10 μM, all of the compounds except for **5r** were shown to be nontoxic to PC12 cells, and most of the compounds showed no obvious effect on the cell viability of HepG2 cells.

Table 2. Inhibition rate (%)[a] of PC12 and HepG2 cells

Compd.	PC12		HepG2	
	1 μM	10 μM	1 μM	10 μM
5a	-2.83	1.37	11.31	8.97
5b	3.08	3.4	5.9	24.32

5c	3.53	-18.19	16.22	23.66
5d	-0.15	-6.72	6.69	21.48
5e	7.29	-2.94	13.48	76.04
5f	-4.47	-13.85	16.26	41.39
5g	0.47	22.86	19.73	34.08
5h	-7.71	6.61	5.92	60.58
5i	0.79	-2.53	6.63	30.60
5j	-4.43	-6.56	19.02	43.87
5k	-2.83	1.37	10.41	54.34
5l	0.70	7.42	18.52	21.39
5m	-0.55	-0.03	9.17	77.08
5n	6.27	-1.7	6.39	25.66
5o	2.50	-12.49	13.32	10.83
5p	0.29	-9.56	5.74	19.03
5q	3.00	9.01	4.42	18.9
5r	0.46	68.90	13.91	71.92
Tacrine	4.62	10.24	5.78	15.05

[a] The values are the mean ± SD of three independent experiments.

2.6. ADMET prediction

The in *silico* prediction of physicochemical, ADME and toxicity properties of compounds **5b** and **5q** were calculated using the ACD/Labs Percepta Platform [23] (License#58830) and results were compared to those obtained for donepezil and tacrine.

As demonstrated in Table 3, the druglikeness of compound **5b** and **5q** was similar to donepezil and different from tacrine, with no violations of Lipinsky's rule of 5 (except rotatable bonds). Regardless of the poor solubility, compounds **5b** and **5q** were showed to be highly or moderately permeable based on predicted Log P, permeability across Caco-2 monolayers (Pe) and human intestinal absorption (HIA) test. The drug safety profile of **5b** and **5q** was also projected using Program ACD/Percepta, based on probabilistic predictors. The metabolic stability, the inhibition of hERG (the human Ether-a-go-go-Related Gene) and the mutagenic profile were calculated and the results converted in the

so called classification scores (Table 4). As depicted in Table 3 compounds **5b** and **5q** were predicted as undefined (score > 0.33 and < 0.67) in HLM (human liver microsomes) and hERG, which were similar with donepezil and tacrine. However, tacrine was predictable to be mutagenic (score > 0.67), and compounds **5b** and **5q** were predicted as undefined (score > 0.33 and < 0.67).

Table 3. Physico-chemistry properties and ADMET profile of compounds **5b**, **5q**, donepezil and tacrine calculated using the Program ACD/Percepta.

Predicted properties	Compounds			
	5b	**5q**	Donepezil	Tacrine
MW (g/mol)	449.58	469.61	379.49	198.26
H-Donors	0	0	0	2
H-Acceptor	5	4	4	2
Rot. Bonds	12	11	6	0
Rings	4	5	4	3
Lipinski	1	1 violation	0	0
Solubility	0.05 mg/ml	0.02 mg/ml	6.43 mg/ml	6 mg/ml
Log P	5.77	7.05	4.23	2.86
Caco-2	$P_e=30\times10^{-6}$ cm/s	$Pe=2\times10^{-6}$ cm/s	$P_e=194\times10^{-6}$ cm/s	$P_e=63\times10^{-6}$ cm/s
PPB	99%	99%	95%	78%
CNS	-3.48	-4.57	-2.65	-2.59
HIA	100%	100%	100%	100%
HLM	0.62	0.57	0.54	0.52
AMES	0.43	0.44	0.26	0.85
hERG	0.58	0.54	0.53	0.45

Table 4. The meaning of classification score range values for ADMET properties using Program ACD/Percepta.

