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Original article

Design, synthesis and evaluation of genistein-*O*-alkylbenzylamines as potential multifunctional agents for the treatment of Alzheimer's disease

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ABSTRACT

A series of genistein derivatives with carbon spacer-linked alkylbenzylamines were designed, synthesized and tested as multifunctional agents for the treatment of Alzheimer's disease (AD). The results showed that most of these compounds exhibited good acetylcholinesterase (AChE) inhibitory activity, with moderate-to-good anti-oxidative activity. Specifically, compounds **10b**, **19d** and **25d** exhibited significant inhibition of β-amyloid (Aβ) aggregation and exhibited metal chelating properties. In particular, **25d** inhibited: self-induced $A\beta_{1-42}$ aggregation, Cu^{2+}-induced $A\beta_{1-42}$ aggregation, and human AChE-induced $A\beta_{1-40}$ aggregation by 35%, 77.8%, and 36.2%, respectively. Moreover, both kinetic analysis of AChE inhibition and the molecular modeling study suggested that **25d** binds simultaneously to catalytic active site and peripheral anionic site of AChE. More importantly, compound **25d** disassembled the well-structured Aβ fibrils generated by Cu^{2+}-induced Aβ aggregation by 72.1%. Furthermore, the step-down passive avoidance test showed this compound significantly reversed scopolamine-induced memory deficit in mice. These results suggest that **25d** may be a promising multifunctional agent for AD treatment.

1. Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disease characterized by progressive and irreversible cognitive impairments, and severe behavioral abnormalities, and ultimately causing death [1]. Although many factors have been implicated in AD over the last decades, its etiology is not still completely known. Thus far, low levels of acetylcholine (ACh), oxidative stress, the disequilibrium of biometals and β-amyloid (Aβ) deposits have been considered to play definitive roles in AD pathogenesis, and several hypotheses based on these factors have been proposed to explain the mechanism of AD pathogenesis [2]. The cholinergic hypothesis for AD suggests that cognitive deterioration is mainly caused by low levels of acetylcholine (ACh) because maintaining ACh levels (via acetylcholinesterase [AChE] inhibitors) has been shown to alleviate these symptoms [3]. AChE also accelerates the aggregation of amyloid fibrils, which is thought to involve the peripheral anionic site (PAS) of AChE [4]. The ability for AChE inhibitors to simultaneously bind to its catalytic and peripheral binding sites has thus become an area of very active research [5]. Furthermore, several studies have suggested that inhibition of a similar form of AChE, butyrylcholinesterase (BuChE), is a desirable activity in anti-Alzheimer compounds [6]. For example, rivastigmine, active on both enzymes, is currently in use. However, serious inhibition of BuChE also may contribute to the peripheral side effects of cholinesterase inhibitors, such as the dual AChE and BuChE inhibitor, tacrine, which has shown severe hepatotoxicity as well as other adverse effects [7].

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; *Hu*AChE, human AChE; *Ee*AChE, *Electrophorus electricus* AChE; Aβ, β-amyloid peptide; CAS, catalytic active site; PAS, peripheral anionic site; MOM, methoxymethyl ether; PBS, phosphate-buffered saline; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; PDB, Protein Data Bank; *Tc*AChE, *Torpedo californica* AChE; ROS, reactive oxygen species; ORAC-FL, Oxygen Radicals Absorbance Capacity by Fluorescence; AAPH, 2,2′-Azobis(amidinopropane) dihydrochloride; ThT, thioflavin T; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid.

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Therefore, the development of AChE inhibitors for the treatment of AD, with expectation of their fewer side effects, may be a more suitable approach as a therapeutic strategy.

Increasing evidence supports the significant impact of oxidative stress in the pathogenesis of AD [8]. Recent studies have indicated that oxidative damage may promote the appearance of amyloid plaques and neurofibrillary (NFT) tangles in AD [9]. Therefore, drugs aimed at clearing or preventing the formation of the free radicals may be useful for either the prevention or effective treatment of AD. Another hypothesis indicates that metal ions (Cu^{2+}, Fe^{2+}, Zn^{2+} and Al^{3+}) may play an important role in the pathogenesis of AD [10]. Metals have been observed to progressively accumulate in AD patients during disease progression from moderate-to-severe AD. The abnormal accumulation of metals is closely associated with the formation of Aβ plaques and neurofibrillary tangles [11]. Abnormally high brain levels of redox-active metal ions, like Cu^{2+} and Fe^{2+}, may contribute to the production of reactive oxygen species (ROS), which promotes oxidative stress thus contributing to AD pathogenesis [12]. Therefore, lowering the concentration of brain metals by chelating metals represents an additional rational approach for the treatment of AD.

According to the amyloid hypothesis, aggregation and deposition of Aβ peptide is considered to be crucial for the pathogenesis of AD because its accumulation may result in a cascade of biochemical events leading to neuronal dysfunction [13]. Aβ_{1-40} and Aβ_{1-42} are the main isoforms of Aβ peptides. Although the amount of Aβ_{1-42} is only 10% of Aβ_{1-40}, Aβ_{1-42} tends to aggregate more rapidly and displays stronger neuronal toxicity than Aβ_{1-40} [14]. Preventing the formation and aggregation of Aβ is a potential therapeutic strategy for AD treatment.

Current treatments of AD focus on the symptomatic aspects of its pathology. These drugs, which are currently approved by the FDA, inhibit AChE to increase the level of ACh. These AChE inhibitors include: tacrine (now discontinued), donepezil, rivastigmine and galantamine. Alternatively, memantine has also been used to antagonize *N*-methyl-D-aspartate (NMDA) receptors to prevent aberrant neuronal stimulation [15]. In clinical trials, however, the impact of these single target drugs on this disease has been modest and transient because of the multifactorial nature of AD. However, observational studies have suggested that combination treatments may increase the time before patients require nursing home care [16]. Nevertheless, studies thus far have not provided convincing evidence that these agents can prevent, halt, or reverse the disease [15]. Therefore, despite the difficulties, the development of agents that affect two or more biological activities that correlate with those causing AD has drawn considerable attention for their advancement in the treatment of AD [17–20]. The discovery of a lead compound with the potential to form part of a multifunctional drug is a crucial step in the search for clinical candidate for the treatment of AD.

Genistein (4′,5,7-trihydroxyisoflavone) is the most abundant isoflavone in soybeans and also produced abundantly by red clover containing plants (*eg. Trifolium pratense*), possessing a broad range of pharmacological properties, such as estrogen activity, anti-inflammatory, anti-oxidative and metal chelating effects, and neuroprotective effects against Aβ [21–25]. These results indicate that genistein may be used as a starting compound in the design of multifunctional drugs for the treatment of AD. However, the lack of AChE inhibitory activity and low bioavailability of genistein restricts its clinical activity as an anti-AD drug [26]. The 1-benzylpiperidine fragment of donepezil has been shown as the cholinesterase inhibitory pharmacophore [4]. Recently, many studies have revealed compounds that bear an amine functional group on the alkyl side chain consist of a lipophilic moiety and often a tertiary amino group, which were found to be the key requirement for adequate AChE inhibition [27]. Therefore, we aimed to use a multi-target-directed drug design strategy to combine genistein with appropriate secondary amines using carbon spacers of different lengths. These new molecules may simultaneously possess a dual binding site for AChE inhibition, anti-oxidative and metal chelating effects, and inhibit Aβ aggregation.

In this study, a series of genistein-*O*-alkylbenzylamine derivatives were designed, synthesized and evaluated for their biological activity, including inhibition of cholinesterase, antioxidant and metal chelating effects, inhibitory effects on Aβ aggregation, and neuroprotective effects in the mouse scopolamine model of memory impairment. The chemical design strategy for genistein derivatives is depicted in Fig. 1.

2. Results and discussion

2.1. Chemistry

The synthesis of genistein-*O*-alkylbenzylamine derivatives (**8**–**10**, **18**–**20** and **24**–**26**) occurred via the general pathway, for which genistein was used as the starting material (Schemes 1–3). The 7-OH group of genistein is more acidic than the 4′-OH group [28]. However, the 4′-phenolate or 4′-OH group is a better nucleophile than the 7-phenolate or 7-OH group, respectively. The hydrogen bond-stabilized 5-OH group is the less nucleophilic group of genistein [29]. The difference in reactivity thus allows for easy regioselectivity, for substitutions at 4′-OH or/and 7-OH positions. Synthesis of 7-*O*-modified genistein derivatives **8**–**10** is shown in Scheme 1. Compounds **2**–**4** were the key intermediates, usually prepared from alkylation of the 7-OH group by using haloalkanes in the presence of a base, as previously described [30,31]. Thus, genistein was alkylated with the equivalent amount of KOH in *N*,*N*-dimethylformamide followed by the addition of excessive amounts of 1,3-dibromopropane, 1,4-dibromobutane or 1,6-dibromohexane furnished intermediates **2**–**4**. Subsequently, compounds **2**–**4** were reacted with two equivalent secondary amines **7a**–**d**, anhydrous K_2CO_3 and the catalytic amount of KI in CH_3CN, at 60–65 °C. The amines **7a**–**d** were synthesized by reductive amination of methylamine or ethylamine with benzaldehydes in the presence of $NaBH_4$ [32]. This process produced the desired target compounds, **8a**–**d**, **9a**–**d**, and **10a**–**d** in moderate-to-good yields.

For the synthesis of 4′-*O*-modified genistein derivatives, **18**–**20**, the 7-OH group of genistein was protected (Scheme 2). Firstly, genistein was reacted with KOH, and then chloromethyl methyl ether (MOM-Cl) was added (dropwise) to produce compound **11**, of which the 7-OH group was protected by MOMCl [33]. Compound **11** was then reacted with excessive amounts of 1,3-dibromopropane, 1,4-dibromobutane or 1,6-dibromohexane in the presence of K_2CO_3 and the catalytic amount of KI in CH_3CN at 65 °C to obtain the intermediates, **12**–**14**. In turn, these intermediates were reacted with the corresponding compounds, **7a**–**d**, respectively, according to the procedure adopted for **8a**–**d** to provide the key intermediates **15a**–**d**, **16a**–**d** and **17a**–**d**. Finally, HCl-mediated removal of methoxymethyl ether (MOM) protecting group gave the corresponding target compounds, **18a**–**d**, **19a**–**d and 20a**–**d**.

The new compounds, **21**–**23**, were the key intermediates for the synthesis of 7,4′-*O*-modified genistein derivatives, **24**–**26** (Scheme 3), which were prepared by treatment of genistein with 4 equivalent of 1,3-dibromopropane, 1,4-dibromobutane or 1,6-dibromohexane under reflux for 8–10 h in acetone. This step was carried out in the presence of anhydrous K_2CO_3 and the catalytic amount of KI. Because the 5-OH group was stabilized by the hydrogen bond, the trisubstituted compounds were not produced under these conditions. Subsequently, compounds **21**–**23** were

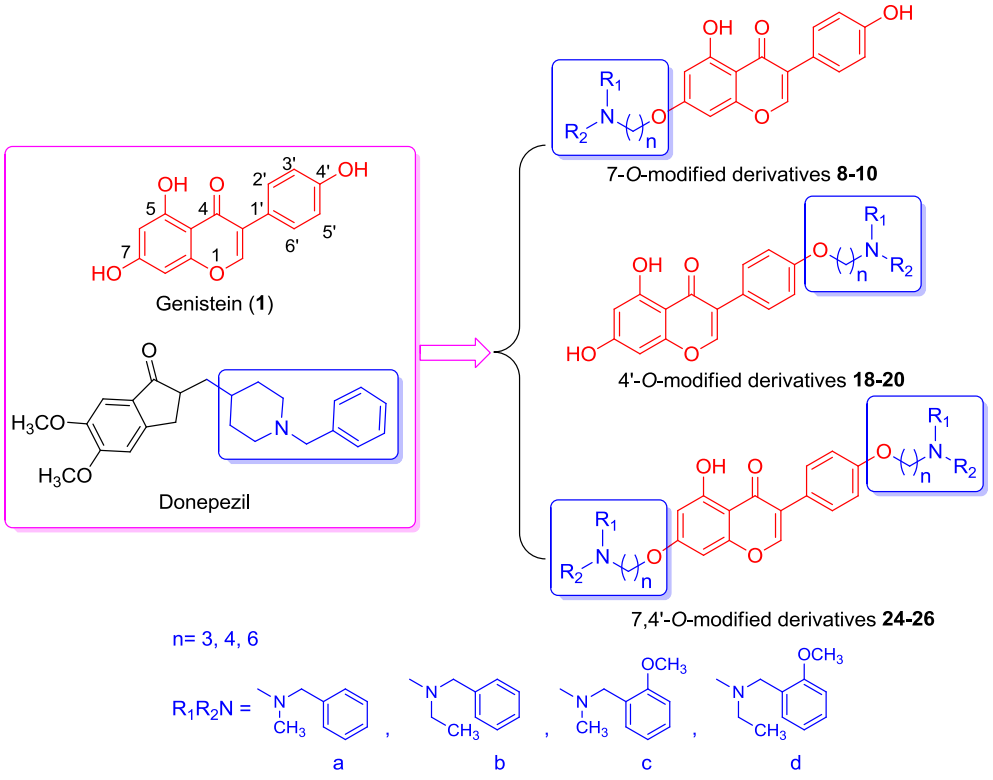
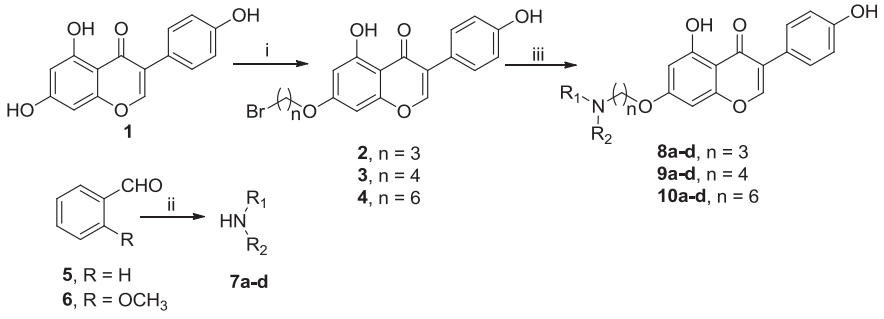
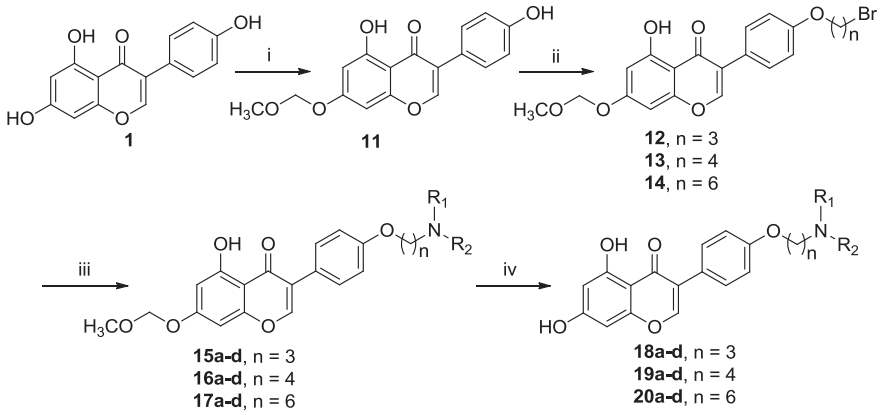


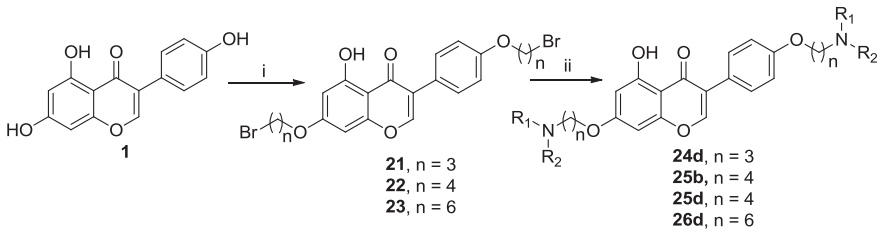
Fig. 1. Design strategy for the genistein-*O*-alkylbenzylamine derivatives.



