Bioorganic & Medicinal Chemistry 21 (2013) 7275–7282

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis and evaluation of novel 4-dimethylamine flavonoid derivatives as potential multi-functional anti-Alzheimer agents

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ARTICLE INFO

Article history:
Received 7 August 2013
Revised 24 September 2013
Accepted 25 September 2013
Available online 4 October 2013

Keywords:
Flavonoid derivatives
Anti-Alzheimer agent
Cholinesterase
Self-induced Aβ aggregation
Antioxidation

ABSTRACT

A series of 4-dimethylamine flavonoid derivatives **5a**–**5r** were designed, synthesized and evaluated as potential multi-functional anti-Alzheimer agents. The results showed that most of the synthesized compounds exhibited high acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity at the micromolar range (IC_{50}, 1.83–33.20 μM for AChE and 0.82–11.45 μM for BChE). A Lineweaver–Burk plot indicated a mixed-type inhibition for compound **5j** with AChE, and molecular modeling study showed that **5j** targeted both the catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE. Besides, the derivatives showed potent self-induced Aβ aggregation inhibitory activity at 20 μM with percentage from 25% to 48%. In addition, some compounds (**5j**–**5q**) showed potent oxygen radical absorbance capacity (ORAC) ranging from 1.5- to 2.6-fold of the Trolox value. These compounds should be further investigated as multi-potent agents for the treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder that is characterized by dementia, memory loss and other cognitive impairments.[1,2] The etiology of AD is unclear, but there are common indicators that seem to play a significant role in the disease, such as low levels of acetylcholine, β-amyloid (Aβ) deposits, τ-protein aggregation and oxidative stress.[3–5]

Current treatment of AD focuses on increasing neurotransmitter acetylcholine levels at cerebral cortex synapses by inhibiting acetylcholinesterase (AChE) activity with drugs such as tacrine, donepezil, rivastigmine and galantamine.[6] Unfortunately, AChE inhibition alone is a palliative treatment and its clinical effectiveness is still under debate.[7] Due to the multi-pathogenesis of AD, a strategy called multi-target-directed ligands (MTDLs) has recently emerged, this strategy is to develop novel anti-AD agents with multiple potencies,[8] including cholinesterase inhibition, anti-oxidation, Aβ deposit inhibition and so on, and the strategy of combining these potencies in one structure was confirmed to be successful.[9,10]

The two types of cholinesterase (ChE) found in the central nervous system, AChE and butyrylcholinesterase (BChE), are both able to hydrolyze acetylcholine, during the development of AD, BChE activity increases by 40–90% in the temporal cortex and hippocampus, while at the same time AChE activity decreases.[11,12] Therefore, the concurrent inhibition of both AChE and BChE should provide additional benefits in the treatment of AD.

The production and accumulation of Aβ in the brain is generally considered to be a central event in the pathogenesis of AD. Many studies have shown that either the oligomeric or fibril form of Aβ is more toxic to neurons than the monomeric form.[13] Therefore, Aβ aggregation is currently another potential target of AD.[14–16]

Oxidative stress has been strongly implicated in the pathophysiology of neurodegenerative disorders such as AD.[17] It has been shown that oxidative damage caused by excessive reactive oxygen species (ROS) is present in the brains of AD patients.[18] Therefore designing antioxidants targeted towards ROS may offer an attractive therapeutic approach for AD.

Flavonoids are a class of polyphenolic compounds present in plants, including many that are commonly consumed by humans such as fruits, vegetables, wine and tea.[19] They express a wide range of biological activities, such as antioxidant, anti-inflammatory, neuroprotection, vasodilator, and inhibition of Aβ aggregation. Flavonoids exhibit especially low toxicity and so they have been considered as leading anti-AD compounds.[20] During recent years, some research groups have attempted to optimize flavonoids' chemical structures in order to develop new drugs to treat AD (Fig. 1A and B).[21,22]

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http://dx.doi.org/10.1016/j.bmc.2013.09.061

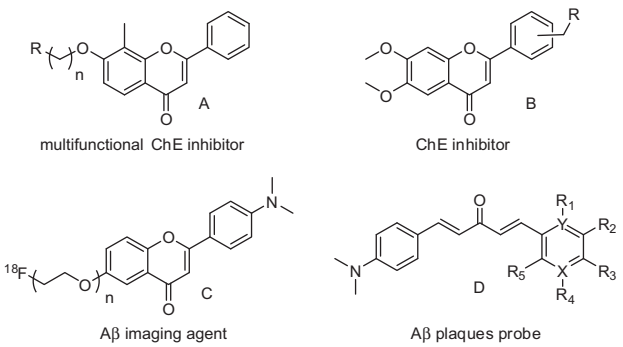


Figure 1. Flavonoids and N,N-dimethylated curcumin derivatives as new drugs or imaging agents in the treatment of AD.

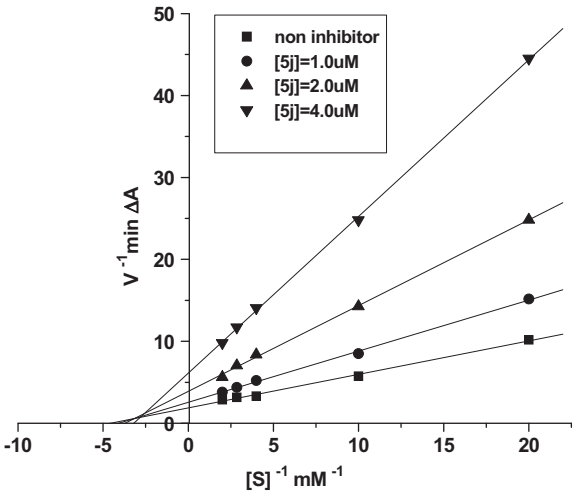


Figure 2. Lineweaver–Burk plot for the inhibition of acetylcholinesterase by compound **5j**.