Classification scores	Predicted ADMET properties		
	HLM	hERG	AMES
≤ 0.33	Stable	Non-inhibitor	Non-mutagenic
>0.33 and ≤ 0.67	Undefined	Undefined	Undefined
> 0.67	Undefined	Inhibitor	Mutagenic

3. Conclusion

In conclusion, we have developed a series of 7-aminoalkyl-substituted flavonoid derivatives **5a-5r** as AChE and BChE inhibitors. These synthetic derivatives feature a higher inhibitory potency for both AChE and BChE compared to other compounds we published previously as well as Rivastigmine. Compounds **5b**, **5c**, **5e** and **5q** (IC_{50} < 1.0 μM) were found to exhibit the best AChE inhibitory activity. Compounds **5b** and **5q** (IC_{50} < 1.0 μM) exhibited the highest inhibitory effect for BChE. Kinetic analysis studies revealed that compound **5q** features a mixed-type inhibition activity on both AChE and BChE. Furthermore, molecular modeling studies indicate that compound **5q** is able to bind to both CAS and PAS in AChE. Moreover, at a concentration of 10 μM, most of these compounds demonstrate no obvious toxicity effects on the cell viability of PC12 and HepG2. Our results indicated this new type of flavonoid derivatives may provide a useful template for further optimization in the field of AD pharmacotherapy.

4. Experimental section

4.1 Chemistry

Melting points (mp) were determined using an X-6 hot stage microscope and were not corrected. ^{1}H and ^{13}C NMR spectra were recorded using TMS as the internal standard in $CDCl_3$ with a Bruker AV-300 spectrometer. MS spectra were recorded on a Shimadzu LCMS-2010A instrument with an ESI mass selective detector. Elemental analyses were performed on a Gmbe VarioEL Elemental Instrument. Flash column chromatography was performed with silica gel (200 - 300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd.

4.2 General procedures for the synthesis of intermediate 3

To a mixture of acetophenone **2** (3.0 mmol) and different benzaldehyde (3.0 mmol) in ethanol (25 mL)

was added 10% sodium hydrate aqueous (5 mL), the mixture was stirred for 24 h at room temperature. The solution was acidified with 1N HCl and filtered, and the filter cake was recrystallized from ethanol got **3** which was used without further purification.

4.3 General procedures for the preparation of intermediate 4

To a solution of intermediate **3** (2.0 mmol) and anhydrous K_2CO_3 (1.38 g, 10.0 mmol) in acetone (10 mL), 1, 8-dibromooctane (4.0 mmol) was added. After reflux for 10 h until the starting material **3** disappeared, the solvent was removed under vacuum, the residue was poured into water and extracted with EtOAc, the solution was dried over Na_2SO_4 and then concentrated, the compounds **4** were crystallized from ethanol which were used without further purification.

4.4 General procedures for the preparation of compounds 5a-5r (except 5h, 5j and 5l)

To a solution of **4** (0.5 mmol) in CH_3CN (10 mL) was added pyrrolidine (1.0 mmol) and anhydrous K_2CO_3 (2.5 mmol). After stirring at 60 °C for 8 h, the solvent was removed under vacuum, the mixture was diluted with $CHCl_3$ and then washed with water and brine. The organic layer was dried over Na_2SO_4, filtered, concentrated in vacuo, and purified by column chromatography with $CHCl_2$/MeOH/NH_4OH (30:1:0.5%) elution, and then crystallized from ethanol.

4.5 General procedures for the preparation of compounds 5h, 5j and 5i

Compounds **5g**, **5i** or **5k** (1 mmol) and a mixture of acetic acid (7 mL) and concentrated hydrochloric (3.7 mL) were heated on a steam bath for 1.5 h. The solution was brought to neutrality with NaOH and then diluted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, concentrated in vacuo, and purified by column chromatography with $CHCl_2$/MeOH (15:1) elution.

*4.5.1 2-phenyl-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5a**)*

Light yellow solid (56% yield), mp 96-98 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, J = 7.2 Hz, 2H), 7.71 (d, J = 8.3 Hz, 1H), 7.46 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.3 Hz, 1H), 6.83 (s, 1H), 6.80 – 6.71 (m,

2H), 4.08 (t, *J* = 6.5 Hz, 2H), 2.66 (d, *J* = 49.4 Hz, 6H), 1.95 – 1.79 (m, 6H), 1.64 (s, 2H), 1.48 (d, *J* = 7.4 Hz, 2H), 1.38 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.06, 168.64, 167.07, 147.89, 132.46, 131.31, 129.60, 128.86, 125.79, 114.56, 112.68, 111.75, 96.99, 68.92, 56.30, 54.00, 29.28, 29.14, 28.88, 27.31, 25.86, 23.38. MS (ESI^+): m/z: 420.1$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{33}NO_3$: C, 77.29; H, 7.93; N, 3.34. Found: C, 77.45; H, 7.90; N, 3.46.