Scheme 1. Synthesis of 7-*O*-modified genistein derivatives **8**–**10**. *Reagents and conditions*: (i) KOH, DMF, at room temperature (rt), for 5 h, then $Br(CH_2)_nBr$, at rt, for 24 h; (ii) $MeNH_2$ or $EtNH_2$, $NaBH_4$, CH_3OH, at rt, for 5–6 h; (iii) R_1R_2NH (**7a**–**d**), K_2CO_3, KI, CH_3CN, at 65 °C, for 12–15 h.



Scheme 2. Synthesis of 4′-*O*-modified genistein derivatives **18**–**20**. *Reagents and conditions*: (i) KOH, DMF, for 1 h, then CH_3OCH_2Cl, at rt, for 12 h; (ii) $Br(CH_2)_nBr$, K_2CO_3, KI, CH_3CN, at 65 °C, for 12–15 h; (iii) R_1R_2NH (**7a**–**d**), K_2CO_3, KI, CH_3CN, at 65 °C, for 12–15 h; (iv) 10% HCl/EtOH, reflux for 3 h.



Scheme 3. Synthesis of 7, 4′-*O*-modified genistein derivatives **24**–**26**. *Reagents and conditions*: (i) $Br(CH_2)_nBr$, K_2CO_3, KI, acetone, reflux for 8 h; (ii) *N*-benzylethylamine (**7b**) or *N*-(2-methoxybenzyl)ethylamine (**7d**), K_2CO_3, KI, CH_3CN, at 65 °C, for 12–15 h.

reacted with *N*-benzylethylamine (**7b**) or *N*-(2-methoxybenzyl) ethylamine (**7d**) to provide the final compounds, **24**–**26**.

All new compounds were purified using recrystallization or chromatography, and the analytical and spectroscopic data confirmed their structures, as detailed in the experimental section.

2.2. Pharmacology

2.2.1. Inhibition studies on AChE and BuChE

To study the multipotent profiles of the three novel series of genistein derivatives (**8**–**10a**–**d**, **18**–**20a**–**d**, **24d**, **25b**, **25d** and **26d**), they were first evaluated as inhibitors of AChE according to Ellman's method [34] but with some modifications. The selectivity of the compounds was also tested by determining their inhibitory activities against BuChE. AChE and BuChE activity was measured from rat cortex homogenate and rat serum, respectively. Moreover, the most potent and representative compounds were selected and re-evaluated using *Electrophorus electricus* AChE (*Ee*AChE) and human erythrocyte AChE (*Hu*AChE). Donepezil was used as reference compound.

The target compounds showed different amounts of inhibitory activity to AChE with IC_{50} values ranging from the submicromolar to micromolar range (Table 1). Compound **25d** exhibited the highest potency to inhibit AChE ($IC_{50} = 0.09 \pm 0.02$ μM). The general trend for AChE inhibition was 7,4′-*O*-modified > 7-*O*-modified >4′-*O*-modified genistein derivatives (with the only exception of **9a**, which was much less potent than **19a**), and all synthetic derivatives exhibited much higher inhibitory activity against AChE compared

Table 1
Inhibition of AChE and BuChE activity, and oxygen radical absorbance capacity (ORAC, Trolox Equivalents) by genistein derivatives, genistein and donepezil.

Compound	IC_{50} (μM) ± SD[a]			BuChE inhibition (%)[e]	ORAC[f]
	Rat AChE[b]	*Ee*AChE[c]	*Hu*AChE[d]		
8a	36.29 ± 2.28	NT[g]	NT[g]	7.4	0.89 ± 0.08
8b	1.42 ± 0.07	NT[g]	NT[g]	9.9	1.12 ± 0.06
8c	13.55 ± 1.08	NT[g]	NT[g]	n.a.[h]	1.05 ± 0.10
8d	0.47 ± 0.04	NT[g]	NT[g]	n.a.[h]	1.02 ± 0.15
9a	54.50 ± 7.70	NT[g]	NT[g]	8.9	1.00 ± 0.17
9b	2.42 ± 0.13	NT[g]	NT[g]	10.4	2.40 ± 0.11
9c	5.55 ± 1.04	NT[g]	NT[g]	n.a.[h]	1.56 ± 0.10
9d	0.83 ± 0.09	NT[g]	NT[g]	7.7	1.06 ± 0.04
10a	4.24 ± 0.20	NT[g]	NT[g]	n.a.[h]	0.65 ± 0.02
10b	0.35 ± 0.01	4.98 ± 0.38	5.80 ± 0.65	n.a.[h]	1.20 ± 0.06
10c	3.19 ± 0.08	NT[g]	NT[g]	na[h]	2.70 ± 0.12
10d	0.53 ± 0.05	NT[g]	NT[g]	n.a.[h]	0.56 ± 0.01
18a	49.25 ± 3.49	NT[g]	NT[g]	n.a.[h]	0.46 ± 0.01
18b	42.83 ± 3.78	NT[g]	NT[g]	11.6	0.58 ± 0.07
18c	37.43 ± 1.05	NT[g]	NT[g]	n.a.[h]	0.62 ± 0.02
18d	16.01 ± 0.80	NT[g]	NT[g]	17.6	0.64 ± 0.02
19a	3.79 ± 0.59	NT[g]	NT[g]	10.8	0.32 ± 0.03
19b	12.80 ± 1.41	NT[g]	NT[g]	n.a.[h]	0.60 ± 0.03
19c	5.31 ± 0.54	NT[g]	NT[g]	9.7	1.10 ± 0.08
19d	1.45 ± 0.09	15.90 ± 2.10	3.88 ± 0.64	6.7	1.10 ± 0.05
20a	3.54 ± 0.24	NT[g]	NT[g]	7.7	0.30 ± 0.01
20b	38.45 ± 1.07	NT[g]	NT[g]	n.a.[h]	0.29 ± 0.01
20c	5.62 ± 0.79	NT[g]	NT[g]	n.a.[h]	0.40 ± 0.02
20d	1.69 ± 0.10	NT[g]	NT[g]	n.a.[h]	0.38 ± 0.01
24d	0.21 ± 0.03	0.16 ± 0.04	NT[g]	12.5	0.49 ± 0.03
25b	0.90 ± 0.06	NT[g]	NT[g]	10.3	0.26 ± 0.01
25d	0.09 ± 0.02	0.14 ± 0.003	0.35 ± 0.03	20.0	0.30 ± 0.01
26d	0.51 ± 0.08	3.79 ± 0.17	NT[g]	11.6	0.13 ± 0.01
Genistein	n.a.[h]	n.a.[h]	n.a.[h]	n.a.[h]	5.00 ± 0.32
Donepezil	0.015 ± 0.002	0.12 ± 0.01	0.011 ± 0.001	$IC_{50} = 20.7 \pm 1.36$ μM	NT[g]

[a] IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of 3 independent experiments, each performed in triplicate (SD = standard deviation).
[b] From 5% rat cortex homogenate.
[c] From *Electrophorus electricus*.
[d] From human erythrocytes.
[e] BuChE from rat serum was used and tested compounds were used at 50 μM.
[f] The mean ± SD of the 3 independent experiments. Data are expressed as μM of Trolox equivalent/μM of tested compound.
[g] NT = not tested.
[h] n.a. = no active. Compounds defined "no active" means a percent inhibition of less than 5.0% at a concentration of 50 μM in the assay conditions.

with the original lead compound, genistein. The results showed that the introduction of the *O*-alkylbenzylamine group to genistein significantly increased the inhibitory activity. The length of the linker and the benzylamine moiety were varied to optimize the interaction of the inhibitors with both the catalytic and peripheral binding sites of AChE. The potency of the derivatives to inhibit AChE was related to the length of alkylene. In general, 7-*O*-modified genistein derivatives with a 6-methylene linker between benzylamine and 7-OH of genistein showed better inhibition of AChE than those with a 3-methylene or 4-methlene chain. For example, compounds **10a**–**c** with a linker of 6 methylene groups were more potent than compounds **8a**–**c** and **9a**–**c**. However, 4′-*O*-modified derivatives with a 4-methylene linker between benzylamine and 4′-OH of genistein showed better inhibition of AChE than those with a 3-methylene or 6-methylene chain. The 7,4′-*O*-modified compound, **25d**, had an IC_{50} of 0.09 ± 0.02 μM, and with a 4-methylene double linkage, this compound displayed the most potent AChE inhibitory activity. Compared with **25d**, the activity of **24d** (with a 3-methylene linker) was 2-fold lower (IC_{50} = 0.21 ± 0.03 μM), and the activity of **26d** (with a 6-methylene linker) was 5-fold lower (IC_{50} = 0.51 ± 0.08 μM). Therefore, the alkyl chain with 4 or 6 methylenes may have been beneficial for AChE inhibitory activity. The results indicated that genistein-*O*-alkylbenzylamine derivatives required side chains of a suitable length to bind to the AChE. The results also revealed that the benzylamine moiety was crucial for AChE inhibition. The substituents on the terminal side chain of nitrogen also played a significant role in the inhibitory activity. AChE inhibitory activity was generally improved with compounds possessing an *N*-ethyl group (**8c**–**d**, **9c**–**d**, **10c**, **18c**–**d**, **19d**, **20d**) compared with compounds possessing an *N*-methyl group (**8a**–**b**, **9a**–**b**, **10a**, **18a**–**b**, **19b**, **20b**). In contrast, AChE inhibitory activity was ameliorated with compounds exhibiting an *N*-2-methoxybenzyl group compared with compounds bearing an *N*-benzyl group (except **10b**). Compound **10b** possessed an *N*-benzyl group but was slightly more potent (IC_{50} = 0.35 ± 0.01 μM) than **10d** (IC_{50} = 0.53 ± 0.05 μM), which had an *N*-2-methoxybenzyl group. Taken together, the results suggested that *N*-(2-methoxybenzyl) ethylamine may be the optimal substitution pattern for AChE inhibition. Compounds **10b**, **19d**, and **25d** displayed the most potent inhibitory activity for rat AChE (IC_{50} = 0.35 ± 0.01 μM, 1.45 ± 0.09 μM and 0.09 ± 0.02 μM, respectively). Furthermore, they were re-evaluated using *Ee*AChE and *Hu*AChE. Inhibition of *Ee*AChE and *Hu*AChE by compounds **10b**, **19d** and **20d** was generally less potent than that of rat AChE (Table 1).

Almost all genistein-*O*-alkylbenzylamine derivatives (50 μM) were inactive or weak on BuChE activity. This result showed that these target compounds were potent AChE inhibitors with high selectivity toward AChE. This selectivity profile may be a limitation, but this also may be beneficial to diminish peripheral cholinergic side effects and provide lower toxicity. For instance, severe side effects of AChE inhibitors, such as tacrine, have been suggested to be attributed to their poor selectivity [35].

Based on the above assay results, we next selected the most potent AChE inhibitor, **25d**, for kinetic analysis to investigate the type of inhibition.

2.2.2. Kinetic studies for the inhibition of AChE

To gain further insight into the mechanism of action of this family of compounds, a kinetics study was carried out with compound **25d** using *Ee*AChE. Graphical analysis of the reciprocal Lineweaver–Burk plots (Fig. 2) showed that with increasing concentrations of **25d**, both the slopes (decreased V_{max}) and intercepts (higher K_m) were increased. This pattern indicated a mixed-type inhibition. Replots of the slope versus concentration of compound **25d** gave an estimate of the inhibition constant (K_i = 0.14 μM). This result indicated that compound **25d** was able to bind at both the catalytic and peripheral site of AChE.

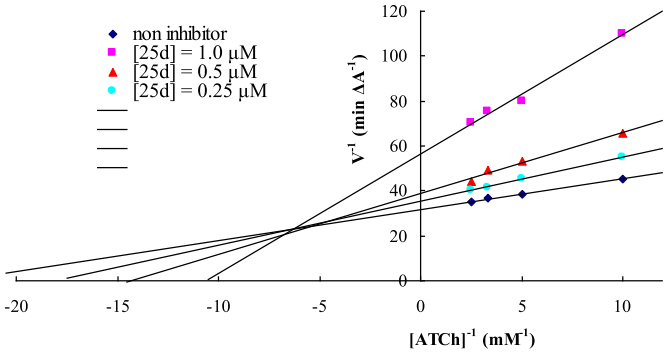


Fig. 2. Kinetic study on the mechanism of *Ee*AChE inhibition by compound **25d**. Merged Lineweaver–Burk reciprocal plots of AChE initial velocity with increasing substrate concentration (0.1–0.4 mM) in the absence or presence of **25d**. Lines were derived from a weighted least-squares analysis of data points.

2.2.3. Molecular modeling study

To explore a possible interacting mode of the genistein derivatives with *Tc*AChE (PDB code: 1EVE), a molecular modeling study was performed using the docking program, AutoDock 4.2 package with Discovery Studio 2.0 [9]. Results showed that

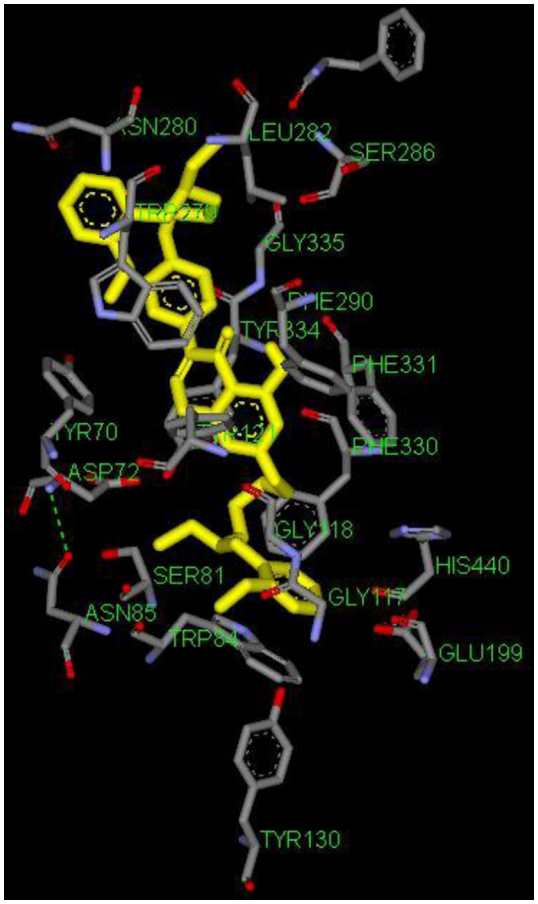


Fig. 3. Representation of compound **25d** (yellow stick) interacting with residues in the binding site of *Tc*AChE (PDB code: 1EVE), highlighting the protein residues that participate in the main interactions with the inhibitor. Picture was generated with Discovery Studio 2.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