Our research group has been involved in the synthesis of flavonoids and the development of ChE inhibitors for many years.[23,24] We recently found compounds containing a N,N-dimethylated flavonoid moiety during routine screening for compounds with biological activity. These compounds have significant antioxidant activity and inhibit Aβ self-induced aggregation. It has previously been reported that N,N-dimethylated flavonoid and curcumin derivatives (Fig. 2C and D) display excellent binding affinities with Aβ during in vitro binding assays.[25,26] Therefore, in the present study, we assessed a series of new N,N-dimethylated flavonoids as multifunctional anti-AD agents. We hypothesized that these flavonoids function as inhibitors of ChE, Aβ self-induced aggregation and ROS. The flavonoids were designed by conjugating an N,N-dimethylated flavonoid with a terminal amine using a long chain linker. It is possible that the N,N-dimethylated flavonoids inhibit AChE activity through binding with the catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE, blocking Aβ self-aggregation and scavenging ROS.

2. Results and discussion

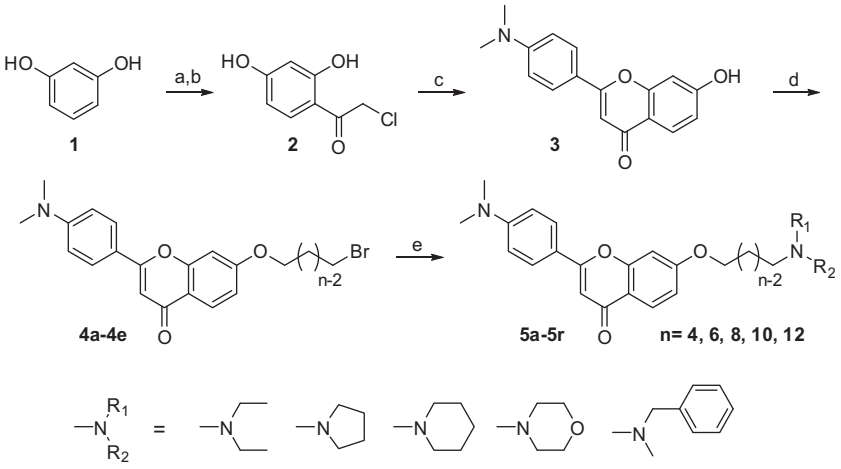
2.1. Chemistry

Eighteen flavonoid derivatives (**5a**–**5r**) with linkers of varying chain lengths were prepared as shown in Scheme 1. Condensation of 1,3-benzenediol (**1**) with chloroacetonitrile catalyzed by $ZnCl_2$ and HCl gas was followed by hydrolysis in water and produced ketone in 87% yield. Treatment of compound **2** with 4-dimethylaminobenzaldehyde in the presence of excess NaOH in H_2O/EtOH was followed by acidification with aqueous HCl and resulted in the expected compound (**3**) in 92% yield. The alkylation of **3** with different α, ω-dibromoalkanes in acetone provided **4a**–**4e** in satisfactory yields. Finally, the reaction of **4a**–**4e** with secondary amines produced the targets **5a**–**5r** in 45–88% yields. The structures and purities of the target compounds were confirmed by ^{1}H NMR, ^{13}C NMR, MS and elemental analysis.

2.2. In vitro inhibition studies on AChE and BChE

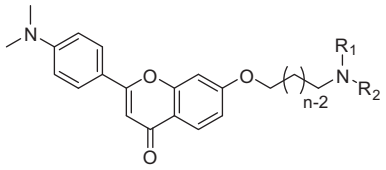
To determine the potential efficacy of the target compounds **5a**–**5r** for the treatment of AD, their ChE inhibitory activity was determined by the Ellman method,[27] with tacrine and rivastigmine as reference compounds. The IC_{50} values for ChE inhibition and their selectivity index are summarized in Table 1. The results showed that most of the tested compounds (**5a**–**5r**) had significant ChE inhibitory activity and selectivity for AChE. Compound **5j** (n = 8), with a diethylamino group, showed the most potent inhibition for AChE with an IC_{50} value of 1.83 μM. Compound **5o** (n = 10) has pyrrolidine moiety and exhibited the strongest inhibition to BChE with an IC_{50} value of 0.82 μM.

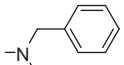
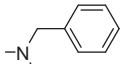
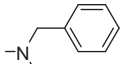
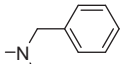
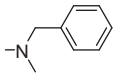
Compounds with the same chain length were compared for their ability to inhibit AChE and BChE. The AChE inhibition activity



Scheme 1. Synthesis of flavonoid derivatives. Reagents and conditions: (a) $ClCH_2CN$, EtOEt, HCl, 0 °C; (b) HCl, H_2O, reflux; (c) 4-dimethylaminobenzaldehyde, 10% NaOH, rt, EtOH; (d) dibromoalkane, K_2CO_3, acetone, reflux; (e) HNR_1R_2, K_2CO_3, CH_3CN, 60 °C.

Table 1
Inhibition of ChE activity, selectivity index and inhibition of Aβ(1–42) self-induced aggregation



Compds	*n*	$-NR_1R_2$	IC_{50}[a] for AChE (μM)	IC_{50}[b] for AChE (μM)	Selectivity index[c]	Aβ(1–42)[d] inhibition
5a	4		2.24 ± 0.14	7.88 ± 1.51	3.5	33.23 ± 1.44
5b	4		2.77 ± 0.02	6.14 ± 0.27	2.2	36.56 ± 4.94
5c	4		3.04 ± 0.21	6.12 ± 0.72	2.0	30.64 ± 0.65
5d	4		>50	10.10 ± 1.32	<0.2	44.67 ± 2.68
5e	4		8.29 ± 2.11	>50	>6.0	39.13 ± 2.38
5f	6		3.11 ± 0.15	4.61 ± 0.43	1.5	38.12 ± 5.67
5g	6		9.18 ± 1.14	11.45 ± 1.90	1.2	32.61 ± 1.95
5h	6		6.73 ± 0.18	5.52 ± 0.18	0.8	25.37 ± 5.92
5i	6		7.85 ± 2.30	>50	6.4	46.55 ± 0.82
5j	8		1.83 ± 0.15	5.01 ± 0.35	2.7	45.80 ± 2.77
5k	8		1.99 ± 0.08	2.60 ± 0.03	1.3	42.15 ± 4.63
5l	8		7.88 ± 1.38	8.72 ± 1.16	1.1	34.81 ± 2.16
5m	8		23.99 ± 1.06	>50	2.1	39.52 ± 6.57
5n	10		3.74 ± 0.10	2.31 ± 0.07	0.6	33.37 ± 3.79
5o	10		2.26 ± 0.07	0.82 ± 0.04	0.4	38.60 ± 4.68
5p	10		>50	2.42 ± 0.10	<0.05	40.73 ± 1.79
5q	10		11.40 ± 1.22	>50	4.4	29.36 ± 5.71
5r	12		33.20 ± 2.06	>50	1.5	48.13 ± 3.70
Rivastigmine	—	—	5.56 ± 1.50	1.71 ± 0.03	0.3	—
Tacrine	—	—	0.219 ± 0.002	0.047 ± 0.001	0.2	—
Curcumin	—	—	—	—	—	51.04 ± 3.51