*4.5.2 2-(2-methoxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5b**)*

Yellow solid (58% yield), mp 101-103 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (d, *J* = 7.7 Hz, 1H), 7.64 (d, *J* = 9.0 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.01 (t, *J* = 7.5 Hz, 1H), 6.87 (d, *J* = 8.3 Hz, 1H), 6.68 (s, 2H), 3.99 (t, *J* = 6.2 Hz, 2H), 3.86 (s, 3H), 2.47 (s, 4H), 2.44 – 2.37 (m, 2H), 1.76 (s, 6H), 1.47 (dd, *J* = 15.7, 7.4 Hz, 4H), 1.33 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.83, 168.29, 166.75, 158.52, 147.83, 131.74, 131.07, 125.54, 121.31, 120.69, 114.69, 112.42, 110.59, 105.73, 96.84, 68.86, 56.65, 55.47, 54.23, 29.48, 29.23, 29.07, 28.90, 27.63, 25.86, 23.37. MS (ESI^+): m/z: 450.3$[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{35}NO_4$: C, 74.80; H, 7.85; N, 3.12. Found: C, 75.06; H, 7.93; N, 3.04.

*4.5.3 2-(3-methoxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5c**)*

Yellow solid (62% yield), mp 72-74 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 7.63 (d, *J* = 9.1 Hz, 1H), 7.41 (d, *J* = 8.2 Hz, 2H), 7.31 (t, *J* = 8.2 Hz, 1H), 6.89 (d, *J* = 9.1 Hz, 1H), 6.73 – 6.66 (m, 3H), 4.00 (t, *J* = 6.5 Hz, 2H), 3.83 (s, 3H), 2.65 (s, 4H), 2.57 – 2.50 (m, 2H), 1.87 – 1.75 (m, 6H), 1.57 (d, *J* = 6.7 Hz, 2H), 1.42 – 1.31 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.95, 168.59, 167.08, 159.69, 147.96, 133.63, 129.77, 125.72, 124.03, 116.34, 115.26, 114.46, 112.69, 111.55, 96.98, 68.94, 56.39, 55.32, 54.08, 29.33, 29.16, 28.88, 28.17, 27.40, 25.85, 23.37. MS (ESI^+): m/z: 450.2$[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{35}NO_4$: C, 74.80; H, 7.85; N, 3.12. Found: C, 74.80; H, 7.93; N, 3.26.

*4.5.4 2-(4-methoxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5d**)*

Yellow solid (65% yield), mp 77-79 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8.8 Hz, 2H), 7.67 (d, J = 9.1 Hz, 1H), 6.96 (d, J = 8.9 Hz, 2H), 6.78 (s, 1H), 6.75 – 6.69 (m, 2H), 4.05 (t, J = 6.5 Hz, 2H), 3.86 (s, 3H), 2.57 (s, 4H), 2.51 – 2.44 (m, 2H), 1.87 – 1.77 (m, 6H), 1.61 – 1.52 (m, 2H), 1.52 – 1.43 (m, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.84, 168.27, 166.80, 160.77, 146.85, 133.09, 125.59, 125.21, 114.88, 114.41, 112.44, 111.95, 96.94, 68.90, 56.58, 55.35, 54.19, 29.42, 29.19, 28.92, 28.72, 27.54, 25.88, 23.40. MS (ESI$^+$): m/z: 450.2[M+H]$^+$. Elemental Anal. Calcd for $C_{28}H_{35}NO_4$: C, 74.80; H, 7.85; N, 3.12. Found: C, 74.71; H, 7.91; N, 3.32.

*4.5.5 2-(3,4-dimethoxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5e**)*

Yellow solid (63% yield), mp 69-71 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.27 (m, 2H), 6.78 (d, J = 8.4 Hz, 1H), 6.58 (dd, J = 10.7, 2.1 Hz, 3H), 3.91 (dd, J = 8.1, 4.8 Hz, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.55 (s, 4H), 2.46 – 2.40 (m, 2H), 1.75 – 1.66 (m, 6H), 1.52 – 1.44 (m, 2H), 1.38 – 1.31 (m, 2H), 1.23 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.51, 168.02, 166.69, 150.43, 148.84, 146.71, 125.51, 125.37, 125.27, 114.64, 113.42, 112.34, 112.03, 111.03, 96.84, 68.81, 56.33, 55.81, 54.02, 29.28, 29.10, 28.85, 28.26, 27.34, 25.79, 23.30. MS (ESI$^+$): m/z: 480.3[M+H]$^+$. Elemental Anal. Calcd for $C_{29}H_{37}NO_5$: C, 72.62; H, 7.78; N, 2.92. Found: C, 72.43; H, 7.67; N, 3.09.