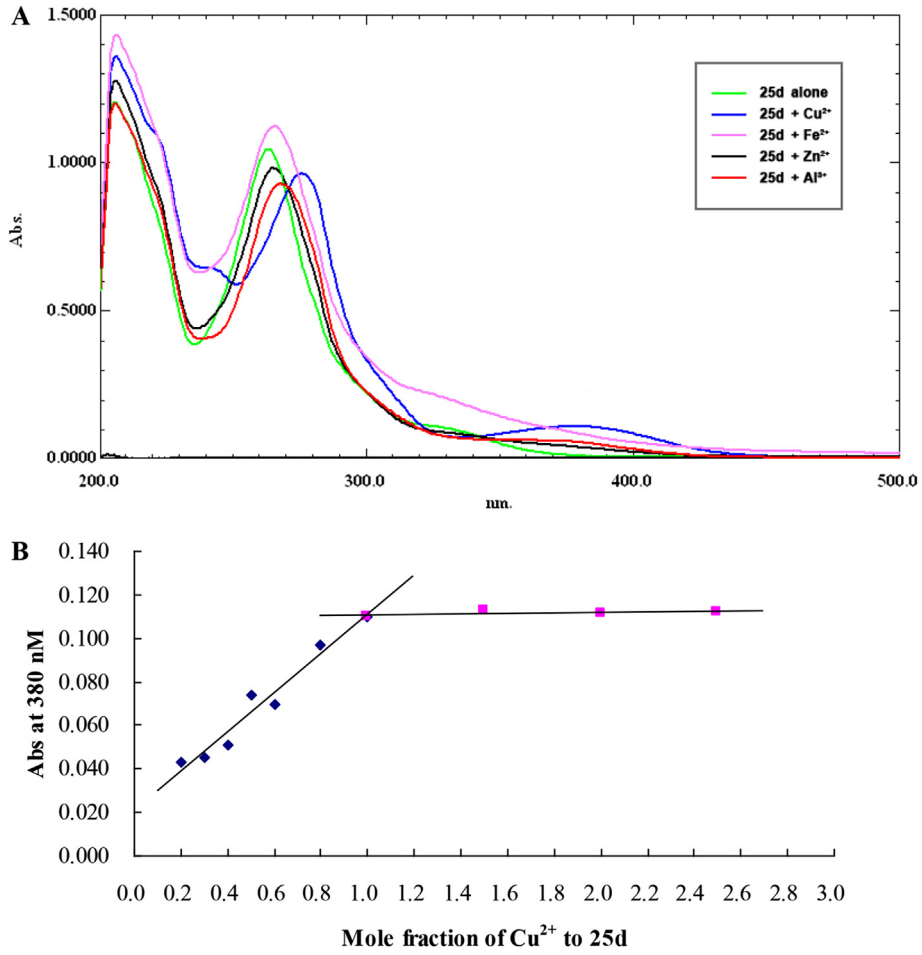


Fig. 4. (A) UV spectrum of compound **25d** (37.5 μM in methanol) alone or in the presence of $CuCl_2$, $FeSO_4$, $ZnCl_2$ or $AlCl_3$ (37.5 μM for all metals in methanol). (B) Determination of the stoichiometry of complex-Cu^{2+} by using the molar ratio method of titrating the methanol solution of compound **25d** with ascending amounts of $CuCl_2$. The final concentration of tested compound was 37.5 μM, and the final concentration of Cu^{2+} ranged from 7.5 to 93.75 μM.

compound **25d** occupied the entire enzymatic catalytic active site (CAS), the mid-gorge sites and the peripheral anionic site (PAS), and binds simultaneously to both the catalytic and peripheral site (Fig. 3). In the *Tc*AChE–**25d** complex, the 7-substituted terminal *N*-(2-methoxybenzyl) ethylamine moiety of **25d** was observed to bind to the CAS via a π–π interaction with Trp84, and potentially induce a hydrophobic interaction with residues Tyr130 and His440. The 4-substituted terminal *N*-(2-methoxybenzyl) ethanamine moiety occupied the PAS of AChE and exhibited a potential hydrophobic interaction with residues Trp279 and Leu282. In addition, the long chain of methylene and skeleton of genistein folded in a conformation in the gorge that allowed them to interact with Gly335, Phe290, Tyr334, Tyr70, Asp72, Tyr121 and Phe330 via the hydrophobic interaction. No direct hydrogen bond interaction was observed between compound **25d** and *Tc*AChE. However, similar to the donepezil complex, water-bridged hydrogen bonding may have occurred under biological circumstances between compound **25d** and *Tc*AChE. The simultaneous binding of **25d** with the active gorge and peripheral site of *Tc*AChE provided an explanation for its highly potent inhibitory activity for AChE and thus revealed a mixed-type inhibition for this compound.

2.2.4. Evaluation of compounds for antioxidant activity via the oxygen radical absorbance capacity by fluorescence (ORAC-FL) method

The antioxidant activities of all the target compounds were evaluated by following the ORAC-FL method [36]. Peroxyl radicals were thermally generated from 2,2-azobis-(amidinopropane) dihydrochloride and reacted with fluorescein to form nonfluorescent products at 535 nm. The antioxidant capacity of genistein derivatives was determined by their competition with fluorescein in the radical capture, using a fluorescence microplate reader. Their ability to scavenge radicals was provided as a Trolox (a vitamin E analog) equivalent, with their relative potency at 5 μM compared with Trolox (Table 1). Genistein was also tested, with an ORAC-FL value of 5.0 trolox equivalents. All of the target compounds demonstrated weak to moderate antioxidant activity. Compounds **9b** and **10c** showed the most potent antioxidant activity of this family with ORAC-FL values of 2.4 and 2.7 trolox equivalents, respectively. The free 4′-OH of genistein was found to be crucial to the radical scavenging ability when the ORAC-FL values were compared between the 7-*O*-modified derivatives (**8a**–**d**, **9a**–**d**, **10a**–**d**) the 4′-*O*-modified derivatives (**18a**–**d**, **19a**–**d**, **20a**–**d**) and the 7,4′-*O*-modified derivatives (**24d**, **25b**, **25d**, **26d**).

2.2.5. Studies of metal-chelating properties

The chelating ability of compounds **10b**, **19d**, and **25d** for the biologically relevant metal ions: Cu^{2+}, Fe^{2+}, Zn^{2+}, and Al^{3+}, was studied by ultraviolet–visible (UV–vis) spectrometry. As an example, a series of UV–vis spectra of compound **25d** is shown in Fig. 4A (the spectra of compound **10b** and **19d** are given in Supporting Information). In the absence of metal ions, the UV–vis spectrum of compound **25d** showed the absorption maximum at 263 nm and a shoulder at 325 nm. When $CuCl_2$ was added, a red

shift in the maximum absorption from 263 nm to 275 nm and 325 nm to 380 nm occurred, indicating the formation of a **25d**–Cu^{2+} complex. When $AlCl_3$ was added, a slight shift in the maximum absorption from 263 to 268 nm was observed, suggesting that **25d** can bind Al^{3+}. However, no significant shift was seen with the addition of $FeSO_4$ or $ZnCl_2$. Similar results were observed for compound **10b** and **19d**. The stoichiometry of the **25d**–Cu^{2+} complex was determined using the molar ratio method, by preparing solutions of compound **25d** with increasing amounts of $CuCl_2$. The UV spectra were used to obtain the absorbance of the **25d** complex and differing concentrations of $CuCl_2$ at 380 nm. The results showed that absorbance linearly increased initially and then plateaued (Fig. 4B). The two straight lines intersected at a mole fraction of 0.99, revealing a 1:1 stoichiometry for complex **25d**–Cu^{2+}.

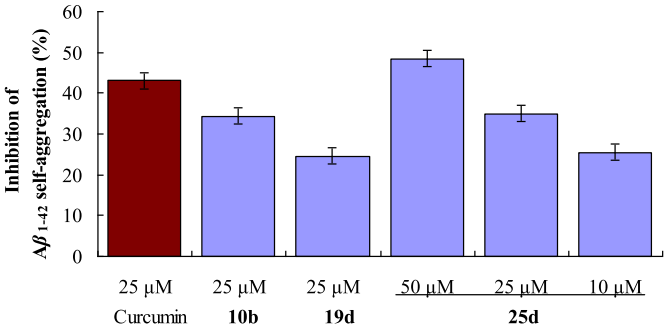


Fig. 5. Inhibition of self-induced $A\beta_{1-42}$ aggregation by compounds **10b**, **19d**, **25d** or curcumin. The thioflavin-T fluorescence method was used. Compounds **10b** and **19d** were tested at 25 μM, and compound **25d** was tested at 10, 25 and 50 μM.

2.2.6. *Effects on the Aβ aggregation*

A number of dual binding site AChE inhibitors have been found to exhibit a significant inhibitory activity on Aβ self-aggregation [37]. Therefore, compounds **10b**, **19d** and **25d**, which showed the highest potency for AChE inhibition, were selected to assess their ability to inhibit self-induced $A\beta_{1-42}$ aggregation via the thioflavin T (ThT) fluorescence method [35,38]. Curcumin, a known active natural product that inhibits self-induced Aβ aggregation, was used as reference compound [39]. Compounds **10b**, **19d** and **25d** (all at 25 μM) were moderately potent with inhibition ratios of 34.3%, 24.6% and 35%, respectively (Table 2), compared with 25 μM curcumin (43.1%). The marketed AD drug, donepezil, did not show any significant inhibitory activity under the same experimental conditions (Table 2).

Compound **25d** also showed a concentration-dependent inhibitory effect on self-induced $A\beta_{1-42}$ aggregation (Fig. 5).

Aβ deposition has been linked to AChE expression [4]. Furthermore, the PAS of AChE can bind to Aβ, accelerating the formation of amyloid fibrils. Therefore, inhibition of AChE, particularly the inhibition of PAS of AChE, may affect Aβ aggregation. The kinetic study showed that compounds **10b**, **19d** and **25d** exhibited a mixtype inhibition and were able to bind both the catalytic and peripheral site of AChE. Therefore, to further explore the dual action of these compounds, we examined their capacity to inhibit *Hu*AChE-induced $A\beta_{1-40}$ via the ThT-based fluorometric assay [40]. Compound **25d** (100 μM) prevented *Hu*AChE-induced $A\beta_{1-40}$ aggregation by 36.2 ± 0.8%, which was higher than that of donepezil (25.0 ± 0.6%) (Table 2). Compared with donepezil (25.0 ± 0.6%), compounds **10b** and **19d** had slightly lower inhibitory activity (18.7 ± 0.31% and 22 ± 1.1%, respectively) against *Hu*AChE-induced $A\beta_{1-40}$ aggregation, indicating that this new family of genistein derivatives had higher affinity for CAS than for PAS of AChE.

Cu^{2+} causes Aβ aggregation in solution, and the interaction of peptides with Cu^{2+} is known to be pH dependent. Cu^{2+}-induced Aβ aggregation is more significant at pH 6.6 than pH 7.4 [41]. To investigate the effects of compounds on Cu^{2+}-induced $A\beta_{1-42}$ aggregation, we performed 2 individual studies (in weak acidic buffered aqueous solution): (1) inhibitory activity of Cu^{2+}-induced $A\beta_{1-42}$ aggregation by chelators and (2) disaggregation effects of chelators on Cu^{2+}-induced $A\beta_{1-42}$ aggregates. ThT fluorescence assay was used to identify the degree of $A\beta_{1-42}$ aggregation [42]. For the ThT fluorescence assay, curcumin was used as the reference compound. $A\beta_{1-42}$ was first treated with one equivalent of Cu^{2+} for 2 min at room temperature and then incubated with or without the testing compounds for 24 h at 37 °C. Cu^{2+} accelerated the aggregation of $A\beta_{1-42}$ (Fig. 6). In contrast, fluorescence was markedly reduced by 72.1 ± 1.1%, 71.9 ± 3.2%, or 77.8 ± 4.0% when $A\beta_{1-42}$ was treated with Cu^{2+} and compounds **10b**, **19d**, or **25d**, respectively. Donepezil did not show any significant inhibitory activity under the same experimental conditions. These results suggested that compounds **10b**, **19d** and **25d** inhibited Cu^{2+}-induced $A\beta_{1-42}$ aggregation by chelating with Cu^{2+}. In particular, **25d** significantly inhibited Cu^{2+}-induced $A\beta_{1-42}$ aggregation in a concentration-dependent manner (Fig. 6). For the disaggregation studies, compounds **10b**, **19d** or **25d** were added to Aβ fibrils, which were

Table 2
Inhibition of self-induced $A\beta_{1-42}$ aggregation, Cu^{2+}-induced $A\beta_{1-42}$ aggregation, and *Hu*AChE-induced $A\beta_{1-40}$ aggregation by compounds **10b**, **19d**, **25d** and reference compounds.

Comp.	% inhibition of Aβ aggregation[a]		
	Self-induced[b,e]	Cu^{2+}-induced[c,e]	*Hu*AChE-induced[d,e]
10b	34.3 ± 1.2	72.1 ± 1.1	18.7 ± 0.31
19d	24.6 ± 1.6	71.9 ± 3.2	22.0 ± 1.1
25d	35.0 ± 1.0	77.8 ± 4.0	36.2 ± 0.8
Curcumin	43.1 ± 1.5	64.0 ± 2.3	NT[f]
Donepezil	n.a.[g]	n.a.[g]	25.0 ± 0.6

[a] For Inhibition of Aβ aggregation, the thioflavin-T fluorescence method was used.
[b] Inhibition of self-induced $A\beta_{1-42}$ aggregation (25 μM) by tested inhibitors at 25 μM.
[c] Inhibition of Cu^{2+}-induced $A\beta_{1-42}$ aggregation. The concentration of tested compounds and Cu^{2+} was 25 μM.
[d] Inhibition of *Hu*AChE-induced $A\beta_{1-40}$ aggregation. The concentration of tested inhibitor and $A\beta_{1-40}$ was 100 and 230 μM, respectively, and the $A\beta_{1-40}$/AChE ratio was equal to 100/1.
[e] Data are presented as the mean ± SD of 3 independent experiments.
[f] NT = not tested.
[g] n.a. = no active. Compounds defined "no active" meant that percent inhibition was less than 5% at 25 μM.

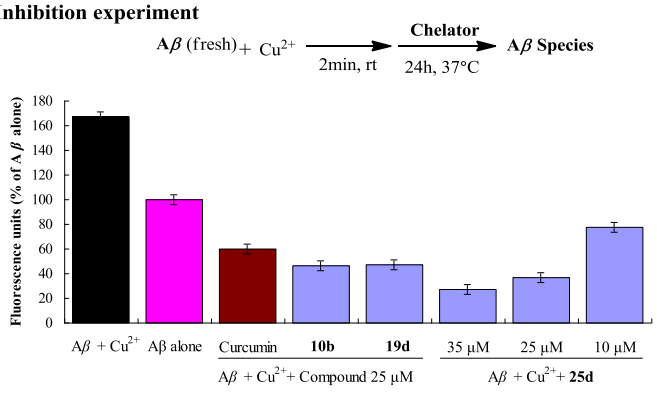


Fig. 6. Inhibition of Cu^{2+}-induced $A\beta_{1-42}$ aggregation by compounds **10b**, **19d**, **25d** and Curcumin. The thioflavin-T fluorescence method was used.

generated by reacting $A\beta_{1-42}$ with one equivalent of Cu^{2+} for 24 h at 37 °C (Fig. 7). The ThT binding assay showed that **10b**, **19d** and **25d** (all at 25 μM) markedly disassembled Aβ fibrils by 36.1 ± 1.3% 39.2 ± 2.0%, and 72.1 ± 2.8%, respectively (Fig. 7).

2.2.7. In vivo assay

Evaluation of the effect of genistein derivatives in animal models of learning and memory impairment is lacking. Scopolamine induces memory deficits and has been used extensively to evaluate potential therapeutic agents for treating AD [43]. Therefore, we investigated the protective effects of genistein derivatives against scopolamine-induced memory deficits via the passive avoidance task. The step-down latency of mice treated with scopolamine alone (control group) was significantly ($p < 0.05$) shorter than that of vehicle-treated mice (normal group). Treatment with compounds **10b** or **19d** (2, 6 and 18 mg/kg), or **25d** (3, 9 and 27 mg/kg) increased the latency time in a dose-dependent manner. In particular, the effect of compounds **19d** (6 mg/kg) and **25d** (9 mg/kg) was comparable with that of donepezil (5 mg/kg) (see Fig. 8). Compounds **10b**, **19d** and **25d** inhibited AChE activity and reversed scopolamine-induced memory deficit in the step-down passive avoidance test. These results indicated that the synthesized genistein-*O*-alkylbenzylamine derivatives may have reversed cognitive deficit by increasing brain cholinergic activity due to the inhibition of AChE.