[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] Selectivity Index = IC_{50} (BChE)/IC_{50} (AChE).
[d] The values are the mean ± SD of three independent experiments and the measurements were carried out in the presence of 20 μM compounds.

of compounds with a diethylamine or pyrrolidine group (**5a**, **5b**, **5j**, **5k**, **5n**, **5o**) were more effective than compounds with a piperidine or benzyl methylamine group (**5c**, **5e**, **5l**, **5m**, **5p**, **5q**). The BChE inhibition activity of compounds with an aminoalkyl substituted group is not so obvious. Compounds with a benzyl methylamine group (**5e**, **5i**, **5m**, **5q**, **5r**) exhibted the weakest inhibition activities for both AChE and BChE, and they showed selectivity for AChE.

Compounds with the same aminoalkyl substituted group, but different chain lengths were also compared. The optimal chain length for inhibiting AChE and BChE was eight methylene groups (**5j**, **5k**) on the flavonoid compound and ten (**5n**, **5o**, **5p**) methylene groups on the aminoalkyl substituted moiety compound. The variation of chain length for the inhibitors had more influence on their inhibition to AChE than BChE. This may be caused by conformational differences between these two enzymes. The active site of BChE is wider than the active site of AChE and therefore, BChE is able to accomodate inhibitors with variable linker lengths. The length of the alkyl chain may also affect the selectivity for AChE

(except compounds with a benzyl methylamine group **5e**, **5i**, **5m**, **5q** and **5r**). Compounds with four, six and eight methylene groups (**5a**, **5b**, **5f**, **5g**, **5j**, **5k**) showed selectivity for AChE rather than the compounds with ten methylene groups (**5n**, **5o**, **5p**).

2.3. Kinetic characterization of AChE inhibition

The compound that appeared to be the most successful at inhibiting AChE was **5j**. We further investigated **5j** using graphical analysis of steady state inhibition data (Fig. 2). The Lineweaver–Burk plots show an increasing slope (decreased V_{max}) and an increasing intercept (higher K_m) for higher inhibitor concentrations, indicating a mixed-type inhibition. Replots of the slope versus concentration of compound **5j** gave an extimate of the inhibition constant, K_i of 0.386 μM. This suggests that compound **5j** was able to bind both CAS and PAS of AChE and supports the results of the molecular modeling study.

2.4. Molecular modeling study

In order to obtain more information about the binding interactions between compound **5j** and *Tc*AChE (PDB code: 1ACJ), docking studies were carried out using the AUTODOCK 4.0 package and PyMOL software as shown in Figure 3.[28,29]

The docking result demonstrates that compound **5j** exhibits multiple binding modes with AChE. In the **5j**–*Tc*AChE complex, compound **5j** occupied the entire enzymatic CAS, mid-gorge and PAS. The charged nitrogen of diethylamine resulted in a cation–π interaction with Trp84 with a distance of 4.2 Å at the CAS. The flavonoid moiety stacked against the Trp279 through π–π stacking with a distance of 3.8 Å at the PAS. The docking studies show that our compounds exhibit a mixed type of inhibition, consistent with our kinetic analysis result.

Since the crystal structure of BChE from equine serum has not been reported and the sequence of equine BChE is highly similar to human BChE, the crystal structure of *Hu*BChE was used in the docking study. Similar interactions were found in **5o** in complex with *Hu*BChE (PDB code: 1POI), a cation–π interaction with Trp82 was observed at the CAS.

2.5. Inhibition of self-mediated Aβ(1–42) aggregation

The derivatives **5a**–**5r** were tested for their ability to inhibit self-mediated aggregation of Aβ(1–42) using the thioflavin T assay.[30] The results suggest that these compounds prevent self-mediated Aβ aggregation with percentages of inhibition ranging from 25% to 48% at 20 μM. These are lower inhibition levels than that of curcumin (51%; Table 1). The most potent compounds were **5d**, **5i**, **5j**, **5k**, **5p** and **5r**, with percentages of inhibition greater than 40%. However, the inhibitory potency of each derivative did not depend on the chain length of the connecting linker. The current data are not sufficient to establish a structure-activity relationship for the activity toward anti-Aβ aggregation, and further investigation is required to determine the activity mechanism.

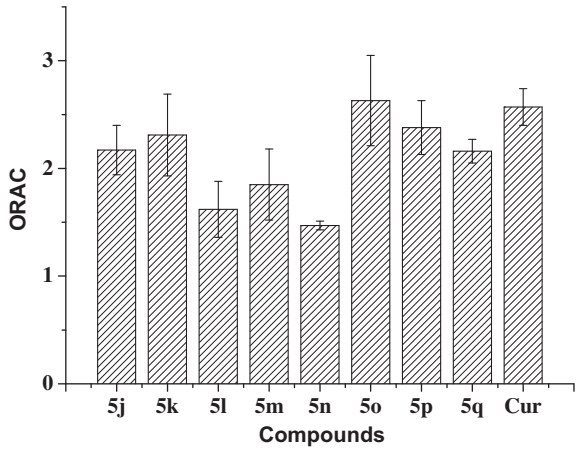


Figure 4. Oxygen radical absorbance capacity (ORAC) of compounds **5a**–**5r**. Data are expressed as (μmol Trolox)/(μmol tested compound).

2.6. Studies of antioxidation activity

The peroxyl radicals reduced by the selected compounds **5j**–**5q** were examined by using the ORAC (oxygen radical absorbance capacity) assay,[31] with curcumin as a reference compound (Fig. 4). The ability of compounds **5j**–**5q** to scavenge radicals at concentrations between 1–5 μM was compared with that of the highly potent compound Trolox and is expressed as their Trolox equivalent. These compounds exhibited significant radical-capture capacity. They showed potent peroxyl radical absorbance capacities ranging from 1.5 to 2.6 times the Trolox value. The 4-dimethylamine flavonoid moiety appears to be crucial to the compound's radical scavenging ability.