*4.5.6 7-(8-(pyrrolidin-1-yl)octyloxy)-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (**5f**)*

Yellow solid (51% yield), mp 73-75 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, J = 8.5 Hz, 1H), 6.98 (s, 2H), 6.65 – 6.52 (m, 3H), 3.92 (t, J = 6.3 Hz, 2H), 3.81 (d, J = 3.4 Hz, 9H), 2.45 (s, 4H), 2.40 – 2.33 (m, 2H), 1.70 (s, 6H), 1.41 (d, J = 34.2 Hz, 4H), 1.25 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.51, 168.16, 166.86, 153.09, 147.22, 139.48, 127.75, 125.46, 114.42, 112.45, 111.76, 108.43, 96.88, 68.86, 60.85, 56.47, 56.02, 54.09, 29.36, 29.14, 28.87, 28.68, 27.46, 25.81, 23.30. MS (ESI$^+$): m/z:

510.4[M+H]$^+$. Elemental Anal. Calcd for $C_{30}H_{39}NO_6$: C, 70.70; H, 7.71; N, 2.75. Found: C, 70.53; H, 7.55; N, 2.41.

*4.5.7 2-(2-(benzyloxy)phenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5g**)*

Yellow oil (53% yield); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, J = 7.8 Hz, 1H), 7.59 (d, J = 9.2 Hz, 1H), 7.36 –7.7.32 (m, 4H), 7.26 (d, J = 7.1 Hz, 1H), 7.24 – 7.17 (m, 2H), 6.96 (t, J = 7.5 Hz, 1H), 6.85 (d, J = 8.3 Hz, 1H), 6.64 –6.61 (m, 2.1 Hz, 2H), 5.08 (s, 2H), 3.94 (s, 2H), 2.54 (s, 4H), 2.47 – 2.40 (m, 2H), 1.77 –1.70 (m, 6H), 1.49 (d, J = 6.6 Hz, 2H), 1.35 (d, J = 6.3 Hz, 2H), 1.26 (d, J = 3.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.91, 168.41, 166.87, 157.68, 148.03, 136.62, 131.97, 131.04, 128.69, 128.00, 127.16, 125.68, 121.85, 121.08, 114.81, 112.51, 112.37, 105.92, 96.95, 70.36, 68.91, 56.47, 54.12, 29.38, 29.19, 28.91, 28.40, 27.47, 25.88, 23.39. MS (ESI$^+$): m/z: 526.3[M+H]$^+$. Elemental Anal. Calcd for $C_{34}H_{39}NO_4$: C, 77.68; H, 7.48; N, 2.66. Found: C, 77.55; H, 7.70; N, 2.55.

*4.5.8 2-(2-hydroxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5h**)*

Light yellow solid (42% yield), mp 120-122 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ^{1}H NMR (300 MHz, MeOD) δ 8.10 (d, J = 7.9 Hz, 1H), 7.58 (d, J = 8.6 Hz, 1H), 7.28 (s, 1H), 7.15 (t, J = 7.8 Hz, 1H), 6.93 – 6.60 (m, 4H), 4.05 (t, J = 6.3 Hz, 2H), 3.09 – 2.99 (m, 2H), 1.97 (t, J = 7.0 Hz, 4H), 1.81 – 1.69 (m, 2H), 1.62 (s, 2H), 1.43 (s, 2H), 1.33 (s, 4H), 1.19 (s, 6H). ^{13}C NMR (75 MHz, CDCl3) δ 184.70, 170.04, 169.01, 158.96, 148.72, 132.80, 126.54, 120.86, 120.57, 116.52, 115.61, 114.12, 108.34, 98.10, 70.18, 56.35, 55.14, 33.08, 30.11, 29.98, 27.54, 27.17, 26.93, 23.95. MS (ESI+): m/z: 436.2[M+H]+. Elemental Anal. Calcd for $C_{27}H_{33}NO_4$: C, 74.45 H, 7.64; N, 3.22. Found: C, 74.59; H, 7.39; N, 3.37.