3. Conclusion

In summary, 28 new genistein-*O*-alkylbenzylamine derivatives (**8**–**10a**–**d**, **18**–**20a**–**d**, **24d**, **25b**, **25d** and **26d**) have been designed, synthesized and evaluated as novel multifunctional agents against AD. Most of these compounds were potent in inhibiting AChE activity and displayed high selectivity for AChE over BuChE. Compounds **10b**, **19d** and **25d** exhibited the highest potency for AChE inhibition potency, particularly **25d** (IC_{50} = 0.09 ± 0.02 μM). Both the inhibition kinetic analysis and molecular modeling study suggested that compound **25d** showed a mixed-type inhibition, binding to both CAS and PAS of AChE, and inducing a strong inhibitory effect. Compounds **10b** and **19d** displayed antioxidant activity, and compound **25d** had relatively lower effects. Importantly, all 3 compounds moderately inhibited self-induced Aβ aggregation, however, exhibited higher Cu^{2+}-induced Aβ aggregation inhibitory activity than that of curcumin. Despite the small inhibition of AChE-induced Aβ aggregation by compound **25d**, this compound proved to be more potent than donepezil. Moreover, compound **10b**, **19d** and **25d** were found to bind Cu^{2+} and assemble into metal complexes, which disassembled well-structured Aβ fibrils as well as inhibit Cu^{2+}-induced Aβ aggregation. Under *in vivo* conditions, mice treated with **10b**, **19d** or **25d** displayed significantly longer step-down latency times than the control group. Taken together, among the synthesized compounds, compound **25d** exhibited the most promising effects. This compound displayed the highest inhibition of AChE, exhibited good antioxidant and metal-chelating activities, significantly inhibited Aβ aggregation, induced a disaggregation of Aβ fibrils generated by Cu^{2+}-induced Aβ aggregation, and reversed cognitive deficit, possibly by increasing brain cholinergic activity due to the inhibition of AChE. Such multifunctional properties promote compound **25d** as an interesting candidate for further studies directed to the development of novel multifunctional agents for the treatment of AD. Further studies to evaluate **25d** *in vivo* are in progress.

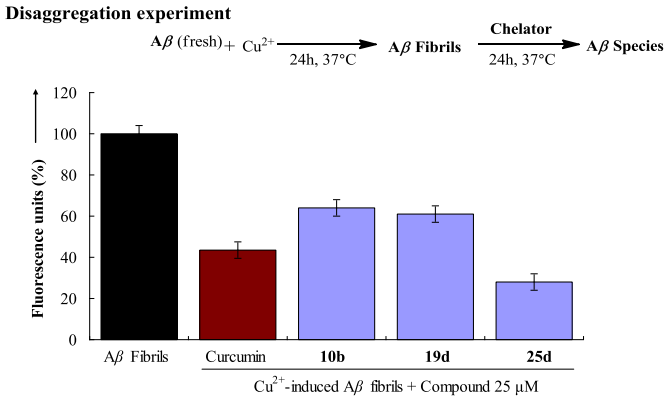


Fig. 7. The disaggregation of Cu^{2+}-induced $A\beta_{1-42}$ aggregation by compounds **10b**, **19d**, **25d** and Curcumin. The measurements was carried out in the presence of 25 μM test compound. The thioflavin-T fluorescence method was used.

4. Experimental section

4.1. Chemistry

Melting points were recorded on YRT-3 melting-point apparatus (China) and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ solutions using a Varian INOVA 400 NMR spectrometer. Chemical shifts are reported in parts per millions (ppm) relative to tetramethylsilane (TMS). Mass spectra were recorded on Agilent-6210 TOF LC–MS Spectrometer. Elemental analyses were performed on a Carlo Erba 1106 apparatus. Genistein was commercially available and purchased from Nanjing Zelang Medical Technology Co., Ltd. All commercially available and anhydrous solvents were of the highest quality and were used without further purification. All the reactions were monitored by thin-layer chromatography (TLC) on silica gel GF254 plates from Qingdao Haiyang Chemical Co. Ltd. (China), and then visualized in an iodine chamber or with an UV lamp (254 nm). Where appropriate, crude products were purified by column chromatography using silica gel (230–400 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. (China).

*4.1.1. General procedure for the synthesis of **2**–**4** [30,31]*

To a solution of genistein (6.0 g, 22.20 mmol) in DMF (50 mL), powdered KOH (1.26 g, 22.20 mmol) was added. After the mixture was stirred at room temperature for 1 h, the appropriate dibromoalkane derivative (26.0 mmol) and KI (296 mg, 1.78 mmol) were added. Then the reaction mixture was stirred at room temperature for 24 h, diluted with water (200 mL), treated with 10% HCl aqueous solution until an acidic pH was reached. The solid formed was collected by filtration, washed with water, dried, and purified by flash chromatography on silica gel.

*4.1.1.1. 7-(3-Bromopropoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**2**).* It was synthesized from 1,3-dibromopropane. Elution with CH_2Cl_2/acetone (100:1) afforded **2** as an almost white solid; 57.5% yield; mp 124–126 °C. (lit., [31] 124–126 °C).

*4.1.1.2. 7-(4-Bromobutoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**3**).* It was synthesized from 1,4-dibromobutane. Elution with CH_2Cl_2/acetone (100:1) afforded **3** as an almost white solid; 67.8% yield; mp 130–132 °C. (lit., [30] 130–131 °C).

*4.1.1.3. 7-((6-Bromohexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**4**).* It was synthesized from 1,6-dibromohexane. Elution with CH_2Cl_2/acetone (100:1) afforded **4** as an almost white solid; 53.5% yield; mp 124–126 °C. (lit. [44] 121–123 °C).

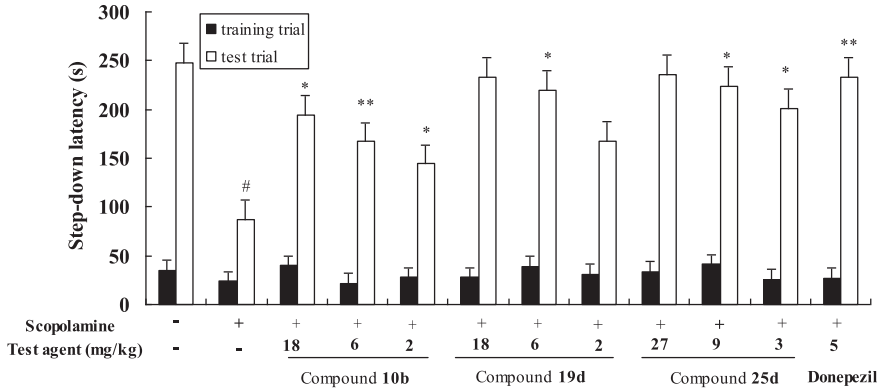


Fig. 8. Effects of compound **10b**, **19d** and **25d** on scopolamine-induced memory deficit in the step-down passive avoidance test. Compounds **10b**, **19d** (both at 2, 6 and 18 mg/kg, *p.o.*), **25d** (3, 9, 27 mg/kg, *p.o.*), or donepezil (5 mg/kg, *p.o.*) were orally given 1 h before treatment with scopolamine. After 30 min, the mice were treated with scopolamine (3 mg/kg, *i.p.*) and tested in the step-down passive avoidance. Values are expressed as the mean ± SEM ($n = 10$). #$p < 0.05$ vs normal group. *$p < 0.05$ and **$p < 0.01$ vs scopolamine-treated control group.

*4.1.2. General procedure for the synthesis of secondary amines (**7a–d**)*

Compounds **7a**–**d** were prepared as previously described [32].

*4.1.3. General procedure for preparation of 7-O-modified genistein derivatives (**8a–d, 9a–d and 10a–d**)*

To a mixture of the corresponding secondary amines **7a**–**d** (0.77 mmol), anhydrous K_2CO_3 (106.2 mg, 0.77 mmol) and KI (8.6 mg, 0.052 mmol) in CH_3CN (10 mL) were added the appropriate intermediates **2**–**4** (0.60 mmol). The reaction mixture was warmed to 60–65 °C and stirred for 12–15 h under an argon atmosphere. After complete reaction, the solvent was evaporated under reduced pressure. Then water (30 mL) was added to the residue and the mixture was extracted with dichloromethane (30 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride (30 mL), dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography using mixtures of CH_2Cl_2/CH_3OH as eluent, obtaining the corresponding 7-*O*-modified genistein derivatives.

*4.1.3.1. 7-(3-(Benzyl(methyl)amino)propoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**8a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 7-(3-bromopropoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**2**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 200:1 gave **8a** as an almost white solid; 52.8% yield; mp 134–136 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.83 (s, 1H, 2-H), 7.36 (d, J = 8.0 Hz, 2H, 2′, 6′-H), 7.31–7.25 (m, 5H, 5 × Ar–H), 6.85 (d, J = 8.0 Hz, 2H, 3′, 5′-H), 6.34 (d, J = 2.0 Hz, 1H, 8-H), 6.33 (d, J = 2.0 Hz, 1H, 6-H), 4.08 (t, J = 6.0 Hz, 2H, CH_2O), 3.56 (s, 2H, $PhCH_2$), 2.58 (t, J = 6.8 Hz, 2H, CH_2N), 2.26 (s, 3H, CH_3N), 2.04–2.00 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{26}H_{26}NO_5$ $[M+H]^+$: 432.1811, found: 432.1808; Anal. calcd. For ($C_{26}H_{25}NO_5$): C, 72.37; H, 5.84; N, 3.25; found: C, 72.56; H, 5.80; N, 3.13.

*4.1.3.2. 7-(3-(Benzyl(ethyl)amino)propoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**8b**).* It was obtained from *N*-benzylethylamine (**7b**) and 7-(3-bromopropoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**2**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 200:1 gave **8b** as an almost white solid; 56.4% yield; mp 100–102 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.85 (s, 1H, 2-H), 7.39 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.34–7.21 (m, 5H, 5 × Ar–H), 6.88 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.34 (d, J = 2.0 Hz, 1H, 8-H), 6.32 (d, J = 2.0 Hz, 1H, 6-H), 4.04 (t, J = 6.4 Hz, 2H, CH_2O), 3.61 (s, 2H, $PhCH_2$), 2.63 (t, J = 6.8 Hz, 2H, CH_2N), 2.58 (t, J = 6.8 Hz, 2H, CH_2N), 1.97 – 1.94 (m, 2H, CH_2), 1.07 (t, J = 6.8 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_5$ $[M+H]^+$: 446.1967, found: 446.1972; Anal. calcd. For ($C_{27}H_{27}NO_5$): C, 72.79; H, 6.11; N, 3.14; found: C, 72.70; H, 6.02; N, 3.06.

*4.1.3.3. 5-Hydroxy-3-(4-hydroxyphenyl)-7-(3-((2-methoxybenzyl)(methyl)amino)propoxy)-4H-chromen-4-one (**8c**).* It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 7-(3-bromopropoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**2**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 150:1 gave **8c** as an almost solid; 54.4% yield; mp 119–122 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.82 (s, 1H, 5-OH), 7.81 (s, 1H, 2-H), 7.35 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.33-7.24 (m, 2H, 2 × Ar–H), 6.94–6.87 (m, 2H, 2 × Ar–H), 6.86 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.34(d, J = 2.0 Hz, 1H, 8-H), 6.32 (d, J = 2.0 Hz, 1H, 6-H), 4.07 (t, J = 6.4 Hz, 2H, CH_2O), 3.81 (s, 3H, CH_3O), 3.65 (s, 2H, $PhCH_2$), 2.67 (t, J = 6.4 Hz, 2H, CH_2N), 2.32 (s, 3H, CH_3N), 2.09 – 2.07 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_6$ $[M+H]^+$: 462.1917, found: 462.1920; Anal. calcd. For ($C_{27}H_{27}NO_6$): C, 70.27; H, 5.90; N, 3.03; found: C, 70.40; H, 5.88; N, 2.95.

*4.1.3.4. 7-(3-(Ethyl(2-methoxybenzyl)amino)propoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**8d**).* It was obtained from *N*-(2-methoxybenzyl)ethylamine (**7d**) and 7-(3-bromopropoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**2**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 150:1 gave **8d** as an almost solid; 60.2% yield; mp 127–129 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.84 (s, 1H, 2-H), 7.38 (d, J = 8.0 Hz, 2H, 2′, 6′-H), 7.37–7.36 (m, 1H, Ar–H), 7.24–7.20 (m, 1H, Ar–H), 6.93–6.84 (m, 4H, 2 × Ar–H, 3′, 5′-H), 6.36 (d, J = 2.0 Hz, 1H, 8-H), 6.34 (d, J = 2.0 Hz, 1H, 6-H), 4.06 (t, J = 6.4 Hz, 2H, CH_2O), 3.81 (s, 3H, CH_3O), 3.63 (s, 2H, $PhCH_2$), 2.66 (t, J = 6.8 Hz, 2H, CH_2N), 2.57 (q, J = 6.8 Hz, 2H, CH_2N), 2.01 – 1.95 (m, 2H, CH_2), 1.07 (t, J = 6.8 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_6$ $[M+H]^+$: 476.2073, found: 476.2072; Anal. calcd. For ($C_{28}H_{29}NO_6$): C, 70.72; H, 6.15; N, 2.95; found: C, 70.83; H, 6.22; N, 3.09.

*4.1.3.5. 7-(4-(Benzyl(methyl)amino)butoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**9a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 7-(4-bromobutoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**3**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 120:1 gave **9a** as an almost white solid; 79.5% yield; mp 158–160 °C; ^{1}H NMR (400 MHz,

$CDCl_3$) δ 12.82 (s, 1H, 5-OH), 7.80 (s, 1H, 2-H), 7.36 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.33–7.26 (m, 5H, 5 × Ar–H), 6.87 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.32 (s, 2H, 8-H, 6-H), 3.97 (t, J = 6.4 Hz, 2H, CH_2O), 3.56 (s, 2H, $PhCH_2$), 2.48 (t, J = 7.2 Hz, 2H, CH_2N), 2.25 (s, 3H, CH_3N), 1.85–1.80 (m, 2H, CH_2), 1.75–1.70 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_5$ $[M+H]^+$: 446.1967, found: 446.1960; Anal. calcd. For ($C_{27}H_{27}NO_5$): C, 72.79; H, 6.11; N, 3.14; found: C, 72.58; H, 6.18; N, 3.05.

*4.1.3.6. 7-(4-(Benzyl(ethyl)amino)butoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**9b**).* It was obtained from *N*-benzylethylamine (**7b**) and 7-(4-bromobutoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**3**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 150:1 gave **9b** as an almost white solid; 61.0% yield; mp 109–111 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.81 (s, 1H, 2-H), 7.37–7.24 (m, 7H, 7 × Ar–H), 6.87 (d, J = 8.0 Hz, 2H, 3′, 5′-H), 6.32 (s, 2H, 8-H, 6-H), 3.94 (t, J = 6.4 Hz, 2H, CH_2O), 3.63 (s, 2H, $PhCH_2$), 2.58 (q, J = 6.8 Hz, 2H, CH_2N), 2.53 (t, J = 6.8 Hz, 2H, CH_2N), 1.84–1.77 (m, 2H, CH_2), 1.69–1.66 (m, 2H, CH_2), 1.09 (t, J = 6.8 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_5$ $[M+H]^+$: 460.2124, found: 460.2118; Anal. calcd. For ($C_{28}H_{29}NO_5$): C, 73.18; H, 6.36; N, 3.05; found: C, 73.15; H, 6.42; N, 3.00.

*4.1.3.7. 5-Hydroxy-3-(4-hydroxyphenyl)-7-(4-((2-methoxybenzyl)(methyl)amino)butoxy)-4H-chromen-4-one (**9c**).* It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 7-(4-bromobutoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**3**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 100:1 gave **9c** as an almost white solid; 78.5% yield; mp 123–125 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.82 (s, 1H, 5-OH), 7.71 (s, 1H, 2-H), 7.36-7.27 (m, 4H, 4 × Ar–H), 6.97–6.87 (m, 2H, 2 × Ar–H), 6.86 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.27 (d, J = 2.0 Hz, 1H, 8-H), 6.26 (d, J = 2.0 Hz, 1H, 6-H), 3.90 (t, J = 6.0 Hz, 2H, CH_2O), 3.81 (s, 3H, CH_3O), 3.74 (s, 2H, $PhCH_2$), 2.62 (t, J = 6.8 Hz, 2H, CH_2N), 2.35 (s, 3H, CH_3N), 1.80 (m, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_6$ $[M+H]^+$: 476.2073, found: 476.2071; Anal. calcd. For ($C_{28}H_{29}NO_6$): C, 70.72; H, 6.15; N, 2.95; found: C, 70.88; H, 6.11; N, 2.80.

