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Design, synthesis and biological evaluation of novel dual inhibitors of acetylcholinesterase and β-secretase

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ABSTRACT

To explore novel effective drugs for the treatment of Alzheimer's disease (AD), a series of dual inhibitors of acetylcholineterase (AChE) and β-secretase (BACE-1) were designed based on the multi-target-directed ligands strategy. Among them, inhibitor **28** exhibited good dual potency in enzyme inhibitory potency assay (BACE-1: IC_{50} = 0.567 μM; AChE: IC_{50} = 1.83 μM), and also showed excellent inhibitory effects on Aβ production of APP transfected HEK293 cells (IC_{50} = 98.7 nM) and mild protective effect against hydrogen peroxide (H_2O_2)-induced PC12 cell injury. Encouragingly, intracerebroventricular injection of **28** into amyloid precursor protein (APP) transgenic mice caused a 29% reduction of $Aβ_{1-40}$ production. Therefore, **28** was demonstrated as a good lead compound for the further study and more importantly, the strategy of AChE and BACE-1 dual inhibitors might be a promising direction for developing novel drugs for AD patients.

1. Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia characterized by a progressive deterioration in cognitive function, affecting approximately 3% of the population between the ages of 65–74, and nearly 50% of those 85 years and older. It has become one of the most costly diseases which brings heavy social and financial burden to both society and families.[1]

To date, on the basis of the *cholinergic hypothesis*, the major marketed drugs for AD are acetylcholinesterase (AChE) inhibitors, that is, donepezil, rivastigmine, and galantamine. Unfortunately, these drugs have been approved for the symptomatic treatment of AD as they do not address the underlying neuropathology.[2] In 2004, memantine was approved by FDA to treat moderate to severe AD patients as a NMDA-receptor antagonist. However, it plays a limited role in terms of the progression of the disease. Therefore, extensive research should be carried out to search for more effective therapy and drugs for the treatment of the disease.

Recently, the accumulation of the β-amyloid peptide (Aβ) in the brain has been thought to be a key factor in the pathogenesis of the disease.[3] β-Secretase (BACE-1), an aspartyl protease, is an attractive therapeutic target since it plays a key role in the rate-limiting step of Aβ generation.[4] In addition, BACE-1 gene knockout homozygote mice showed a complete absence of Aβ production and no obvious deficits in basal neurological and physiological functions.[5] Since the identification of BACE-1 in 1999, numerous BACE-1 inhibitors have been synthesized and tested in vitro as well in vivo, showing amyloid lowering properties in animal models (Fig. 1).[6]

Despite of the success, the actual effects of BACE-1 inhibitors for AD patients need to be further evaluated. It is still important to explore novel drug candidate. Considering the complexity of AD, the classic 'one molecule, one target' solution may not be effective enough.[7] The novel multi-target-directed strategy has received attention, since single molecule simultaneously interacts with multiple targets in the complex neurotoxic cascades would achieve better efficacy by a complementary manner. Meanwhile, the hybrid would reduce individual toxicity by special metabolic pathway compared with the combinational drugs. The recent studies following this strategy have led to the synthesis of several chemically diverse structures with dual or multiple biological profiles of AD,[8] including AChE and monoamine oxidase B (MAO-B) dual inhibitors (e.g., Ladostigil),[8e] AChE and serotonin transporter (SER) dual inhibitors (e.g., **3**),[8f] and AChE, BACE, Aβ aggregation inhibiting and antioxidant multiple functional compounds (e.g., Memoquin) (Fig. 2).[8t] Accordingly, considering AChE is the most successful tar-

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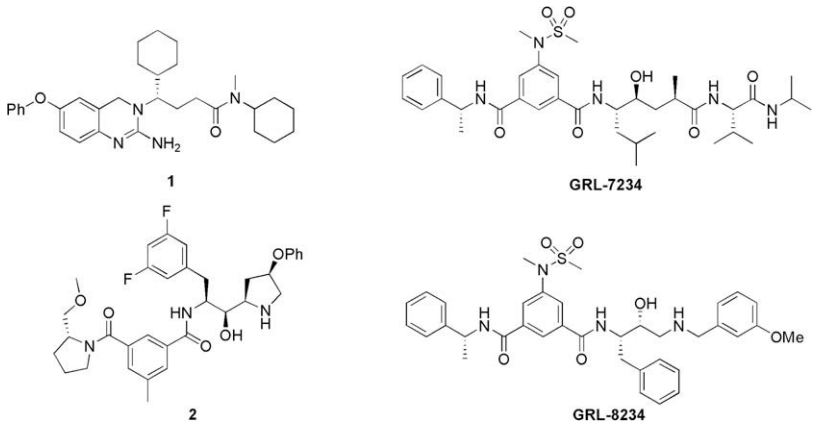