*4.5.9 2-(3-(benzyloxy)phenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5i**)*

Light yellow solid (53% yield), mp 84-86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 (d, J = 9.2 Hz, 1H),

7.47 (t, *J* = 2.0 Hz, 1H), 7.42 – 7.28 (m, 6H), 7.22 (d, *J* = 10.9 Hz, 1H), 6.92 (dd, *J* = 8.0, 2.6 Hz, 1H), 6.69 – 6.62 (m, 3H), 5.04 (s, 2H), 3.96 (t, *J* = 6.5 Hz, 2H), 2.61 – 2.53 (m, 4H), 2.50 – 2.43 (m, 2H), 1.81 – 1.71 (m, 6H), 1.52 (t, *J* = 7.7 Hz, 2H), 1.40 – 1.34 (m, 2H), 1.27 (d, *J* = 4.1 Hz, 4H), 1.18 (s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) *δ*182.96, 168.60, 167.09, 158.93, 147.97, 136.76, 133.69, 129.80, 128.67, 128.12, 127.61, 125.74, 124.37, 117.20, 116.22, 114.46, 112.75, 111.55, 96.97, 70.11, 68.97, 56.43, 54.09, 29.37, 29.20, 28.91, 28.29, 27.45, 25.88, 23.38. MS (ESI^+): m/z: 526.4$[M+H]^+$. Elemental Anal. Calcd for $C_{34}H_{39}NO_4$: C, 77.68; H, 7.48; N, 2.66. Found: C, 78.00; H, 7.57; N, 2.49.

*4.5.10 2-(3-hydroxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5j**)*

Light yellow solid (41% yield), mp 179-181 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 7.85 (s, 1H), 7.60 (d, *J* = 8.6 Hz, 1H), 7.37 (t, *J* = 2.0 Hz, 1H), 7.30 (dt, *J* = 7.7, 1.3 Hz, 1H), 7.22 (t, *J* = 7.8 Hz, 1H), 6.86 (d, *J* = 2.0 Hz, 1H), 6.81 (dd, *J* = 8.0, 1.5 Hz, 1H), 6.75 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.66 (s, 1H), 4.06 (t, *J* = 6.3 Hz, 2H), 3.25 (p, *J* = 1.7 Hz, 4H), 3.21 (s, 2H), 3.07 – 3.01 (m, 2H), 2.02 – 1.96 (m, 4H), 1.77 (t, *J* = 7.2 Hz, 2H), 1.36 (s, 6H), 1.22 (d, *J* = 1.8 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 181.67, 168.05, 166.83, 157.70, 147.18, 133.06, 129.89, 125.53, 117.59, 117.24, 113.71, 111.26, 97.38, 68.87, 53.83, 52.74, 28.48, 26.08, 25.15, 22.71. MS (ESI^+): m/z: 436.1$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{33}NO_4$: C, 74.45; H, 7.64; N, 3.22. Found: C, 74.82; H, 6.68; N, 3.08.

*4.5.11 2-(4-(benzyloxy)phenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5k**)*

Light yellow solid (62% yield), mp 74-76 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.84 (d, *J* = 8.8 Hz, 2H), 7.67 (d, *J* = 9.2 Hz, 1H), 7.39 (ddd, *J* = 10.9, 9.3, 7.4 Hz, 5H), 7.02 (d, *J* = 8.8 Hz, 2H), 6.77 (s, 1H), 6.75 – 6.63 (m, 2H), 5.10 (s, 2H), 4.03 (t, *J* = 6.5 Hz, 2H), 2.59 (d, *J* = 5.5 Hz, 4H), 2.53 – 2.46 (m, 2H), 1.88 – 1.77 (m, 6H), 1.59 – 1.55 (m, 2H), 1.51 – 1.31 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 182.87, 168.28, 166.82, 159.93, 146.89, 136.46, 133.13, 128.68, 128.17, 127.49, 125.61, 125.42, 115.28, 114.85,

112.51, 111.92, 96.92, 70.05, 68.91, 56.55, 54.17, 29.41, 29.20, 28.92, 28.60, 27.52, 25.89, 23.40. MS (ESI^+): m/z: 526.4$[M+H]^+$. Elemental Anal. Calcd for $C_{34}H_{39}NO_4$: C, 77.68; H, 7.48; N, 2.66. Found: C, 77.98; H, 7.28; N, 3.83.