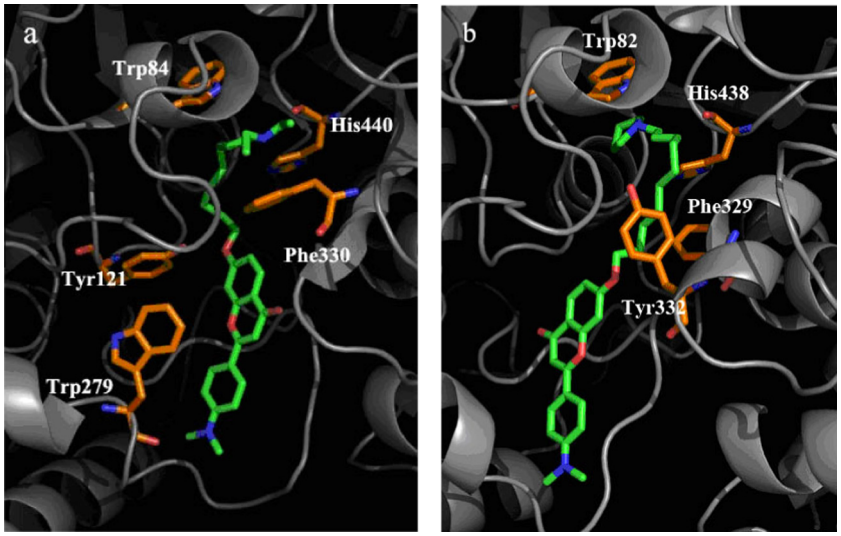


Figure 3. Docking models of compound–enzyme complex: (a) **5j**–*Tc*AChE complex; (b) **5o**–*Hu*BChE complex.

3. Conclusion

In summary, a novel series of 4-dimethylamine flavonoid derivatives **5a**–**5r** were synthesized and characterized. These synthetic compounds exhibit high inhibitory potency for both AChE and BChE, at levels similar to or better than those of Rivastigmine. Compound **5j** groups showed the best AChE inhibitory activity and **5o** exhibited the highest inhibitory effect for BChE. A kinetic analysis and molecular modeling study indicated that compound **5j** was able to bind with both CAS and PAS of the AChE. Additionally, these compounds exhibited potent inhibition of self-induced Aβ aggregation. Compounds **5j**–**5o** also had significant capacity to absorb peroxyl radicals. Our results indicate that this new type of multifunctional flavonoid derivatives may provide a useful template for the development of new anti-AD agents with multiple potencies.

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-400 spectrometer at 400 MHz and 100 MHz, respectively. 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. Synthesis of intermediate 2

To a mixture of resorcinol (4.37 g, 39.7 mmol) and chloroacetonitrile (2.5 mL, 39.7 mmol) in ether (50 mL) was added anhydrous $ZnCl_2$ (0.54 g, 3.96 mmol). The solution was cooled to 0 °C and HCl was bubbled through the reaction for 2 h. The solution was left in the cold-room overnight and HCl was bubbled again for 2 h. The precipitated imine was filtered off and washed three times with ether. The imine was dissolved in 100 mL of 1 N HCl and reflux for 1 h. The solid was filtered off, washed three times with water and dried under vacum to yield pure acetophenone **2** as a white solid which was used without further purification. Yield 6.44 g (87%).

4.3. Synthesis of intermediate 3

To a mixture of acetophenone **2** (3.96 g, 21.2 mmol) and 4-dimethylaminobenzaldehyde (3.16 g, 21.2 mmol) in ethanol (50 mL) was added 10% sodium hydrate aqueous (30 mL), the mixture was stirred for 24 h at room temperature. The solution was acidified with 1 N HCl and filtered, and the filter cake was recrystallized from ethanol got a dark red solid **3** which was used without further purification. Yield 5.48 g (92%). MS (ESI$^+$): *m*/*z*: 282.1[M+H]$^+$.

4.4. General procedures for the preparation of intermediate 4a–4e

To a solution of intermediate **3** (0.56 g, 2.0 mmol) and anhydrous K_2CO_3 (1.38 g, 10.0 mmol) in acetone (10 mL), α,ω-dibromoalkane (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 **4a**–**4e** were crystallized from ethanol which were used without further purification.

4.5. General procedures for the preparation of compound 5a–5r

To a solution of **4a**–**4e** (0.5 mmol) in CH_3CN (10 mL) was added different amines (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.

4.5.1. 7-(4-(Diethylamino)butoxy)-2-(4-(dimethylamino) phenyl)-4*H*-chromen-4-one (5a)

Intermediate **4a** was treated with diethylamine according to general procedure to give the desired product **5a** as yellow solid (61% yield), mp 114–116 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.4 Hz, 1H), 6.84 (s, 1H), 6.79–6.71 (m, 4H), 4.10 (t, *J* = 6.4 Hz, 2H), 3.07 (s, 3H), 2.60–2.48 (m, 6H), 1.86 (dd, *J* = 14.5, 6.9 Hz, 2H), 1.83–1.64 (m, 2H), 1.05 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.60, 167.69, 166.24, 151.09, 145.90, 133.28, 125.39, 120.11, 115.42, 113.87, 112.13, 111.90, 96.85, 68.64, 58.33, 52.46, 46.81, 40.11, 27.08, 23.53, 18.48, 11.67. MS (ESI$^+$): *m*/*z*: 409.1[M+H]$^+$. Elemental Anal. Calcd for $C_{25}H_{32}N_2O_3$: C, 73.50; H, 7.90; N, 6.86. Found: C, 73.54; H, 7.90; N, 6.76.