*4.1.3.8. 7-(4-(Ethyl(2-methoxybenzyl)amino)butoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**9d**).* It was obtained from *N*-(2-methoxybenzyl)ethylamine (**7d**) and 7-(4-bromobutoxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**3**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 120:1 gave **9d** as an almost white solid; 81.7% yield; mp 45–48 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.74 (s, 1H, 2-H), 7.41 (d, J = 7.2 Hz, 1H, Ar–H), 7.32 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.28–7.24(m, 1H, Ar–H), 6.95 (t, J = 7.2 Hz, 1H, Ar–H), 6.88–6.84 (m, 3H, Ar–H, 3′, 5′-H), 6.28 (s, 2H, 8-H, 6-H), 3.89 (t, J = 6.4 Hz, 2H, CH_2O), 3.81 (s, 3H, CH_3O), 3.74 (s, 2H, $PhCH_2$), 2.67–2.61 (m, 4H, 2 × CH_2N), 1.78 – 1.72 (m, 4H, 2 × CH_2), 1.14 (t, J = 6.4 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{29}H_{32}NO_6$ $[M+H]^+$: 490.2230, found: 490.2236; Anal. calcd. For ($C_{29}H_{31}NO_6$): C, 71.15; H, 6.38; N, 2.86; found: C, 71.00; H, 6.46; N, 3.02.

*4.1.3.9. 7-((6-(Benzyl(methyl)amino)hexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**10a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 7-((6-bromohexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**4**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 150:1 gave **10a** as an almost white solid; 57.0% yield; mp 110–111 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.80 (s, 1H, 5-OH), 7.80 (s, 1H, 2-H), 7.38 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.33–7.26 (m, 5H, 5 × Ar–H), 6.86 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.32 (d, J = 2.0 Hz, 1H, 8-H), 6.31 (d, J = 2.0 Hz, 1H, 6-H), 3.92 (t, J = 6.4 Hz, 2H, CH_2O), 3.57 (s, 2H, $PhCH_2$), 2.43 (t, J = 6.8 Hz, 2H, CH_2N), 2.24 (s, 3H, CH_3N), 1.74–1.71 (m, 2H, CH_2), 1.60–1.55 (m, 2H, CH_2), 1.42–1.36 (m, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{29}H_{32}NO_5$ $[M+H]^+$: 474.2280, found: 474.2284; Anal. calcd. For ($C_{29}H_{31}NO_5$): C, 73.55; H, 6.60; N, 2.96; found: C, 73.52; H, 6.69; N, 3.16.

*4.1.3.10. 7-((6-(Benzyl(ethyl)amino)hexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**10b**).* It was obtained from *N*-benzylethylamine (**7b**) and 7-((6-bromohexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**4**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 100:1 gave **10b** as an almost white solid; 67.7% yield; mp 42–44 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.81 (s, 1H, 2-H), 7.38 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.37–7.26 (m, 5H, 5 × Ar–H), 6.87 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.33 (d, J = 2.0 Hz, 1H, 8-H), 6.32 (d, J = 2.0 Hz, 1H, 6-H), 3.94 (t, J = 6.4 Hz, 2H, CH_2O), 3.64 (s, 2H, $PhCH_2$), 2.58 (q, J = 6.8 Hz, 2H, CH_2N), 2.48 (t, J = 7.2 Hz, 2H, CH_2N), 1.75–1.70 (m, 2H, CH_2), 1.56–1.53 (m, 2H, CH_2), 1.41–1.34 (m, 4H, 2 × CH_2), 1.08 (t, J = 6.8 Hz, 3H, CH_3); $^{13C\ NMR\ (100\ MHz,\ CDCl_3)}$ δ 180.8, 164.9, 162.3, 157.7, 157.1, 152.5, 137.5, 130.1, 129.4, 128.2, 127.2, 123.5, 121.8, 116.0, 105.9, 98.5, 92.5, 68.4, 57.4, 52.5, 46.6, 28.7, 27.0, 25.9, 25.6, 10.7; HR-ESI-MS: Calcd. for $C_{30}H_{34}NO_5$ $[M+H]^+$: 488.2437, found: 488.2429; Anal. calcd. For ($C_{30}H_{33}NO_5$): C, 73.90; H, 6.82; N, 2.87; found: C, 73.76; H, 6.90; N, 3.05.

*4.1.3.11. 5-Hydroxy-3-(4-hydroxyphenyl)-7-((6-((2-methoxybenzyl)(methyl)amino)hexyl)oxy)-4H-chromen-4-one (**10c**).* It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 7-((6-bromohexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**4**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 120:1 gave **10c** as an almost white solid; 68.8% yield; mp 45–47 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.80 (s, 1H, 5-OH), 7.76 (s, 1H, 2-H), 7.38-7.26 (m, 4H, 4 × Ar–H), 6.97–6.87 (m, 4H, 4 × Ar–H), 6.26 (s, 2H, 8-H, 6-H), 3.85 (t, J = 6.4 Hz, 2H, CH_2O), 3.81(s, 3H, CH_3O), 3.73 (s, 2H, $PhCH_2$), 2.55 (t, J = 7.2 Hz, 2H, CH_2N), 2.33 (s, 3H, CH_3N), 1.66–1.64 (m, 4H, 2 × CH_2), 1.36 (s, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{30}H_{34}NO_6$ $[M+H]^+$: 504.2386, found: 504.2378; Anal. calcd. For ($C_{30}H_{33}NO_6$): C, 71.55; H, 6.61; N, 2.78; found: C, 71.43; H, 6.73; N, 2.90.

*4.1.3.12. 7-((6-(Ethyl(2-methoxybenzyl)amino)hexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**10d**).* It was obtained from *N*-(2-methoxybenzyl)ethylamine (**7d**) and 7-((6-bromohexyl)oxy)-5-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (**4**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 100:1 gave **10d** as an almost white solid; 79.2% yield; mp 41–43 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.78 (s, 1H, 5-OH), 7.75 (s, 1H, 2-H), 7.51 (d, J = 7.2 Hz, 1H, Ar–H), 7.37–7.31 (m, 3H, 3 × Ar–H), 7.01–6.90 (m, 4H, 4 × Ar–H), 6.21 (s, 2H, 8-H, 6-H), 3.96 (t, J = 6.4 Hz, 2H, CH_2O), 3.85 (s, 3H, CH_3O), 3.79 (s, 2H, $PhCH_2$), 2.84 (brs, 2H, CH_2N), 2.72 (brs, 2H, CH_2N), 1.71 (brs, 2H, CH_2), 1.62 (brs, 2H, CH_2), 1.31–1.25 (m, 7H, 2 × CH_2, CH_3); HR-ESI-MS: Calcd. for $C_{31}H_{36}NO_6$ $[M+H]^+$: 518.2543, found: 518.2540; Anal. calcd. For ($C_{31}H_{35}NO_6$): C, 71.93; H, 6.82; N, 2.71; found: C, 72.00; H, 6.87; N, 2.58.

*4.1.4. 5-Hydroxy-3-(4-hydroxyphenyl)-7-(methoxymethoxy)-4H-chromen-4-one (**11**)*

Intermediate **11** was synthesized as previously described [33].

*4.1.5. General procedure for the synthesis of **12**–**14***

To a mixture of the intermediate **11** (1.00 g, 3.18 mmol), anhydrous K_2CO_3 (440 mg, 3.18 mmol) and KI (53 mg, 0.32 mmol) in CH_3CN (30 mL) the appropriate dibromoalkane derivative (6.36 mmol) was added. The reaction mixture was warmed to 60–

65 °C and stirred for 12–15 h under an argon atmosphere. After complete reaction, the solvent was evaporated under reduced pressure. Water (30 mL) was added to the residue and the mixture was extracted with dichloromethane (30 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride, dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography to give the intermediates **12**–**14.**

*4.1.5.1. 3-(4-(3-Bromopropoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**12**).* It was synthesized from 1,3-dibromopropane. Elution with CH_2Cl_2 afforded **12** as an almost white solid; 62.1% yield; mp 98–100 °C. (lit. [44] 94–97 °C).

*4.1.5.2. 3-(4-(4-Bromobutoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**13**).* It was synthesized from 1,4-dibromobutane. Elution with CH_2Cl_2 afforded **13** as an almost white solid; 61.5% yield; mp 102–104 °C. (lit. [44] 100–102 °C).

*4.1.5.3. 3-(4-((6-Bromohexyl)oxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**14**).* It was synthesized from 1,6-dibromohexane. Elution with CH_2Cl_2 afforded **14** as an almost white solid; 52.1% yield; mp 79–80 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.45 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.97 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.4 Hz, 1H, 8-H), 6.50 (d, J = 2.4 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 4.00 (t, J = 6.4 Hz, 2H, CH_2O), 3.50 (s, 3H, CH_3O), 3.44 (t, J = 6.4 Hz, 2H, CH_2Br), 1.92–1.89 (m, 2H, CH_2), 1.84–1.80 (m, 2H, CH_2), 1.53–1.51 (m, 4H, 2 × CH_2).

*4.1.6. General procedure for preparation of 7-O-MOM-4′-O-modified genistein derivatives (**15a–d, 16a–d and 17a–d**)*

To a mixture of the corresponding secondary amines **7a**–**d** (0.77 mmol), anhydrous K_2CO_3 (106.2 mg, 0.77 mmol) and KI (8.6 mg, 0.052 mmol) in CH_3CN (10 mL) were added the appropriate intermediates **12**–**14** (0.60 mmol). The reaction mixture was warmed to 60–65 °C and stirred for 12–15 h under an argon atmosphere. After complete reaction, the solvent was evaporated under reduced pressure. Water (30 mL) was added to the residue and the mixture was extracted with dichloromethane (30 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride (30 mL), dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography using mixture of CH_2Cl_2/CH_3OH as eluent, obtaining the corresponding 7-*O*-MOM-4′-*O*-modified genistein derivatives.

*4.1.6.1. 3-(4-(3-(Benzyl(methyl)amino)propoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**15a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 3-(4-(3-bromopropoxy) phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**12**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 100:1 gave **15a** as a light yellow oil; 61.9% yield; ^{1}H NMR(400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.31–7.24 (m, 5H, 5 × Ar–H), 6.96 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 4.06 (t, J = 6.4 Hz, 2H, CH_2O), 3.53 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.58 (t, J = 6.8 Hz, 2H, CH_2N), 2.24 (s, 3H, CH_3N), 2.03–2.00 (m, 2H, CH_2).

*4.1.6.2. 3-(4-(3-(Benzyl(ethyl)amino)propoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**15b**).* It was obtained from *N*-benzylethylamine (**7b**) and 3-(4-(3-bromopropoxy) phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**12**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 80:1 gave **15b** as a light yellow oil; 73.6% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.35–7.22 (m, 5H, 5 × Ar–H), 6.94 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 4.03 (t, J = 6.4 Hz, 2H, CH_2O), 3.62 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.65 (t, J = 6.8 Hz, 2H, CH_2N), 2.56 (q, J = 6.4 Hz, 2H, CH_2N), 1.99–1.96 (m, 2H, CH_2), 1.07 (t, J = 6.4 Hz, 3H, CH_3).

*4.1.6.3. 5-Hydroxy-3-(4-(3-((2-methoxybenzyl)(methyl)amino)propoxy)phenyl)-7-(methoxy methoxy)-4H-chromen-4-one (**15c**).* It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 3-(4-(3-bromopropoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**12**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 80:1 gave **15c** as a light yellow oil; 86.7% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.34 (d, J = 7.2 Hz, 1H, Ar–H), 7.26–7.24 (m, 1H, Ar–H), 6.97 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.92–6.86 (m, 2H, 2 × Ar–H), 6.58 (d, J = 1.6 Hz, 1H, 8-H), 6.50 (s, J = 1.6 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 4.08 (t, J = 6.4 Hz, 2H, CH_2O), 3.84 (s, 3H, CH_3O), 3.61 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.66 (t, J = 6.8 Hz, 2H, CH_2N), 2.33 (s, 3H, CH_3N), 2.09–2.07 (m, 2H, CH_2).

*4.1.6.4. 3-(4-(3-(Ethyl(2-methoxybenzyl)amino)propoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**15d**).* It was obtained from *N*-(2-methoxybenzyl)-ethylamine (**7d**) and 3-(4-(3-bromopropoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**12**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **15d** as a light yellow oil; 89.0% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.45–7.42 (m, 3H, 3 × Ar–H), 7.23 (t, J = 8.0 Hz, 1H, Ar–H), 6.95–6.91 (m, 3H, 3 × Ar–H), 6.86 (d, J = 8.0 Hz, 1H, Ar–H), 6.58 (d, J = 1.6 Hz, 1H, 8-H), 6.50 (d, J = 1.6 Hz, 1H, 6-H), 5.24(s, 2H, OCH_2O), 4.05 (t, J = 6.4 Hz, 2H, CH_2O), 3.82 (s, 3H, CH_3O), 3.70 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.72 (brs, 2H, CH_2N), 2.63 (brs, 2H, CH_2N), 2.03 (brs, 2H, CH_2), 1.11 (brs, 3H, CH_3).

*4.1.6.5. 3-(4-(4-(Benzyl(methyl)amino)butoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**16a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 3-(4-(4-bromobutoxy) phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**13**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 80:1 gave **16a** as a light yellow oil; 81.6% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.87 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.32–7.25 (m, 5H, 5 × Ar–H), 6.95 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.4 Hz, 1H, 8-H), 6.50 (d, J = 2.4 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 3.99 (t, J = 6.8 Hz, 2H, CH_2O), 3.51 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.45 (t, J = 7.2 Hz, 2H, CH_2N), 2.22 (s, 3H, CH_3N), 1.85–1.82 (m, 2H, CH_2), 1.73–1.69 (m, 2H, CH_2).

*4.1.6.6. 3-(4-(4-(Benzyl(ethyl)amino)butoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**16b**).* It was obtained from *N*-benzylethylamine (**7b**) and 3-(4-(4-bromobutoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**13**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **16b** as a light yellow oil; 84.8% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.36–7.24 (m, 5H, 5 × Ar–H), 6.94 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.4 Hz, 1H, 8-H), 6.50 (d, J = 2.4 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 3.96 (t, J = 6.4 Hz, 2H, CH_2O), 3.59 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.55–2.51 (m, 4H, 2 × CH_2N), 1.83–1.78 (m, 2H, CH_2), 1.68–1.67 (m, 2H, CH_2), 1.06 (t, J = 6.8 Hz, 3H, CH_3).

*4.1.6.7. 5-Hydroxy-3-(4-(4-((2-methoxybenzyl)(methyl)amino)butoxy)phenyl)-7-(methoxymethoxy)-4H-chromen-4-one (**16c**).*

It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 3-(4-(4-bromobutoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**13**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 120:1 gave **16c** as a light yellow oil; 54.4% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.36 (d, J = 7.2 Hz, 1H, Ar–H), 7.26–7.23 (m, 1H, Ar–H), 6.97–6.87 (m, 4H, 4 × Ar–H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 4.01 (t, J = 6.4 Hz, 2H, CH_2O), 3.83 (s, 3H, CH_3O), 3.62 (s, 2H, $PhCH_2$), 3.50(s, 3H, CH_3O), 2.55 (t, J = 7.2 Hz, 2H, CH_2N), 2.30 (s, 3H, CH_3N), 1.87–1.78 (m, 4H, 2 × CH_2).