*4.5.12 2-(4-hydroxyphenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5l**)*

Light yellow solid (55% yield), mp 70-72 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.85 (d, J = 8.7 Hz, 2H), 7.68 (d, J = 8.6 Hz, 1H), 6.98 (d, J = 1.9 Hz, 1H), 6.91 (s, 1H), 6.90 – 6.78 (m, 3H), 4.17 (t, J = 6.3 Hz, 2H), 3.08 (s, 4H), 2.99 – 2.88 (m, 2H), 2.07 – 1.96 (m, 4H), 1.86 (dd, J = 14.3, 6.7 Hz, 2H), 1.67 (d, J = 7.1 Hz, 2H), 1.55 (s, 2H), 1.44 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.99, 168.28, 167.37, 160.13, 146.14, 133.47, 124.95, 123.33, 115.80, 114.25, 113.29, 112.64, 96.56, 68.75, 55.24, 53.55, 28.85, 28.63, 26.50, 26.40, 25.54, 22.63. MS (ESI^+): m/z: 436.2$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{33}NO_4$: C, 74.45; H, 7.64; N, 3.22. Found: C, 74.79; H, 7.47; N, 3.16.

*4.5.13 7-(8-(pyrrolidin-1-yl)octyloxy)-2-p-tolyl-4H-chromen-4-one (**5m**)*

Light yellow solid (64% yield), mp 75-77 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, J = 8.2 Hz, 2H), 7.69 (d, J = 8.8 Hz, 1H), 7.24 (s, 1H), 6.80 (s, 1H), 6.74 (d, J = 8.2 Hz, 2H), 4.06 (t, J = 6.5 Hz, 2H), 2.62 (s, 4H), 2.58 – 2.49 (m, 2H), 2.40 (s, 3H), 1.84 (t, J = 5.1 Hz, 6H), 1.58 (d, J = 6.5 Hz, 2H), 1.50 – 1.32 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.05, 168.51, 166.97, 147.53, 140.09, 131.34, 129.69, 129.65, 125.72, 114.74, 112.57, 112.03, 96.98, 68.93, 56.54, 54.16, 29.40, 29.19, 28.92, 28.51, 27.50, 25.89, 23.40, 21.63. MS (ESI^+): m/z: 434.3$[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{35}NO_3$: C, 77.56; H, 8.14; N, 3.23. Found: C, 77.46; H, 7.95; N, 3.27.

*4.5.14 2-(4-chlorophenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5n**)*

Orange solid (43% yield), mp 89-91 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.82 (d, J = 8.5 Hz, 2H), 7.69

(d, *J* = 9.1 Hz, 1H), 7.40 (d, *J* = 8.5 Hz, 2H), 6.80 – 6.70 (m, 3H), 4.06 (t, *J* = 6.5 Hz, 2H), 2.53 (s, 4H), 2.48 – 2.42 (m, 2H), 1.85 – 1.77 (m, 6H), 1.58 – 1.45 (m, 4H), 1.36 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.87, 168.59, 167.20, 148.04, 135.45, 132.40, 131.00, 129.13, 125.87, 114.42, 112.81, 110.27, 97.00, 69.02, 56.67, 54.26, 29.48, 29.23, 28.94, 28.91, 27.61, 25.90, 23.39. MS (ESI^+): m/z: 454.2$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{32}ClNO_3$: C, 71.43; H, 7.10; N, 3.09. Found: C, 71.25; H, 7.03; N, 3.34.

*4.5.15 2-(4-bromophenyl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5o**)*

Light yellow solid (47% yield), mp 108-111 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.5 Hz, 2H), 7.69 (d, *J* = 9.1 Hz, 1H), 7.57 (d, *J* = 8.6 Hz, 2H), 6.76 – 6.72 (m, 3H), 4.07 (t, *J* = 6.5 Hz, 2H), 3.05 (s, 2H), 2.88 – 2.79 (m, 2H), 2.04 (s, 4H), 1.86 – 1.77 (m, 4H), 1.48 – 1.33 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.85, 168.57, 167.17, 148.11, 132.58, 132.09, 131.38, 125.86, 123.90, 114.42, 112.81, 110.31, 97.04, 68.93, 55.95, 53.78, 29.08, 29.06, 28.85, 27.00, 26.65, 25.83, 23.39. MS (ESI^+): m/z: 498.1$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{32}BrNO_3$: C, 65.06; H, 6.47; N, 2.81. Found: C, 64.88; H, 6.57; N, 2.71.