Figure 1. Representative potent BACE-1 inhibitors.

get for symptomatic treatment of AD and BACE-1 is a crucial factor of Aβ formation for the pathogenesis, our efforts were focused on designing and synthesizing dual inhibitors that can simultaneously work on both AChE and BACE-1. Inhibition of AChE activity would alleviate clinical symptom in short-term therapy, while inhibition of BACE-1 would have an additive effect by preventing the formation of Aβ and further slowing the progression of the long-term disease. This therapy may likely result in a comprehensive suppression of AD by synergistic manner and obtain higher therapeutic effectiveness. More recently, Lorna Piazzi and co-workers have reported the design and evaluation of the first dual inhibitors of AChE and BACE-1 (e.g., **4**), which further strengthen our hypothesis.[8y] Herein, we displayed the discovery of novel dual inhibitors of AChE and BACE-1, which demonstrated not only in vitro enzymes inhibitory potency and cellular activity, but also in vivo functional efficacy.

2. Chemistry

The synthesis of the compounds **9**–**14** was accomplished as shown in Scheme 1.[9] Removing the *N*-Boc group of the γ-lactone **6** in the presence of 30% CF_3COOH in CH_2Cl_2, the resulting amine was subsequently reacted with 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid and 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid using EDCI and HOBt as the coupling agents to yield compounds **7** and **8**, respectively.[10] Hydrolysis of

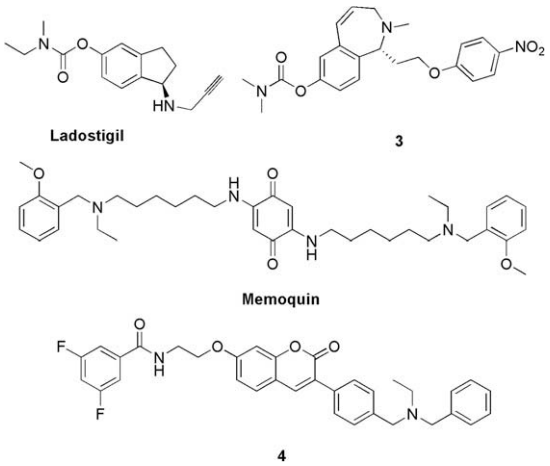
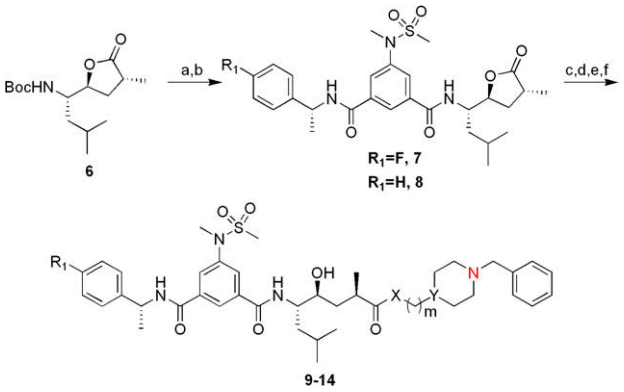


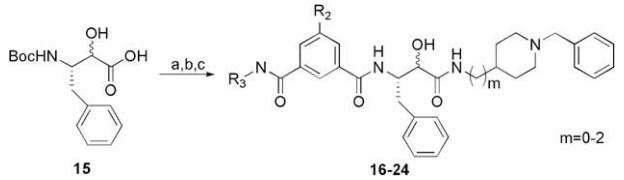
Figure 2. Reported hybrids with dual or multiple biological profiles.