*4.1.6.8. 3-(4-(4-(Ethyl(2-methoxybenzyl)amino)butoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**16d**).* It was obtained from *N*-(2-methoxybenzyl)-ethylamine (**7d**) and 3-(4-(4-bromobutoxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**13**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 120:1 gave **16d** as a light yellow oil; 52.6% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83(s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.47-7.43 (m, 3H, 3 × Ar–H), 7.26–7.23 (m, 1H, Ar–H), 6.97–6.93 (m, 3H, 3 × Ar–H), 6.88 (d, J = 8.0 Hz, 1H, Ar–H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.22 (s, 2H, OCH_2O), 3.98 (t, J = 6.0 Hz, 2H, CH_2O), 3.83 (s, 3H, CH_3O), 3.76 (s, 2H, $PhCH_2$), 3.49 (s, 3H, CH_3O), 2.68–2.64 (m, 4H, 2 × CH_2N), 1.82–1.78 (m, 4H, 2 × CH_2), 1.15 (brs, 3H, CH_3).

*4.1.6.9. 3-(4-((6-(Benzyl(methyl)amino)hexyl)oxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**17a**).* It was obtained from *N*-benzylmethylamine (**7a**) and 3-(4-((6-bromohexyl) oxy) phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**14**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **17a** as a light yellow oil; 85.2% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.33-7.25 (m, 5H, 5 × Ar–H), 6.96 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 3.98 (t, J = 6.4 Hz, 2H, CH_2O), 3.52 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.41 (t, J = 7.2 Hz, 2H, CH_2N), 2.22 (s, 3H, CH_3N), 1.82–1.78 (m, 2H, CH_2), 1.59–1.56 (m, 2H, CH_2), 1.49–1.44 (m, 2H, CH_2), 1.42–1.36 (m, 2H, CH_2).

*4.1.6.10. 3-(4-((6-(Benzyl(ethyl)amino)hexyl)oxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4H-chromen-4-one (**17b**).* It was obtained from *N*-benzylethylamine (**7b**) and 3-(4-((6-bromohexyl) oxy) phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**14**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **17b** as a light yellow oil; 92.0% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.87 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.35–7.23 (m, 5H, 5 × Ar–H), 6.96 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.57 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.23 (s, 2H, OCH_2O), 3.97 (t, J = 6.4 Hz, 2H, CH_2O), 3.59 (s, 2H, $PhCH_2$), 3.49 (s, 3H, CH_3O), 2.54 (q, J = 6.8 Hz, 2H, CH_2N), 2.45 (t, J = 6.8 Hz, 2H, CH_2N), 1.80–1.76 (m, 2H, CH_2), 1.54–1.51 (m, 2H, CH_2), 1.46–1.43 (m, 2H, CH_2), 1.38–1.33 (m, 2H, CH_2), 1.05 (t, J = 6.8 Hz, 3H, CH_3).

*4.1.6.11. 5-Hydroxy-3-(4-((6-((2-methoxybenzyl)(methyl)amino) hexyl)oxy)phenyl)-7-(methoxy methoxy)-4H-chromen-4-one (**17c**).* It was obtained from *N*-(2-methoxybenzyl)-methylamine (**7c**) and 3-(4-((6-bromohexyl)oxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**14**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 80:1 gave **17c** as a light yellow oil; 83.3% yield; ^{1}H NMR(400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.37 (d, J = 7.2 Hz, 1H, Ar–H), 7.26–7.24 (m, 1H, Ar–H), 6.96 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.95–6.93 (m, 1H, Ar–H), 6.88 (d, J = 8.8 Hz, 1H, Ar–H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 3.99 (t, J = 6.4 Hz, 2H, CH_2O), 3.82 (s, 3H, CH_3O), 3.63 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.49 (t, J = 7.2 Hz, 2H, CH_2N), 2.30 (s, 3H, CH_3N), 1.82–1.79 (m, 2H, CH_2), 1.64–1.63 (m, 2H, CH_2), 1.51–1.46 (m, 2H, CH_2), 1.43–1.38 (m, 2H, CH_2).

*4.1.6.12. 3-(4-((6-(Ethyl(2-methoxybenzyl)amino)hexyl)oxy)phenyl)-5-hydroxy-7-(methoxy methoxy)-4H-chromen-4-one (**17d**).* It was obtained from *N*-(2-methoxybenzyl)-ethylamine (**7d**) and 3-(4-((6-bromohexyl)oxy)phenyl)-5-hydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (**14**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 50:1 gave **17d** as a light yellow oil; 91.0% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.88 (s, 1H, 2-H), 7.45-7.43 (d, J = 8.8 Hz, 3H, 3 × Ar–H), 7.22 (t, J = 7.6 Hz, 1H, Ar–H), 6.97-6.92 (m, 3H, 2 × Ar–H), 6.86 (d, J = 7.6 Hz, 1H, Ar–H), 6.58 (d, J = 2.0 Hz, 1H, 8-H), 6.50 (d, J = 2.0 Hz, 1H, 6-H), 5.24 (s, 2H, OCH_2O), 3.97 (t, J = 6.4 Hz, 2H, CH_2O), 3.82 (s, 3H, CH_3O), 3.63 (s, 2H, $PhCH_2$), 3.50 (s, 3H, CH_3O), 2.56 (brs, 2H, CH_2N), 2.49 (brs, 2H, CH_2N), 1.81–1.75 (m, 2H, CH_2), 1.50–1.48 (m, 2H, CH_2), 1.48–1.43 (m, 2H, CH_2), 1.39–1.34 (m, 2H, CH_2), 1.07 (t, J = 7.2 Hz, 3H, CH_3).

*4.1.7. General procedure for preparation of 4′-O-modified genistein derivatives (**18a–d, 19a–d and 20a–d**)*

7-*O*-MOM-4′-*O*-modified genistein derivatives **15a–d**, **16a–d** or **17a–d** (1.0 mmol) were dissolved in ethanol (9 mL), and 10% HCl solution (3 mL) was added. The reaction mixture was refluxed for 2–3 h under an argon atmosphere. After complete reaction, ethanol was evaporated under reduced pressure. The residue was treated with aqueous saturated $NaHCO_3$ solution until an alkaline pH was reached, and extracted with dichloromethane (20 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride, dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography using mixture of CH_2Cl_2/CH_3OH as eluent, obtaining the corresponding 4′-*O*-modified genistein derivatives.

*4.1.7.1. 3-(4-(3-(Benzyl(methyl)amino)propoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**18a**).* It was synthesized from **15a** according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **18a** as an almost white solid; 75.2% yield; mp 95–97 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.88 (s, 1H, 5-OH), 7.73 (s, 1H, 2-H), 7.33–7.28 (m, 7H, 7 × Ar–H), 6.83–6.81 (m, 2H, 3′, 5′-H), 6.19-6.18 (m, 2H, 8-H, 6-H), 4.02 (t, J = 6.4 Hz, 2H, CH_2O), 3.66 (s, 2H, $PhCH_2$), 2.70 (t, J = 6.8 Hz, 2H, CH_2N), 2.30 (s, 3H, CH_3N), 2.06–2.03 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{26}H_{26}NO_5$ $[M+H]^+$: 432.1811, found: 432.1816; Anal. calcd. For ($C_{26}H_{25}NO_5$): C, 72.37; H, 5.84; N, 3.25; found: C, 72.45; H, 5.96; N, 3.16.

*4.1.7.2. 3-(4-(3-(Benzyl(ethyl)amino)propoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**18b**).* It was synthesized from **15b** according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **18b** as an almost white solid; 78.7% yield; mp 59–61 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.89 (s, 1H, 5-OH), 7.75 (s, 1H, 2-H), 7.37–7.27 (m, 7H, 7 × Ar–H), 6.84 (d, J = 8.0 Hz, 2H, 3′, 5′-H), 6.25–6.20 (m, 2H, 8-H, 6-H), 4.00 (t, J = 6.0 Hz, 2H, CH_2O), 3.71 (s, 2H, $PhCH_2$), 2.73 (t, J = 6.8 Hz, 2H, CH_2N), 2.64 (q, J = 6.8 Hz, 2H, CH_2N), 2.04–1.98 (m, 2H, CH_2), 1.11 (t, J = 6.8 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_5$ $[M+H]^+$: 446.1967, found: 446.1966; Anal. calcd. For ($C_{27}H_{27}NO_5$): C, 72.79; H, 6.11; N, 3.14; found: C, 72.73; H, 6.20; N, 3.05.

*4.1.7.3. 5,7-Dihydroxy-3-(4-(3-((2-methoxybenzyl)(methyl)amino) propoxy)phenyl)-4H-chromen-4-one (**18c**).* It was synthesized from **15c** according to the general procedure. Elution with CH_2Cl_2/

MeOH = 80:1 gave **18c** as an almost white solid; 70.2% yield; mp 75–78 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.65 (s, 1H, 2-H), 7.32–7.26 (m, 4H, 4 × Ar–H), 6.93 (t, J = 7.6 Hz, 1H, Ar–H), 6.88 (d, J = 8.8 Hz, 1H, Ar–H), 6.76 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.12 (brs, 1H, 8-H), 6.08 (brs, 1H, 6-H), 4.00 (t, J = 6.0 Hz, 2H, CH_2O), 3.78 (s, 5H, CH_3O, $PhCH_2$), 2.78 (t, J = 6.8 Hz, 2H, CH_2N), 2.35 (s, 3H, CH_3N), 2.09–2.06 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_6$ $[M+H]^+$: 462.1917, found: 462.1912; Anal. calcd. For ($C_{27}H_{27}NO_6$): C, 70.27; H, 5.90; N, 3.03; found: C, 70.22; H, 5.93; N, 3.11.

*4.1.7.4. 3-(4-(3-(Ethyl(2-methoxybenzyl)amino)propoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**18d**).* It was synthesized from **15d** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **18d** as an almost white solid; 81.0% yield; mp 61–63 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.68 (s, 1H, 2-H), 7.39–7.27 (m, 4H, 4 × Ar–H), 6.94 (t, J = 7.2 Hz, 1H, Ar–H), 6.87 (d, J = 8.4 Hz, 1H, Ar–H), 6.82 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.17 (s, 1H, 8-H), 6.16 (s, 1H, 6-H), 3.97 (t, J = 6.0 Hz, 2H, CH_2O), 3.80 (s, 5H, CH_3O, $PhCH_2$), 2.80 (t, J = 6.8 Hz, 2H, CH_2N), 2.72 (q, J = 7.2 Hz, 2H, CH_2N), 2.07–2.03 (m, 2H, CH_2), 1.17 (t, J = 7.2 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_6$ $[M+H]^+$: 476.2073, found: 476.2076; Anal. calcd. For ($C_{28}H_{29}NO_6$): C, 70.72; H, 6.15; N, 2.95; found: C, 70.66; H, 6.16; N, 2.88.

*4.1.7.5. 3-(4-(4-(Benzyl(methyl)amino)butoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**19a**).* It was synthesized from **16a** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **19a** as an almost white solid; 87.7% yield; mp 60–62 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.61 (s, 1H, 2-H), 7.34–7.26 (m, 7H, 7 × Ar–H), 6.71 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.17 (s, 1H, 8-H), 6.11 (s, 1H, 6-H), 3.88 (t, J = 5.6 Hz, 2H, CH_2O), 3.71 (s, 2H, $PhCH_2$), 2.63 (t, J = 8.0 Hz, 2H, CH_2N), 2.32 (s, 3H, CH_3N), 1.86–1.78 (m, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{27}H_{28}NO_5$ $[M+H]^+$: 446.1967, found: 446.1973; Anal. calcd. For ($C_{27}H_{27}NO_5$): C, 72.79; H, 6.11; N, 3.14; found: C, 72.90; H, 6.16; N, 2.95.

*4.1.7.6. 3-(4-(4-(Benzyl(ethyl)amino)butoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**19b**).* It was synthesized from **16b** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **19b** as an almost white solid; 87.0% yield; mp 53–55 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.64 (s, 1H, 2-H), 7.39–7.30 (m, 7H, 7 × Ar–H), 6.74 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.19 (s, 1H, 8-H), 6.15 (s, 1H, 6-H), 3.86 (t, J = 5.2 Hz, 2H, CH_2O), 3.79 (s, 2H, $PhCH_2$), 2.72 (q, J = 7.2 Hz, 2H, CH_2N), 2.67 (t, J = 7.6 Hz, 2H, CH_2N), 1.79–1.76 (m, 4H, 2 × CH_2), 1.18 (t, J = 7.2 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_5$ $[M+H]^+$: 460.2124, found: 460.2122; Anal. calcd. For ($C_{28}H_{29}NO_5$): C, 73.18; H, 6.36; N, 3.05; found: C, 73.06; H, 6.42; N, 2.90.

*4.1.7.7. 5,7-Dihydroxy-3-(4-(4-((2-methoxybenzyl)(methyl)amino)butoxy)phenyl)-4H-chromen-4-one (**19c**).* It was synthesized from **16c** according to the general procedure. Elution with CH_2Cl_2/MeOH = 60:1 gave **19c** as an almost white solid; 76.6% yield; mp 67–69 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.55 (s, 1H, 2-H), 7.36–7.33 (m, 2H, 2 × Ar–H), 7.25 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.97 (t, J = 7.6 Hz, 1H, Ar–H), 6.90 (d, J = 8.4 Hz, 1H, Ar–H), 6.67 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.23 (s, 1H, 8-H), 6.15 (s, 1H, 6-H), 3.91 (s, 2H, CH_2O), 3.82 (s, 5H, CH_3O, $PhCH_2$), 2.77 (t, J = 7.6 Hz, 2H, CH_2N), 2.59 (s, 3H, CH_3N), 1.90–1.87 (m, 2H, CH_2), 1.79–1.76 (m, 2H, CH_2); HR-ESI-MS: Calcd. for $C_{28}H_{30}NO_6$ $[M+H]^+$: 476.2073, found: 476.2080; Anal. calcd. For ($C_{28}H_{29}NO_6$): C, 70.72; H, 6.15; N, 2.95; found: C, 70.80; H, 6.12; N, 3.03.

*4.1.7.8. 3-(4-(4-(Ethyl(2-methoxybenzyl)amino)butoxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**19d**).* It was synthesized from **16d** according to the general procedure. Elution with CH_2Cl_2/MeOH = 80:1 gave **19d** as an almost white solid; 65.5% yield; mp 60–63 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.81 (s, 1H, 5-OH), 7.56 (s, 1H, 2-H), 7.42 (d, J = 7.6 Hz, 1H, Ar–H), 7.33 (t, J = 8.0 Hz, 1H, Ar–H), 7.28 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.98 (t, J = 7.6 Hz, 1H, Ar–H), 6.89 (d, J = 8.0 Hz, 1H, Ar–H), 6.69 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.20 (s, 1H, 8-H), 6.13 (s, 1H, 6-H), 3.97 (s, 2H, CH_2O), 3.82–3.77 (m, 5H, CH_3O, $PhCH_2$), 2.86 (q, J = 6.8 Hz, 2H, CH_2N), 2.80 (t, J = 7.6 Hz, 2H, CH_2N), 1.88 (m, 2H, CH_2), 1.78–1.74 (m, 2H, CH_2), 1.27 (t, J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.1, 167.6, 162.4, 158.7, 158.2, 157.9, 152.2, 131.3, 130.5, 129.8, 129.3, 123.5, 123.2, 122.6, 120.4, 114.2, 110.5, 110.3, 104.2, 100.7, 94.8, 67.2, 55.2, 52.2, 51.0, 46.8, 26.8, 22.3, 10.2; HR-ESI-MS: Calcd. for $C_{29}H_{32}NO_6$ $[M+H]^+$: 490.2230, found: 490.2192; Anal. calcd. For ($C_{29}H_{31}NO_6$): C, 71.15; H, 6.38; N, 2.86; found: C, 71.02; H, 6.45; N, 2.66.