4.5.2. 2-(4-(Dimethylamino)phenyl)-7-(4-(pyrrolidin-1-yl)butoxy)-4*H*-chromen-4-one (5b)

Intermediate **4a** was treated with pyrrolidine according to general procedure to give the desired product **5b** as yellow solid (56% yield), mp 124–126 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.5 Hz, 1H), 6.84 (s, 1H), 6.79–6.69 (m, 4H), 4.10 (t, *J* = 6.4 Hz, 2H), 3.07 (s, 6H), 2.53 (t, *J* = 7.1 Hz, 6H), 1.91–1.70 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.56, 167.66, 166.21, 151.07, 145.88, 133.27, 125.34, 120.08, 115.41, 113.84, 112.13, 111.88, 96.84, 68.55, 56.02, 54.23, 40.09, 27.09, 25.47, 23.42. MS (ESI$^+$): *m*/*z*: 407.1[M+H]$^+$. Elemental Anal. Calcd for $C_{25}H_{30}N_2O_3{\cdot}0.4C_2H_5OH{\cdot}0.1CHCl_3$: C, 71.20; H, 7.50; N, 6.41. Found: C, 71.46; H, 7.23; N, 6.44.

4.5.3. 2-(4-(Dimethylamino)phenyl)-7-(4-(piperidin-1-yl)butoxy)-4*H*-chromen-4-one (5c)

Intermediate **4a** was treated with piperidine according to general procedure to give the desired product **5c** as yellow crystal (67% yield), mp 141–142 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.9 Hz, 2H), 7.69 (d, *J* = 8.5 Hz, 1H), 6.84 (s, 1H), 6.79–6.68 (m, 4H), 4.09 (t, *J* = 6.4 Hz, 2H), 3.07 (s, 6H), 2.65–2.19 (m, 6H), 1.88–1.82 (m, 2H), 1.72–1.68 (m, 2H), 1.64–1.56 (m, 4H), 1.46 (d, *J* = 4.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.50, 167.64, 166.19, 151.04, 145.85, 133.25, 125.29, 120.05, 115.38, 113.80, 112.12, 111.85, 96.82, 68.58, 58.93, 54.63, 40.06, 27.10, 26.04, 24.50, 23.40. MS (ESI$^+$): *m*/*z*: 421.2[M+H]$^+$. Elemental Anal. Calcd for $C_{26}H_{32}N_2O_3$: C, 74.26; H, 7.67; N, 6.66. Found: C, 74.20; H, 7.93; N, 6.26.

4.5.4. 2-(4-(Dimethylamino)phenyl)-7-(4-morpholinobutoxy)-4*H*-chromen-4-one (5d)

Intermediate **4a** was treated with morpholine according to general procedure to give the desired product **5d** as orange solid (85% yield), mp 157–159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 8.5 Hz, 1H), 6.84 (s, 1H), 6.79–6.74 (m, 2H), 6.73–6.71 (m, 2H), 4.10 (t, *J* = 6.4 Hz, 2H), 3.76–3.73 (m, 4H), 3.07 (s, 6H), 2.48–2.41 (m, 6H), 1.91–1.85 (m, 2H), 1.74–1.68 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.53, 167.65, 166.13, 151.09, 145.86, 133.28, 125.38, 120.07, 115.47, 113.88, 112.09, 111.89, 96.84, 77.41, 77.09, 76.78, 68.46, 67.02, 58.44, 53.74, 40.10,

26.84, 22.98. MS (ESI^+): *m*/*z*: 423.2$[M+H]^+$. Elemental Anal. Calcd for $C_{25}H_{30}N_2O_4$: C, 71.07; H, 7.16; N, 6.63. Found: C, 70.71; H, 7.21; N, 6.52.

4.5.5. 7-(4-(Benzyl(methyl)amino)butoxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5e)

Intermediate **4a** was treated with *N*-methyl-1-phenylmethanamine according to general procedure to give the desired product **5e** as orange needle-like crystal (73% yield), mp 106–109 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 9.1 Hz, 1H), 7.34 (d, *J* = 4.4 Hz, 4H), 7.28 (s, 1H), 6.85 (s, 1H), 6.77–6.68 (m, 4H), 4.06 (t, *J* = 6.4 Hz, 2H), 3.52 (s, 2H), 3.07 (s, 6H), 2.46 (t, *J* = 7.1 Hz, 2H), 2.23 (s, 3H), 1.93–1.83 (m, 2H), 1.76–1.68 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.55, 167.67, 166.24, 151.08, 145.90, 139.23, 133.29, 129.05, 128.26, 126.98, 125.32, 120.11, 115.41, 113.84, 112.15, 111.90, 96.87, 68.56, 62.42, 56.69, 42.26, 40.10, 26.74, 23.77. MS (ESI^+): *m*/*z*: 457.2$[M+H]^+$. Elemental Anal. Calcd for $C_{29}H_{32}N_2O_3$: C, 76.29; H, 7.06; N, 6.14. Found: C, 76.23; H, 7.07; N, 6.09.

4.5.6. 7-(6-(Diethylamino)hexyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5f)

Intermediate **4b** was treated with diethylamine according to general procedure to give the desired product **5f** as yellow solid (60% yield), mp 109–112 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 9.0 Hz, 2H), 7.70 (d, *J* = 8.4 Hz, 1H), 6.84 (s, 1H), 6.78–6.69 (m, 4H), 4.07 (t, *J* = 6.5 Hz, 2H), 3.07 (s, 6H), 2.54 (q, *J* = 7.2 Hz, 4H), 2.48–2.40 (m, 2H), 1.87–1.82 (m, 2H), 1.56–1.47 (m, 4H), 1.42–1.36 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.56, 167.68, 166.28, 151.07, 145.90, 133.26, 125.35, 120.12, 115.40, 113.80, 112.13, 111.89, 96.83, 68.74, 52.85, 46.90, 40.10, 28.98, 27.43, 26.99, 26.00, 11.69. MS (ESI^+): *m*/*z*: 437.3$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{36}N_2O_3$: C, 74.28; H, 8.31; N, 6.42. Found: C, 74.53; H, 8.33; N, 6.41.