*4.5.16 7-(8-(pyrrolidin-1-yl)octyloxy)-2-(4-(pyrrolidin-1-yl)phenyl)-4H-chromen-4-one (**5p**)*

Dark red solid (42% yield), mp 96-98 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.6 Hz, 2H), 7.66 (d, *J* = 8.3 Hz, 1H), 6.81 (s, 1H), 6.70 (d, *J* = 8.2 Hz, 2H), 6.56 (d, *J* = 8.6 Hz, 2H), 4.02 (t, *J* = 6.4 Hz, 2H), 3.34 (s, 4H), 2.67 (s, 4H), 2.59 – 2.51 (m, 2H), 2.02 (s, 4H), 1.89 – 1.77 (m, 6H), 1.61 (s, 2H), 1.47 – 1.30 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.46, 167.54, 166.17, 148.68, 145.61, 133.47, 125.29, 119.51, 115.48, 114.30, 112.06, 111.85, 96.78, 68.74, 56.57, 54.17, 47.52, 29.41, 29.20, 28.94, 28.66, 27.53, 25.89, 25.45, 23.36. MS (ESI^+): m/z: 489.4$[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{40}N_2O_3$: C, 76.19; H, 8.25; N, 5.73. Found: C, 76.29; H, 8.13; N, 5.41.

*4.5.17 2-(naphthalen-1-yl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5q**)*

Light yellow solid (50% yield), mp 128-131 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (d, J = 7.0 Hz, 1H), 8.29 (d, J = 8.4 Hz, 1H), 7.87 (dd, J = 8.1, 1.4 Hz, 2H), 7.70 (d, J = 9.1 Hz, 1H), 7.60 (dd, J = 5.0, 1.7 Hz, 2H), 7.58 – 7.50 (m, 2H), 6.76 – 6.69 (m, 2H), 4.02 (t, J = 6.5 Hz, 2H), 2.76 (s, 4H), 2.66 – 2.58 (m, 2H), 1.94 – 1.85 (m, 4H), 1.85 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.49 – 1.40 (m, 2H), 1.34 (d, J = 3.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.83, 168.74, 167.09, 148.75, 133.72, 132.23, 130.23, 129.96, 128.94, 128.39, 126.99, 126.16, 125.85, 125.58, 123.43, 114.68, 112.73, 107.26, 97.08, 68.95, 56.29, 54.02, 29.28, 29.15, 28.88, 27.83, 27.31, 25.86, 23.38. MS (ESI^+): m/z: 470.3$[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{35}NO_3$: C, 79.28; H, 7.51; N, 2.98. Found: C, 79.58; H, 7.19; N, 3.13.

*4.5.18 2-(biphenyl-4-yl)-7-(8-(pyrrolidin-1-yl)octyloxy)-4H-chromen-4-one (**5r**)*

Light yellow solid (45% yield), mp 144-146 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (d, J = 8.3 Hz, 2H), 7.69 – 7.62 (m, 6H), 7.46 (t, J = 7.5 Hz, 2H), 7.38 (d, J = 7.3 Hz, 1H), 6.82 (s, 1H), 6.76 – 6.70 (m, 2H), 4.03 (t, J = 6.5 Hz, 2H), 2.77 (s, 4H), 2.65 – 2.60 (m, 2H), 1.93 – 1.87 (m, 4H), 1.82 (dd, J = 14.2, 7.2 Hz, 2H), 1.68 – 1.61 (m, 2H), 1.45 (d, J = 7.2 Hz, 2H), 1.35 (d, J = 3.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.88, 168.54, 167.06, 147.98, 142.13, 140.17, 131.78, 131.49, 128.95, 127.88, 127.44, 127.06, 125.74, 114.62, 112.68, 111.36, 97.06, 68.96, 56.29, 54.01, 29.30, 29.16, 28.91, 27.84, 27.32, 25.87, 23.40. MS (ESI^+): m/z: 496.3$[M+H]^+$. Elemental Anal. Calcd for $C_{33}H_{37}NO_3$: C, 79.97; H, 7.52; N, 2.83. Found: C, 80.09; H, 7.52; N, 2.85.

4.6 Biological activity

4.6.1 Inhibition assays on AChE and BChE in vitro

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from *electric eel*), butyrylcholinesterase (BChE, E.C.