*4.1.7.9. 3-(4-((6-(Benzyl(methyl)amino)hexyl)oxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**20a**).* It was synthesized from **17a** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **20a** as an almost white solid; 82.0% yield; mp 120–122 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.90 (s, 1H, 5-OH), 7.66 (s, 1H, 2-H), 7.34–7.32 (m, 7H, 7 × Ar–H), 6.85 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.23 (d, J = 2.0 Hz, 1H, 8-H), 6.20 (d, J = 2.0 Hz, 1H, 6-H), 3.84 (t, J = 6.4 Hz, 2H, CH_2O), 3.69 (s, 2H, $PhCH_2$), 2.55 (t, J = 7.2 Hz, 2H, CH_2N), 2.30 (s, 3H, CH_3N), 1.75–1.69 (m, 2H, CH_2), 1.68–1.61 (m, 2H, CH_2), 1.44–1.38 (m, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{29}H_{32}NO_5$ $[M+H]^+$: 474.2280, found: 474.2273; Anal. calcd. For ($C_{29}H_{31}NO_5$): C, 73.55; H, 6.60; N, 2.96; found: C, 73.42; H, 6.76; N, 2.78.

*4.1.7.10. 3-(4-((6-(Benzyl(ethyl)amino)hexyl)oxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**20b**).* It was synthesized from **17b** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **20b** as an almost white solid; 82.4% yield; mp 47–50 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.90 (s, 1H, 5-OH), 7.68 (s, 1H, 2-H), 7.38–7.30 (m, 7H, 7 × Ar–H), 6.86 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.24 (d, J = 2.0 Hz, 1H, 8-H), 6.21 (d, J = 2.0 Hz, 1H, 6-H), 3.85 (t, J = 6.4 Hz, 2H, CH_2O), 3.78 (s, 2H, $PhCH_2$), 2.70 (q, J = 7.2 Hz, 2H, CH_2N), 2.61 (t, J = 7.2 Hz, 2H, CH_2N), 1.74–1.68 (m, 2H, CH_2), 1.67–1.61 (m, 2H, CH_2), 1.45–1.35 (m, 4H, 2 × CH_2), 1.16 (t, J = 7.2 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{30}H_{34}NO_5$ $[M+H]^+$: 488.2437, found: 488.2438; Anal. calcd. For ($C_{30}H_{33}NO_5$): C, 73.90; H, 6.82; N, 2.87; found: C, 73.76; H, 6.86; N, 2.99.

*4.1.7.11. 5,7-Dihydroxy-3-(4-((6-((2-methoxybenzyl)(methyl)amino)hexyl)oxy)phenyl)-4H-chromen-4-one (**20c**).* It was synthesized from **17c** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **20c** as an almost white solid; 87.2% yield; mp 59–61 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.87 (s, 1H, 5-OH), 7.62 (s, 1H, 2-H), 7.36 (t, J = 7.6 Hz, 1H, Ar–H), 7.34 (d, J = 8.0 Hz, 1H, Ar–H), 7.29 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.97 (t, J = 7.6 Hz, 1H, Ar–H), 6.91 (d, J = 8.0 Hz, 1H, Ar–H), 6.81 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.29 (d, J = 1.2 Hz, 1H, 8-H), 6.27 (d, J = 1.2 Hz, 1H, 6-H), 3.93 (s, 2H, $PhCH_2$), 3.82 (s, 3H, CH_3O), 3.78 (t, J = 6.4 Hz, 2H, CH_2O), 2.74 (t, J = 7.6 Hz, 2H, CH_2N), 2.47 (s, 3H, CH_3N), 1.75–1.68 (m, 4H, 2 × CH_2), 1.43–1.38 (m, 4H, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{30}H_{34}NO_6$ $[M+H]^+$: 504.2386, found: 504.2380; Anal. calcd. For ($C_{30}H_{33}NO_6$): C, 71.55; H, 6.61; N, 2.78; found: C, 71.46; H, 6.80; N, 3.01.

*4.1.7.12. 3-(4-((6-(Ethyl(2-methoxybenzyl)amino)hexyl)oxy)phenyl)-5,7-dihydroxy-4H-chromen-4-one (**20d**).* It was synthesized from **17d** according to the general procedure. Elution with CH_2Cl_2/MeOH = 25:1 gave **20d** as an almost white solid; 91.4% yield; mp 51–53 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.86 (s, 1H, 5-OH), 7.63 (s, 1H, 2-H), 7.40 (d, J = 7.2 Hz, 1H, Ar–H), 7.33–7.29 (m, 3H, 3 × Ar–H), 6.96 (t, J = 7.2 Hz, 1H, Ar–H), 6.88 (d, J = 8.0 Hz, 1H, Ar–H), 6.84 (d,

J = 8.8 Hz, 2H, 3′, 5′-H), 6.17 (s, 1H, 8-H), 6.16 (s, 1H, 6-H), 3.88 (s, 2H, $PhCH_2$), 3.83 (t, J = 6.4 Hz, 2H, CH_2O), 3.81 (s, 3H, CH_3O), 2.80 (q, J = 7.2 Hz, 2H, CH_2N), 2.69 (t, J = 7.2 Hz, 2H, CH_2N), 1.70–1.68 (m, 4H, 2 × CH_2), 1.37–1.34 (m, 4H, 2 × CH_2), 1.22 (t, J = 7.2 Hz, 3H, CH_3); HR-ESI-MS: Calcd. for $C_{31}H_{36}NO_6$ $[M+H]^+$: 518.2543, found: 518.2540; Anal. calcd. For ($C_{31}H_{35}NO_6$): C, 71.93; H, 6.82; N, 2.71; found: C, 71.79; H, 6.80; N, 2.58.

*4.1.8. General procedure for synthesis of intermediates (**21**–**23**)*

To a mixture of the genistein (300 mg, 1.11 mmol), anhydrous K_2CO_3 (614 mg, 4.44 mmol) and KI (18 mg, 0.11 mmol) in acetone (10.0 mL), the appropriate dibromoalkane derivative (4.44 mmol) was added. The reaction mixture was stirred and refluxed for 8–10 h under an argon atmosphere. After complete reaction, the solvent was evaporated under reduced pressure. Water (30 mL) was added to the residue and the mixture was extracted with dichloromethane (30 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride (30 mL), dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography to give the intermediates **21**–**23**.

*4.1.8.1. 7-(3-Bromopropoxy)-3-(4-(3-bromopropoxy)phenyl)-5-hydroxy-4H-chromen-4-one (**21**).* It was synthesized from 1,3-dibromopropane. Elution with CH_2Cl_2 afforded **21** as an almost white solid; yield 68.7%; mp 132–134 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.87 (s, 1H, 2-H), 7.46 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.99 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.42 (d, J = 2.0 Hz, 1H, 8-H), 6.38 (d, J = 2.0 Hz, 1H, 6-H), 4.19–4.13 (m, 4H, 2 × CH_2O), 3.63–3.59 (m, 4H, 2 × CH_2Br), 2.38–2.31 (m, 4H, 2 × CH_2).

*4.1.8.2. 7-(4-Bromobutoxy)-3-(4-(4-bromobutoxy)phenyl)-5-hydroxy-4H-chromen-4-one (**22**).* It was synthesized from 1,4-dibromobutane. Elution with CH_2Cl_2 afforded **22** as an almost white solid; yield 54.0%; mp 98–100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.86 (s, 1H, 2-H), 7.46**5** (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.96 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.39 (d, J = 2.0 Hz, 1H, 8-H), 6.36 (d, J = 2.0 Hz, 1H, 6-H), 4.08–4.02 (m, 4H, 2 × CH_2O), 3.52–3.49 (m, 4H, 2 × CH_2Br), 2.12–2.02 (m, 4H, 2 × CH_2), 2.00–1.94 (m, 4H, 2 × CH_2).

*4.1.8.3. 7-((6-Bromohexyl)oxy)-3-(4-((6-bromohexyl)oxy)phenyl)-5-hydroxy-4H-chromen-4-one (**23**).* It was synthesized from 1,6-dibromohexane. Elution with CH_2Cl_2 afforded **23** as an almost white solid; yield 75.5%; mp 97–109 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.86 (s, 1H, 2-H), 7.45 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 6.96 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 6.39 (d, J = 2.0 Hz, 1H, 8-H), 6.36 (d, J = 2.0 Hz, 1H, 6-H), 4.04–3.98 (m, 4H, 2 × CH_2O), 3.45–3.38 (m, 4H, 2 × CH_2Br), 1.92–1.80 (m, 8H, 4 × CH_2), 1.59–1.45 (m, 8H, 4 × CH_2).

*4.1.9. General procedure for preparation of 7,4′-O-modified genistein derivatives (**24d, 25b, 25d and 26d**)*

To a mixture of *N*-benzylethylamine (**7b**) or *N*-(2-methoxybenzyl)-ethylamine (**7d**) (0.67 mmol), anhydrous K_2CO_3 (93 mg, 0.67 mmol) and KI (2.82 mg, 0.017 mmol) were added the appropriate intermediates **21**–**23** (0.168 mmol). The reaction mixture was warmed to 60–65 °C and stirred for 12–15 h under an argon atmosphere. After complete reaction, the solvent was evaporated under reduced pressure. Water (20 mL) was added to the residue and the mixture was extracted with dichloromethane (20 mL × 3). The combined organic phases were washed with saturated aqueous sodium chloride (20 mL), dried over sodium sulfate, and filtered. The solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel chromatography using mixtures of CH_2Cl_2/CH_3OH as eluent, obtaining the corresponding 7,4′-*O*-modified genistein derivatives.

*4.1.9.1. 7-(3-(Ethyl(2-methoxybenzyl)amino)propoxy)-3-(4-(3-(ethyl(2-methoxybenzyl)amino) propoxy)phenyl)-5-hydroxy-4H-chromen-4-one (**24d**).* It was obtained from *N*-(2-methoxybenzyl)-ethylamine (**7d**) and 7-(3-bromopropoxy)-3-(4-(3-bromopropoxy) phenyl)-5-hydroxy-4*H*-chromen-4-one (**21**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 50:1 gave **24d** as a light yellow oil; 56.0% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.85 (s, 1H, 2-H), 7.44–7.38 (m, 4H, 4 × Ar–H), 7.21 (t, J = 7.2 Hz, 2H, 2 × Ar–H), 6.95–6.89 (m, 4H, 4 × Ar–H), 6.87–6.84 (m, 2H, 2 × Ar–H), 6.36 (d, J = 2.0 Hz, 1H, 8-H), 6.33 (d, J = 2.0 Hz, 1H, 6-H), 4.09–4.03 (m, 4H, 2 × CH_2O), 3.81 (s, 6H, 2 × CH_3O), 3.63 (s, 4H, 2 × $PhCH_2$), 2.66 (brs, 4H, 2 × CH_2N), 2.58 (brs, 4H, 2 × CH_2N), 1.99 (brs, 4H, 2 × CH_2), 1.09–1.07 (m, 6H, 2 × CH_3); HR-ESI-MS: Calcd. for $C_{41}H_{49}N_2O_7$ $[M+H]^+$: 681.3540, found: 681.3535; Anal. calcd. For ($C_{41}H_{48}N_2O_7$): C, 72.33; H, 7.11; N, 4.11; found: C, 72.12; H, 7.27; N, 3.95.

*4.1.9.2. 7-(4-(benzyl(ethyl)amino)butoxy)-3-(4-(4-(benzyl(ethyl)amino)butoxy)phenyl)-5-hydroxy-4H-chromen-4-one (**25b**).* It was obtained from *N*-benzylethylamine (**7b**) and 7-(4-bromobutoxy)-3-(4-(4-bromobutoxy)phenyl)-5-hydroxy-4*H*-chromen-4-one (**22**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 30:1 gave **25b** as a light yellow oil; 68.6% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.86 (s, 1H, 5-OH), 7.85 (s, 1H, 2-H), 7.44 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 7.33-7.22 (m, 10H, 10 × Ar–H), 6.94 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 6.36 (d, J = 2.0 Hz, 1H, 8-H), 6.34 (d, J = 2.0 Hz, 1H, 6-H), 3.99–3.94 (m, 4H, 2 × CH_2O), 3.60 (s, 2H, $PhCH_2$), 3.58 (s, 2H, $PhCH_2$), 2.57–2.48 (brs, 8H, 4 × CH_2N), 1.84–1.79 (m, 4H, 2 × CH_2), 1.69–1.63 (brs, 4H, 2 × CH_2), 1.06 (brs, 6H, 2 × CH_3); $^{13C\ NMR\ (100\ MHz,\ CDCl_3)}$ δ 180.8, 165.0, 162.6, 159.2, 157.9, 152.6, 130.0, 128.9, 128.8, 128.1, 126.8, 123.6, 122.7, 114.6, 106.1, 98.5, 92.8, 68.3, 67.7, 58.0, 57.9, 52.5, 52.3, 47.2, 47.1, 26.9, 26.6, 23.3, 11.6, 11.5; HR-ESI-MS: Calcd. for $C_{41}H_{49}N_2O_5$ $[M+H]^+$: 649.3641, found: 649.3628; Anal. calcd. For ($C_{41}H_{48}N_2O_5$): C, 75.90; H, 7.46; N, 4.32; found: C, 75.75; H, 7.45; N, 4.23.

*4.1.9.3. 7-(4-(Ethyl(2-methoxybenzyl)amino)butoxy)-3-(4-(4-(ethyl(2-methoxybenzyl)amino) butoxy)phenyl)-5-hydroxy-4H-chromen-4-one (**25d**).* It was obtained from *N*-(2-methoxybenzyl)-ethylamine (**7d**) and 7-(4-bromobutoxy)-3-(4-(4-bromobutoxy) phenyl)-5-hydroxy-4*H*-chromen-4-one (**22**) according to the general procedure. Elution with CH_2Cl_2/MeOH = 30:1 gave **25d** as a light yellow oil; 66.4% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.86 (s, 1H, 2-H), 7.45–7.43 (m, 4H, 4 × Ar–H), 7.25–7.23 (m, 2H, 2 × Ar–H), 6.95–6.93 (m, 4H, 4 × Ar–H), 6.87 (d, J = 8.0 Hz, 2H, 3′, 5′-H), 6.36 (d, J = 2.0 Hz, 1H, 8-H), 6.34 (d, J = 2.0 Hz, 1H, 6-H), 4.01–3.96 (m, 4H, 2 × CH_2O), 3.83 (s, 6H, 2 × CH_3O), 3.68 (s, 4H, 2 × $PhCH_2$), 2.60 (brs, 8H, 4 × CH_2N), 1.88–1.81 (m, 4H, 2 × CH_2), 1.73 (brs, 4H, 2 × CH_2), 1.11 (brs, 6H, 2 × CH_3); $^{13C\ NMR\ (100\ MHz,\ CDCl_3)}$ δ 180.7, 164.8, 162.5, 159.0, 157.8, 157.7, 157.6, 152.5, 130.8, 130.5, 129.9, 128.5, 128.2, 123.4, 122.7, 120.4, 120.3, 114.5, 110.3, 110.2, 106.0, 98.5, 92.6, 68.2, 67.6, 55.3, 52.4, 50.9, 50.8, 47.5, 47.4, 26.9, 26.6, 22.9, 22.7, 11.2, 11.0; HR-ESI-MS: Calcd. for $C_{43}H_{53}N_2O_7$ $[M+H]^+$: 709.3753, found: 709.3754; Anal. calcd. For ($C_{43}H_{52}N_2O_7$): C, 72.86; H, 7.39; N, 3.95; found: C, 72.67; H, 7.50; N, 3.82.