4.5.7. 2-(4-(Dimethylamino)phenyl)-7-(6-(pyrrolidin-1-yl)hexyloxy)-4*H*-chromen-4-one (5g)

Intermediate **4b** was treated with pyrrolidine according to general procedure to give the desired product **5g** as orange solid (88% yield), mp 139–141 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.9 Hz, 2H), 7.64 (d, *J* = 9.0 Hz, 1H), 6.77 (s, 1H), 6.67 (d, *J* = 9.3 Hz, 4H), 3.99 (t, *J* = 6.5 Hz, 2H), 2.99 (s, 6H), 2.48 (d, *J* = 6.4 Hz, 4H), 2.46–2.39 (m, 2H), 1.86–1.67 (m, 6H), 1.56–1.50 (m, 2H), 1.48–1.42 (m, 2H), 1.41–1.31 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.47, 167.62, 166.24, 151.03, 145.83, 133.24, 125.26, 120.02, 115.34, 113.82, 112.12, 111.84, 96.78, 68.70, 56.51, 54.23, 40.04, 28.91, 27.40, 25.94, 23.37. MS (ESI^+): *m*/*z*: 435.2$[M+H]^+$. Elemental Anal. Calcd for $C_{27}H_{34}N_2O_3{\cdot}0.4C_2H_5OH{\cdot}0.1CHCl_3$: C, 72.07; H, 7.91; N, 6.03. Found: C, 72.55; H, 7.70; N, 6.25.

4.5.8. 2-(4-(Dimethylamino)phenyl)-7-(6-(piperidin-1-yl)hexyloxy)-4*H*-chromen-4-one (5h)

Intermediate **4b** was treated with piperidine according to general procedure to give the desired product **5h** as orange crystal (62% yield), mp 120–122 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.9 Hz, 2H), 7.67 (d, *J* = 8.3 Hz, 1H), 6.81 (s, 1H), 6.74–6.68 (m, 4H), 4.04 (t, *J* = 6.5 Hz, 2H), 3.04 (s, 6H), 2.36–2.26 (m, 6H), 1.87–1.78 (m, 2H), 1.64–1.32 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.54, 167.65, 166.25, 151.03, 145.85, 133.26, 125.30, 120.04, 115.35, 113.82, 112.13, 111.85, 96.78, 68.71, 59.55, 54.69, 40.08, 28.93, 27.48, 26.93, 26.02, 25.97, 24.51. MS (ESI^+): *m*/*z*: 449.3$[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{36}N_2O_3$: C, 74.97; H, 8.09; N, 6.24. Found: C, 74.59; H, 8.39; N, 6.37.

4.5.9. 7-(6-(Benzyl(methyl)amino)hexyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5i)

Intermediate **4b** was treated with *N*-methyl-1-phenylmethanamine according to general procedure to give the desired product **5i** as orange crystal (70% yield), mp 103–105 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.3 Hz, 1H), 7.33 (d, *J* = 4.3 Hz, 4H), 7.25 (d, *J* = 4.5 Hz, 1H), 6.84 (s, 1H), 6.78–6.72 (m, 4H), 4.06 (t, *J* = 6.5 Hz, 2H), 3.50 (s, 2H), 3.07 (s, 6H), 2.47–2.32 (m, 2H), 2.21 (s, 3H), 1.90–1.81 (m, 2H), 1.65–1.54 (m, 2H), 1.52–1.39 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.57, 167.68, 166.29, 151.06, 145.89, 139.25, 133.29, 129.08, 128.23, 126.92, 125.34, 120.08, 115.39, 113.86, 112.16, 111.89, 96.82, 68.75, 62.40, 57.37, 42.35, 40.11, 28.99, 27.37, 27.14, 25.94. MS (ESI^+): *m*/*z*: 485.3$[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{36}N_2O_3$: C, 76.83; H, 7.49; N, 5.78. Found: C, 76.42; H, 7.47; N, 5.69.

4.5.10. 7-(8-(Diethylamino)octyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5j)

Intermediate **4c** was treated with diethylamine according to general procedure to give the desired product **5j** as yellow solid (65% yield), mp 100–102 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 8.4 Hz, 1H), 6.84 (s, 1H), 6.77–6.70 (m, 4H), 4.07 (t, *J* = 6.5 Hz, 2H), 3.07 (s, 6H), 2.57 (q, *J* = 7.1 Hz, 4H), 2.49–2.42 (m, 2H), 1.89–1.80 (m, 2H), 1.48 (d, *J* = 7.1 Hz, 4H), 1.37–1.31 (m, 8H), 1.05 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.58, 167.71, 166.32, 151.09, 145.92, 133.26, 125.37, 120.16, 115.40, 113.79, 112.13, 111.91, 96.84, 68.81, 52.94, 46.86, 40.10, 29.52, 29.30, 28.97, 27.63, 26.89, 25.93, 11.57. MS (ESI^+): *m*/*z*: 465.3$[M+H]^+$. Elemental Anal. Calcd for $C_{29}H_{40}N_2O_3{\cdot}0.4C_2H_5OH$: C, 74.09; H, 8.85; N, 5.80. Found: C, 74.02; H, 8.68; N, 5.81.

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

Intermediate **4c** was treated with pyrrolidine according to general procedure to give the desired product **5k** as yellow solid (61% yield), mp 89–91 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.9 Hz, 2H), 7.67 (d, *J* = 8.5 Hz, 1H), 6.81 (s, 1H), 6.76–6.65 (m, 4H), 4.04 (t, *J* = 6.5 Hz, 2H), 3.04 (s, 6H), 2.51 (s, 4H), 2.46–2.37 (m, 2H), 1.87–1.74 (m, 6H), 1.57–1.43 (m, 4H), 1.35 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.53, 167.68, 166.31, 151.08, 145.90, 133.25, 125.33, 120.14, 115.39, 113.77, 112.12, 111.90, 96.84, 68.80, 56.67, 54.24, 40.07, 29.48, 29.24, 29.01, 28.96, 27.64, 25.91, 23.40. MS (ESI^+): *m*/*z*: 463.3$[M+H]^+$. Elemental Anal. Calcd for $C_{29}H_{38}N_2O_3$: C, 75.29; H, 8.28; N, 6.06. Found: C, 74.98; H, 8.28; N, 5.93.