3.1.1.8, from *equine serum*), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine chloride (ATC), butylthiocholine chloride (BTC) were purchased from Sigma Aldrich. Test compounds were dissolved in a minimum volume of DMSO (1%) and then diluted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0) to provide a final concentration range.

All the assays were under 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 8.0, using a Shimadzu UV-2450 Spectrophotometer. Enzyme solutions were prepared to give 2.0 units/mL in 2 mL aliquots. The assay medium contained phosphate buffer, pH 8.0 (1 mL), 50 μL of 0.01 M DTNB, 10 μL of enzyme, and 50 μL of 0.01 M substrate (ATC). The substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor after 15 min of incubation time. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals at 37 °C. Calculations were performed according to the method of the equation in Ellman et al.[21] *In vitro* BChE assay use the similar method described above. Each concentration was assayed in triplicate.

4.6.2 Kinetic characterization of AChE and BChE inhibition

Kinetic characterization of AChE was performed using a reported method.[24,25] Six different concentrations of substrate were mixed in the 1 mL 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0), containing 50 μL of DTNB, 10 μL AChE, and 50 μL substrate. Test compound was added into the assay solution and pre-incubated with the enzyme at 37°C for 15 min, followed by the addition of substrate. Kinetic characterization of the hydrolysis of ATC catalyzed by AChE was done spectrometrically at 412 nm. A parallel control with no inhibitor in the mixture, allowed adjusting activities to be measured at various times. BChE assay use the similar method described above.

4.6.3 Molecular modeling

The crystal structure of the torpedo AChE (code ID: 1ACJ) were obtained in the Protein Data Bank

after eliminating the inhibitor and water molecules. The 3D structure of compounds **5q** was built and performed geometry optimization by molecular mechanics. Further preparation of substrates was according to the previous report. [20]

Docking studies were carried out using the AUTODOCK 4.0 program using ADT, polar hydrogen atoms were added and Gasteiger charges were assigned to the enzyme. The resulting enzyme structure was used as an input for the AUTOGRID program. AUTOGRID performed a precalculated atomic affinity grid maps for each atom type in the ligand plus an electrostatics map and a separate desolvation map present in the substrate molecule. All maps were calculated with 0.375 Å spacing between grid points. The centre of the grid box was placed at the bottom of the active site gorge (AChE [2.781 64.383 67.971]. The dimensions of the active site box were set at 50 × 46 × 46 Å. Flexible ligand docking was performed for the compounds. Docking calculations were carried out using the Lamarckian genetic algorithm (LGA) and all parameters were the same for each docking.

4.6.4 MTT assay of HepG2 and PC12 cell viability

MTT assay of HepG2 was conducted according to the previous report. [19]

PC12 cells were grown in DMEM, supplemented with 10% heat-inactivated fetal calf serum at 37 ºC under 5% CO_2 in a humidified chamber. The antiproliferative ability of compounds was evaluated in PC12 cells by the conversion of MTT to a purple formazan precipitate. The cells were seeded into 96-well plates at 5×10^3 cells/well, after 12 h, 1.0, 10 μM of compounds were subsequently added and incubated for 48 h. The cell viability was determined by using MTT colorimetry, measuring the absorption at 590 nm. Controls were taken as having 100% viability. Each concentration was tested in triplicate.

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Conflict of Interest

We declare that we have no conflict of interest.

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Graphical Abstract

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Design, Synthesis and Evaluation of 7-Aminoalkyl-Substituted Flavonoid Derivatives with Improved Cholinesterase Inhibitory Activities

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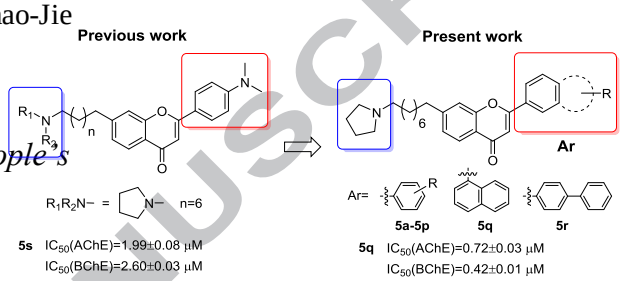

Eighteen 7-aminoalkyl-substituted flavonoid derivatives were synthesized and evaluated as potential cholinesterase inhibitors, these compounds (5a-5r) exhibited better inhibitory activity than our previously reported compounds. They showed a mixed-type inhibition for AChE and did not affect PC12 and HepG2 cell viability at the concentration of 10 μM.