*4.1.9.4. 7-((6-(Ethyl(2-methoxybenzyl)amino)hexyl)oxy)-3-(4-((6-(ethyl(2-methoxybenzyl)amino) hexyl)oxy)phenyl)-5-hydroxy-4H-chromen-4-one (**26d**).* It was obtained from *N*-(2-methoxybenzyl) n-ethylamine (**7d**) and 7-((6-bromohexyl)oxy)-3-(4-((6-bromohexyl)oxy) phenyl)-5-hydroxy-4*H*-chromen-4-one (**23**) according to the general procedure. Elution with CH_2Cl_2/

MeOH = 50:1 gave **26d** as a light yellow oil; 53.0% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.86 (s, 1H, 5-OH), 7.87 (s, 1H, 2-H), 7.71–7.67 (m, 2H, 2 × Ar–H), 7.45 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 7.37 (t, J = 7.6 Hz, 2H, 2 × Ar–H), 7.03 (t, J = 7.6 Hz, 2H, 2 × Ar–H), 6.97–6.92 (m, 4H, 4 × Ar–H), 6.39 (d, J = 2.4 Hz, 1H, 8-H), 6.35 (d, J = 2.4 Hz, 1H, 6-H), 4.15 (s, 4H, 2 × $PhCH_2$), 4.02–3.96 (m, 4H, 2 × CH_2O), 3.87 (s, 6H, 2 × CH_3O), 2.99 (brs, 4H, 2 × CH_2N), 2.87 (m, 4H, 2 × CH_2N), 1.86–1.76 (m, 8H, 4 × CH_2), 1.54–1.47 (m, 4H, 2 × CH_2), 1.45–1.34 (m, 10H, 2 × CH_3, 2 × CH_2); HR-ESI-MS: Calcd. for $C_{47}H_{61}N_2O_7$ $[M+H]^+$: 765.4479, found: 765.4468; Anal. calcd. For ($C_{47}H_{60}N_2O_7$): C, 73.79; H, 7.91; N, 3.66; found: C, 73.90; H, 7.75; N, 3.58.

4.2. Biological evaluation

4.2.1. Inhibition experiments of AChE and BuChE

To assess the inhibitory activity of the compounds toward AChE or BuChE, we followed the spectrophotometric method of Ellman, using AChE from 5% rat cortex homogenate or purified AChE from both *E. electricus* (Sigma–Aldrich Co.) and human erythrocytes (Sigma–Aldrich Co.) or BuChE from rat serum [33,45]. The brain homogenate was preincubated for 5 min with tetraisopropyl pyrophosphoramide (*iso*-OMPA, selective inhibitor of BuChE, 4.0 mmol/L) (Sigma–Aldrich Co.) before use. For rat AChE or BuChE inhibition assays, a reaction mixture (100 μL) containing acetylthiocholine iodide (1 mmol/L, 30 μL) (J&K Scientific) or butyrylthiocholine iodide (1 mmol/L, 30 μL) (TCI Shanghai Development), phosphate-buffered solution (0.1 mmol/L, pH = 7.4, 40 μL), 5% homogenate or 25% serum (10 μL) and different concentrations of test compounds (20 μL) was incubated at 37 °C for 15 min. Then 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, 0.2%, 30 μL) (J&K Scientific) was added to produce the yellow anion of 5-thio-2-nitrobenzoic acid. Changes in absorbance were detected at 405 nm in a Varioskan Flash Multimode Reader (Thermo Scientific). For *E. electricus* AChE and human AChE inhibition assay, *Ee*AChE or *Hu*AChE (0.05 U/mL, final concentration) was used and the assay was carried out in a phosphate buffer (0.01 mmol/L, pH = 8.0). Changes in absorbance were detected at 412 nm [34,46]. The other procedure was the same as above. Compounds inhibiting AChE or BuChE activity would reduce the color generation. Thus, IC_{50} values were calculated as the concentration of compound that produces 50% AChE or BuChE activity inhibition. Donepezil was applied as positive drug. All samples were assayed in triplicate.

4.2.2. Kinetic characterization of AChE inhibition

Kinetic characterization of AChE inhibition was performed based on a reported method using purified AChE from *E. electricus* (*Ee*AChE) [46]. The assay solution (100 μL) consists of 0.1 M phosphate buffer (pH 8.0), with the addition of 30 μL of 0.2% DTNB, 10 μL of 0.5 units/mL *Ee*AChE, and 20 μL of substrate (ATCh). Three different concentrations of inhibitors were added to the assay solution and pre-incubated for 15 min at 37 °C with the *Ee*AChE followed by the addition of substrate in different concentrations. Kinetic characterization of the hydrolysis of ATCh catalyzed by *Ee*AChE was done spectrometrically at 412 nm. The parallel control experiments were performed without inhibitor in the assay. The plots were assessed by a weighted least square analysis that assumed the variance of v to be a constant percentage of v for the entire data set. Slopes of these reciprocal plots were then plotted against the concentration of **25d** in a weighted analysis, and K_i was determined as the intercept on the negative x-axis.

4.2.3. Molecular docking

The crystal structure of AChE complexed with donepezil (code ID: 1EVE) was obtained from the Protein Data Bank after eliminating the original inhibitors and water molecules. The 3D Structure of **25d** was built and performed geometry optimization by molecular mechanics. After addition of Gasteiger charges, removal of hydrogen atoms, addition of their atomic charges to skeleton atoms, and the assignment of proper atomic types, the further preparation of the inhibitor was accomplished. Autotors was then used to define the rotatable bonds in the ligands. Docking studies were performed using the AUTODOCK 4.2 program. By using Autodock Tools (ADT; version 1.5.6), polar hydrogen atoms were added to amino acid residues, and Gasteiger charges were assigned to all atoms of the enzyme. The resulting enzyme structure was used as an input for the AUTOGRID program. AUTOGRID performed a pre-calculated atomic affinity grid maps for each atom type in the ligand, plus an electrostatics map and a separate desolvation map presented in the substrate molecule. All maps were calculated with 0.375 Å spacing between grid points. The center of the grid box was placed at the center of donepezil with coordinates $x = 2.023$, $y = 63.295$, $z = 67.062$. The dimensions of the active site box were set at 50 × 50 × 50 Å. Flexible ligand docking was performed for the compounds. Each docked system was performed by 100 runs of the AUTODOCK search by the Lamarckian genetic algorithm (LGA). Other than the referred parameters above, the other parameters were accepted as default. A cluster analysis was performed on the docking results using a root mean square (RMS) tolerance of 1.0 and the lowest energy conformation of the highest populated cluster was selected for analysis. Graphic manipulations and visualizations were done by Autodock Tools or Discovery Studio 2.0 software.

4.2.4. Antioxidant activity assay

The antioxidant activity was determined by the oxygen radical absorbance capacity fluorescein (ORAC-FL) method partially modified by Fang Lei et al. [47]. 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH) was purchased from Accela ChemBio Co., Ltd. 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), and fluorescein (FL) were purchased from TCI (Shanghai) Development. All the assays were conducted with 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant (20 μL) and fluorescein (120 μL, 150 nM final concentration) were placed in the wells of a black 96-well plate. The mixture was pre-incubated for 15 min at 37 °C, and then AAPH solution (60 μL, 12 mM final concentration) was added rapidly using an autosampler. The plate was immediately placed in a Varioskan Flash Multimode Reader (Thermo Scientific) and the fluorescence recorded every minute for 90 min with excitation at 485 nm and emission at 535 nm. The plate was automatically shaken prior to each reading. Trolox was used as standard (1–8 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 concentration (1–10 μM). All the reaction mixture was prepared in duplicate, and at least three independent assays were performed for each sample. Antioxidant curves (fluorescence versus time) were normalized to the curve of the blank in the same assay, and then the area under the fluorescence decay curve (AUC) was calculated. The net AUC of a sample was obtained by subtracting the AUC of the blank. ORAC-FL values were expressed as Trolox equivalents by using the standard curve calculated for each sample, where the ORAC-FL value of Trolox was taken as 1, indicating the antioxidant potency of the tested compounds.

4.2.5. Metal binding studies [9,42]

The metal binding studies were carried out in a Shimadzu UV-2450 spectrophotometer. To investigate the metal binding ability of compound, the UV absorption of the tested compound **10b**, **19d** or **25d**, in the absence or presence of $CuCl_2$, $FeSO_4$, $ZnCl_2$, and $AlCl_3$,

was recorded with wavelength ranging from 200 to 500 nm after incubating for 30 min at room temperature. The final volume of reaction mixture was 1 mL, and the final concentrations of tested compound and metals were 37.5 μM. Numerical subtraction of the spectra of the metal alone and the compound alone from the spectra of the mixture gave the difference UV–vis spectra due to complex formation. The molar ration method was performed to determine the stoichiometry of the complex compound-metal by titrating the methanol solution of tested compound with ascending of $CuCl_2$. The final concentration of tested compound was 37.5 μM, and the final concentration of Cu^{2+} ranged from 7.5 to 93.75 μM. The UV spectra was recorded and treated by numerical subtraction of $CuCl_2$ and tested compound at corresponding concentrations, plotted versus the mole fraction of tested compound.

4.2.6. Determination of the inhibitory effect on the self-induced $A\beta_{1-42}$ aggregation

In order to investigate the self-induced $A\beta_{1-42}$ aggregation, a Thioflavin T-based flurometric assay was performed [35]. Thioflavin T (Basic Yellow 1) was purchased from TCI (Shanghai) Development. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Energy Chemical. β-Amyloid$_{1-42}$ ($A\beta_{1-42}$), supplied as trifluoroacetate salt, was purchased from ChinaPeptides Co., Ltd. Briefly, $A\beta_{1-42}$ was dissolved in HFIP (1 mg/mL) and incubated for 24 h at room temperature, and solvent was evaporated. Then the HFIP pretreated $A\beta_{1-42}$ was resolubilized in dry DMSO to a final stock concentration of 200 μM and was kept frozen at −80 °C until use. Solutions of test compounds were prepared in DMSO in 2.5 mM for storage and diluted with phosphate buffer solution (pH 7.4) before use. For the self-induced assay, $A\beta_{1-42}$ (20 μL, 25 μM, final concentration) was incubated with 20 μL of test compounds at different concentrations ranging from 10 to 50 μM in 50 mM phosphate buffer solution (pH 7.4) at 37 °C for 24 h. To minimize evaporation effect the wells were sealed by a transparent heat-resistant plastic film. After incubation, 160 μL of 5 μM thioflavin T in 50 mM glycine–NaOH buffer (pH 8.5) was added. The final concentration of DMSO in each well was 12.5%. Each assay was run in triplicate. Fluorescence was measured on a Varioskan Flash Multimode Reader (Thermo Scientific) with excitation and emission wavelengths at 446 nm and 490 nm, respectively. The fluorescence intensities were compared and the percent inhibition due to the presence of the inhibitor was calculated by the following formula: $100 - IF_i/(IF_c{*}100)$, where IF_i and IF_c were the fluorescence intensities obtained for $A\beta_{1-42}$ in the presence and in the absence of inhibitors, respectively.

4.2.7. Inhibition of AChE-induced $A\beta_{1-40}$ aggregation

The thioflavin-T (ThT) fluorescence method was used as previously described [40]. Acetylcholinesterase from human erythrocytes was purchased from Sigma Co. β-Amyloid$_{1-40}$ ($A\beta_{1-40}$), supplied as trifluoroacetate salt, was purchased from ChinaPeptides Co., Ltd. HFIP pre-treated $A\beta_{1-40}$ and tested compounds were dissolved in DMSO to obtain 2.3 mM and 1 mM solutions respectively. For the AChE-induced assay, Aliquots of 2 μL of $A\beta_{1-40}$ were incubated for 24 h at room temperature in 0.215 mM sodium phosphate buffer (pH 8.0) at a final concentration of 230 μM. For co-incubations experiments, 16 μL of *Hu*AChE (final concentration of 2.3 μM, $A\beta_{1-40}$/AChE molar ration of 100:1) and AChE in the presence of 2 μL of the tested inhibitor (final concentration 100 μM) in 0.215 M sodium phosphate buffer (pH 8.0) solutions were added. Blanks containing $A\beta_{1-40}$ alone, human AChE alone, and $A\beta_{1-40}$ plus tested inhibitors in 0.215 sodium phosphate buffer (pH 8.0) were prepared. After incubation, 180 μL of 5 μM thioflavin T in 50 mM glycine–NaOH buffer (pH 8.5) was added. Each assay was run in triplicate. The detection method was the same as above. The percent inhibition of the AChE-induced aggregation due to the presence of the tested compound was calculated by the following formula: $100 - IF_i/(IF_c{*}100)$, where IF_i and IF_c were the fluorescence intensities obtained for Aβ plus AChE in the presence and in the absence of inhibitors, respectively, minus the fluorescence intensities due to the respectively blanks.

4.2.8. Effect of test compounds on metal-induced $A\beta_{1-42}$ aggregation and disaggregation experiments by ThT method

Solutions of Cu^{2+} were prepared from standards to concentration of 75 μM using the HEPES buffer (20 mM, pH 6.6, 150 mM NaCl). For the inhibition of copper-induced $A\beta_{1-42}$ aggregation assay [41,42], the $A\beta_{1-42}$ stock solution was diluted in HEPES buffer (20 mM, pH 6.6, 150 mM NaCl). The mixture of the peptide (20 μL, 25 μM, final concentration) and Cu^{2+} (20 μL, 25 μM, final concentration), with or without the tested compound at different concentrations (20 μL, 10–35 μM, final concentration) was incubated at 37 °C for 24 h. After incubation, 190 μL of 5 μM thioflavin T in 50 mM glycine–NaOH buffer (pH 8.5) was added. The final concentration of DMSO in each well was 12.5%. Each assay was run in triplicate. The detection method was the same as that of self-induced $A\beta_{1-42}$ experiment.

For the disaggregation of copper-induced Aβ fibrils experiment, the $A\beta_{1-42}$ stock solution was diluted in HEPES buffer (20 mM, pH 6.6, 150 mM NaCl). The mixture of the $A\beta_{1-42}$ (20 μL, 25 μM, final concentration) with Cu^{2+} (20 μL, 25 μM, final concentration) was incubated 37 °C for 24 h. The tested compound (20 μL, 25 μM, final concentration) was then added and incubated at 37 °C for another 24 h. To minimize evaporation effect the wells were sealed by a transparent heat-resistant plastic film. After incubation, 190 μL of 5 μM thioflavin T in 50 mM glycine–NaOH buffer (pH 8.5) was added. The final concentration of DMSO in each well was 12.5%. Each assay was run in triplicate. The detection method was the same as above.

4.2.9. Step-down passive avoidance test

4.2.9.1. Materials and animals. Donepezil was purchased from Eisai China Inc. Scopolamine was purchase from J&K Scientific. Scopolamine was dissolved in 0.9% saline. Donepezil and test compounds were dissolved in vegetable oil, which was also used for the negative control. Kunming mice at body weight of 18–22 g (six weeks old, either gender) were supplied by the Center of Experimental Animals of Sichuan Academy of Chinese Medicine Sciences (eligibility certification no. SCXK-Sichuan 2008–19). Mice were maintained under standard conditions with a 12 h:12 h light–dark cycle, a temperature and humidity controlled environment with access to food and water ad libitum. The mice were submitted to behavioral tests one day after 7 days of treatment with compounds.

4.2.9.2. Assay method. A modification of step-down passive avoidance test was used to assess learning and memory in mice [48,49]. The apparatus consisted of a grid floor with a wooden block placed in the center. The block served as a shock free zone. The mice underwent two separate trials: a training trial and a test trial 24 h later. For training trial, mice were initially placed on the block and were given an electrical foot shock (0.5 mA, 2 s) through the grid floor on stepping down. We used a total of 120 mice in the passive avoidance test with 10 mice were used per treatment. Compounds **10b** and **19d** (2, 6 and 18 mg/kg, *p.o.*) or **25d** (3, 9, 27 mg/kg, *p.o.*) or donepezil (5 mg/kg, *p.o.*) as a positive control were orally given 1 h before each training trial. After 30 min, memory impairment was induced by administering scopolamine (3 mg/kg, *i.p.*). Twenty-four hours after the training trial, mice were placed on the block and the time for the animal to step down was measured as latency time for test trial. An upper cut-off time was set at 300 s.

4.2.9.3. Statistical analysis. All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using one-way ANOVA with Student's *t* test. A *p* value less than 0.05 denoted the presence of a statistically significant difference.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.045.

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