4.5.12. 2-(4-(Dimethylamino)phenyl)-7-(8-(piperidin-1-yl)octyloxy)-4*H*-chromen-4-one (5l)

Intermediate **4c** was treated with piperidine according to general procedure to give the desired product **5l** as yellow solid (63% yield), mp 130–132 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 8.3 Hz, 1H), 6.84 (s, 1H), 6.79–6.68 (m, 4H), 4.07 (t, *J* = 6.5 Hz, 2H), 3.07 (s, 6H), 2.36 (d, *J* = 28.0 Hz, 4H), 2.29 (d, *J* = 7.9 Hz, 2H), 1.88–1.79 (m, 2H), 1.65–1.58 (m, 4H), 1.56–1.43 (m, 6H), 1.40–1.29 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.55, 167.69, 166.31, 151.08, 145.91, 133.25, 125.34, 120.15, 115.39, 113.77, 112.12, 111.90, 96.84, 68.80, 59.66, 54.68, 40.09, 29.50, 29.25, 28.97, 27.68, 26.94, 26.00, 25.91, 24.51. MS (ESI^+): *m*/*z*: 477.3$[M+H]^+$. Elemental Anal. Calcd for $C_{30}H_{40}N_2O_3{\cdot}0.4C_2H_5OH$: C, 74.72; H, 8.63; N, 5.66. Found: C, 74.97; H, 8.47; N, 5.76.

4.5.13. 7-(8-(Benzyl(methyl)amino)octyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5m)

Intermediate **4c** was treated with *N*-methyl-1-phenylmethanamine according to general procedure to give the desired product **5m** as orange solid (46% yield), mp 80–82 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.8 Hz, 2H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.31 (d,

J = 4.4 Hz, 4H), 7.24 (d, J = 4.3 Hz, 1H), 6.83 (s, 1H), 6.76–6.71 (m, 4H), 4.05 (t, J = 6.5 Hz, 2H), 3.48 (s, 2H), 3.05 (s, 6H), 2.45–2.29 (m, 2H), 2.18 (s, 3H), 1.87–1.78 (m, 2H), 1.51–1.47 (m, 4H), 1.34 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.57, 167.71, 166.33, 151.10, 145.93, 133.27, 129.11, 128.21, 126.93, 125.37, 120.16, 115.41, 113.79, 112.14, 111.92, 96.86, 68.82, 62.31, 57.50, 42.26, 40.10, 29.47, 29.31, 28.98, 27.35, 25.93. MS (ESI^+): m/z: 513.4$[M+H]^+$. Elemental Anal. Calcd for $C_{33}H_{40}N_2O_3{\cdot}0.4C_2H_5OH$: C, 76.44; H, 8.05; N, 5.27. Found: C, 76.46; H, 7.95; N, 5.27.

4.5.14. 7-(10-(Diethylamino)decyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5n)

Intermediate **4d** was treated with diethylamine according to general procedure to give the desired product **5n** as yellow solid (58% yield), mp 101–102 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91–7.76 (m, 2H), 7.76–7.62 (m, 1H), 6.83 (s, 1H), 6.81–6.68 (m, 4H), 4.06 (t, J = 6.6 Hz, 2H), 3.06 (s, 6H), 2.53 (q, J = 7.2 Hz, 4H), 2.43–2.39 (m, 2H), 1.92–1.75 (m, 2H), 1.46–1.42 (m, 4H), 1.40–1.26 (m, 10H), 1.03 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.56, 167.71, 166.33, 151.09, 145.93, 133.25, 125.36, 120.18, 115.41, 113.74, 112.12, 111.91, 96.85, 68.83, 53.03, 46.90, 40.09, 29.63, 29.55, 29.51, 29.33, 28.99, 27.73, 27.03, 25.96, 11.68. MS (ESI^+): m/z: 493.4$[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{44}N_2O_3$: C, 75.57; H, 9.00; N, 5.69. Found: C, 75.25; H, 9.03; N, 5.54.

4.5.15. 2-(4-(Dimethylamino)phenyl)-7-(10-(pyrrolidin-1-yl)decyloxy)-4*H*-chromen-4-one (5o)

Intermediate **4d** was treated with pyrrolidine according to general procedure to give the desired product **5o** as yellow solid (47% yield), mp 98–100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, J = 8.9 Hz, 2H), 7.68 (d, J = 8.3 Hz, 1H), 6.82 (s, 1H), 6.76–6.69 (m, 4H), 4.05 (t, J = 6.5 Hz, 2H), 3.05 (s, 6H), 2.54 (s, 4H), 2.48–2.42 (m, 2H), 1.87–1.75 (m, 6H), 1.58–1.43 (m, 4H), 1.35–1.31 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.58, 167.70, 166.32, 151.07, 145.90, 133.26, 125.35, 120.12, 115.37, 113.81, 112.15, 111.89, 96.82, 68.82, 56.70, 54.24, 40.10, 29.57, 29.50, 29.32, 28.97, 28.93, 27.69, 25.95, 23.38. MS (ESI^+): m/z: 491.3$[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{42}N_2O_3{\cdot}0.5C_2H_5OH$: C, 74.82; H, 8.83; N, 5.45. Found: C, 74.88; H, 8.57; N, 5.71.

4.5.16. 2-(4-(Dimethylamino)phenyl)-7-(10-(piperidin-1-yl)decyloxy)-4*H*-chromen-4-one (5p)

Intermediate **4d** was treated with piperidine according to general procedure to give the desired product **5p** as yellow solid (52% yield), mp 105–107 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 9.0 Hz, 2H), 7.70 (d, J = 8.4 Hz, 1H), 6.84 (s, 1H), 6.74 (m, 4H), 4.07 (t, J = 6.5 Hz, 2H), 3.07 (s, 6H), 2.38 (s, 4H), 2.28 (d, J = 8.1 Hz, 2H), 1.89–1.80 (m, 2H), 1.64–1.57 (m, 4H), 1.55–1.43 (m, 7H), 1.33–1.27 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.61, 167.72, 166.34, 151.09, 145.93, 133.27, 125.39, 120.17, 115.40, 113.79, 112.14, 111.92, 96.84, 68.83, 59.71, 54.68, 40.12, 29.60, 29.51, 29.33, 28.98, 27.77, 26.96, 25.97, 24.50. MS (ESI^+): m/z: 505.3$[M+H]^+$. Elemental Anal. Calcd for $C_{32}H_{44}N_2O_3$: C, 76.15; H, 8.79; N, 5.55. Found: C, 76.29; H, 9.13; N, 5.31.

4.5.17. 7-(10-(Benzyl(methyl)amino)decyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5q)

Intermediate **4d** was treated with *N*-methyl-1-phenylmethanamine according to general procedure to give the desired product **5q** as yellow needle-like crystal (50% yield), mp 82–84 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 8.9 Hz, 2H), 7.70 (d, J = 8.7 Hz, 1H), 7.33 (d, J = 4.4 Hz, 4H), 7.27–7.24 (m, 1H), 6.84 (s, 1H), 6.79–6.71 (m, 4H), 4.07 (t, J = 6.6 Hz, 2H), 3.49 (s, 2H), 3.07 (s, 6H), 2.45–2.30 (m, 2H), 2.20 (s, 3H), 1.90–1.80 (m, 2H), 1.58–1.45 (m, 4H), 1.34 (m, J = 23.5 Hz, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.59, 167.72, 166.34, 151.10, 145.94, 133.26, 129.08, 128.18, 126.86, 125.39, 120.19, 115.41, 113.77, 112.13, 111.93, 96.86, 68.84, 62.34, 57.61, 42.30, 40.11, 29.59, 29.56, 29.52, 29.34, 28.99, 27.45, 25.97. MS (ESI^+): m/z: 541.3$[M+H]^+$. Elemental Anal. Calcd for $C_{35}H_{44}N_2O_3$: C, 77.74; H, 8.20; N, 5.18. Found: C, 77.78; H, 8.19; N, 5.13.

4.5.18. 7-(12-(Benzyl(methyl)amino)dodecyloxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (5r)

Intermediate **4e** was treated with *N*-methyl-1-phenylmethanamine according to general procedure to give the desired product **5r** as yellow crystal (45% yield), mp 66–68 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, J = 8.9 Hz, 2H), 7.67 (d, J = 8.6 Hz, 1H), 7.31 (d, J = 4.4 Hz, 4H), 7.26–7.21 (m, 1H), 6.82 (s, 1H), 6.74–6.66 (m, 4H), 4.03 (t, J = 6.5 Hz, 2H), 3.47 (s, 2H), 3.04 (s, 6H), 2.39–2.31 (m, 2H), 2.17 (s, 3H), 1.87–1.77 (m, 2H), 1.55–1.41 (m, 4H), 1.38–1.24 (m, 14H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.59, 167.70, 166.33, 151.07, 145.91, 139.25, 133.28, 129.10, 128.19, 126.88, 125.36, 120.12, 115.38, 113.83, 112.16, 111.90, 96.82, 68.85, 62.33, 57.63, 42.31, 40.12, 29.67, 29.65, 29.62, 29.60, 29.39, 29.01, 27.50, 27.46, 26.00. MS (ESI^+): m/z: 569.4$[M+H]^+$. Elemental Anal. Calcd for $C_{37}H_{48}N_2O_3$: C, 78.13; H, 8.51; N, 4.93. Found: C, 78.09; H, 8.52; N, 4.85.

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.[27] In vitro BChE assay use the similar method described above. Each concentration was assayed in triplicate.

4.6.2. Kinetic characterization of AChE inhibition

Kinetic characterization of AChE was performed using a reported method.[23] 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.

4.6.3. Molecular modeling

The crystal structure of the torpedo AChE (code ID: 1ACJ) and the human BChE (code ID: 1POI) were obtained in the Protein Data Bank after eliminating the inhibitor and water molecules. The 3D structure of compounds **5j** and **5o** was built and performed geometry optimization by molecular mechanics. Further preparation of substrates included addition of Gasteiger charges, removal of hydrogen atoms and addition of their atomic charges to skeleton

atoms, and finally, assignment of proper atomic types. Autotors was then used to define the rotatable bonds in the ligand.

Docking studies were carried out using the AUTODOCK 4.0 programe 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]; BChE [135.628 111.375 39.905]). 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. To ensure the reliability of the results, the docking procedures were repeated 10 independent times for the compounds and the obtained orientations were analyzed.

4.6.4. Inhibition of self-mediated Aβ(1–42) aggregation

In order to investigate the self-mediated Aβ(1–42) aggregation, a thioflavin-T fluorescence assay was performed.[30] Aβ(1–42) peptide (Anaspec Inc) was dissolved in phosphate buffer (pH 7.40, 0.01 M) to obtain a 20 μM solution. Compounds were firstly prepared in DMSO to obtain a 10 mM solution. The final concentration of Aβ(1–42) and inhibitors were 20 μM. After incubated in 37 °C for 48 h, thioflavin-T (5 μM in 50 mM glycine–NaOH buffer, pH 8.00) was added. Fluorescence was measured at 450 nm (λ_{ex}) and 485 nm (λ_{em}). Each inhibitor was run in triplicate. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated by the following expression: $(1 - I_{Fi}/I_{Fc})^*100\%$ in which I_{Fi} and I_{Fc} were the fluorescence intensities obtained for absorbance in the presence and absence of inhibitors, respectively, after subtracting the fluorescence of respective blanks.

4.6.5. Measurement of the anti-oxidation activity

The antioxidation activity was determined by using the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay. The ORAC-assay measures antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C.

The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant (20 μL) and fluorescein (120 μL, 300 nM final concentration) were placed in the wells of a black 96 well plate, and the mixture was incubated for 15 min at 37 °C. Then AAPH solution (60 μL; 12 mM final concentration) was added rapidly. The plate was immediately placed into a Spectrafluor Plus plate reader (Tecan, Switzerland), and the fluorescence was measured every 60 s for 90 min with exitation at 485 nm and emission at 535 nm. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard (0.625–5 μM, final concentration). A blank (FL + AAPH) using phosphate buffer instead of antioxidant and Trolox calibration were carried out in each assay. The samples were measured at different concentrations (0.625–5 μM), and at least four independent runs were performed for each sample. Fluorescence measurements were normalized based on the curve of the blank (without antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

(1). $AUC = 1 + \sum_{i=1}^{i=90} f_i/f_0$ where f_0 is the initial fluorescence at 0 min, and f_i is the fluorescence at time i. The net AUC for a sample was calculated as following:

(2). Net $AUC = AUC_{antioxidant} - AUC_{blank}$; The ORAC-FL values were calculated as following:

(3). $[(AUC_{sample} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank})] \times$ [(concentration of Trolox/concentration of sample)], and expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were in μM of Trolox equivalent/μM of pure compound.

Acknowledgements

We thank the Natural Science Foundation of China (Nos. 21172053, 21302041), the Postdoctoral Science Foundation of China (No. 2012M521391), and the Postdoctoral Science Foundation of Henan Province (No. BH2011043) for financial support of this study.

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