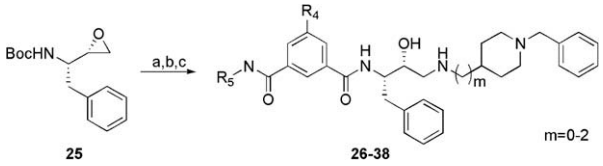
Scheme 1. Synthesis of designed dual inhibitors **9**–**14** bearing HE scaffold. Reagents and conditions: (a) CF_3COOH, CH_2Cl_2, rt; (b) EDCI, HOBt, benzoic acids, DIPEA, DMF, rt; (c) aq LiOH, THF–H_2O; (d) TBDMSCl, imidazole, DMF, rt; (e) EDCI, HOBt, amines, DIPEA, DMF, rt; (f) nBu$_4$N$^+$F$^-$, THF, rt.

γ-lactone with lithium hydroxide, followed by protection of the γ-hydroxyl group with *tert*-butyldimethylsilyl chloride (TBDMSCl), then coupling with different amines using EDCI and HOBt to give the corresponding amides. *N*-Benzylpiperazine and 4-amino-1-benzylpiperidine were commercially available. (1-Benzylpiperidin-4-yl)methylamine and (1-benzylpiperidin-4-yl)ethylamine were synthesized according to the literature.[11] Removal of the silyl protecting group by treatment with tetrabutylammoniumfluoride (nBu$_4$N$^+$F$^-$) in tetrahydrofuran (THF) afforded the target compounds **9**–**14**.

The general synthesis of various inhibitors containing the hydroxymethylcarbonyl (HMC) isostere is outlined in Scheme 2.[12] The Boc-protected α-hydroxyl-β-amino acid **15** was coupled with amines using EDCI, HOBt and DIPEA to provide the amides, which were removed the Boc protecting group in the presence of 4 N HCl/1,4-dioxane. Then the resulting amine hydrochlorides were coupled with acids to provide **16**–**24**.



Scheme 2. Synthesis of designed dual inhibitors **16**–**24** bearing HMC scaffold. Reagents and conditions: (a) EDCI, HOBt, amines, DIPEA, DMF, rt; (b) 4 N HCl/1,4-dioxane; (c) EDCI, HOBt, acids, DIPEA, DMF, rt.



Scheme 3. Synthesis of designed dual inhibitors **26**–**38** bearing HEA scaffold. Reagents and conditions: (a) amines, isopropyl alcohol, 90 °C, 24 h; (b) 4 N HCl/1,4-dioxane; (c) EDCI, HOBt, acids, DIPEA, DMF, MW, 70 °C, 10 min.

Compounds **26**–**38** were obtained from a well-known three-step procedure (Scheme 3) starting from the requisite erythro α-amino epoxide **25**, which was prepared according to the literature procedures.[13] Epoxide opening in sealed tube at 90 °C for 24 h afforded the Boc-protected amino alcohol, which were then deprotected under acidic conditions and underwent amide coupling using facile microwave to provide compounds **26**–**38**.

3. Results and discussion

The way we adopted to explore the dual inhibitors of AChE and BACE-1 was that combining pharmacophores of AChE inhibitors with those of BACE-1 inhibitors through suitable common scaffold. As reported, isophthalamide was a widely used pharmacophore in BACE-1 inhibitors.[14,6d–f,10,13d] And the small molecular weight compound **39** without the P'_1—P'_2 parts also showed moderate activity towards BACE-1. It gave us an implication that the S'_1—S'_2 pockets of BACE-1 were comparably 'tolerant'.[14e] On the other hand, through studying the crystal structure and related literature of AChE, we knew that the AChE has a catalytic site at the bottom of deep and narrow gorge and a peripheral site at the entrance.[15] As the drug donepezil of an AChE inhibitor has been widely used in clinic and studied in academic field, its pharmacophore aroused our great interest. The *N*-benzylpiperidine moiety of donepezil, which forms strong interactions with AChE, locates at the bottom of the catalytic site of AChE. The other indanone moiety of donepezil, which could be substituted in many other highly potent AChE inhibitors,[16,11] locates at the peripheral site. According to these analyses, we decided to use HE isostere which has been studied for a long period to discover BACE-1 inhibitors in our laboratory[14d] as the common scaffold, choose isophthalamide moiety and *N*-benzylpiperidine group at the N-terminus and C-terminus of HE, respectively to generate dual inhibitor (Fig. 3). In this way, the introduction of *N*-benzylpiperidine group to the ligand's P'_2 part may cause little effect to its BACE-1 activity. Meanwhile, the designed hybrid may also display certain AChE activity if effective interactions occur between the *N*-benzylpiperidine group and the catalytic site of AChE and proper location of isophthalamide moiety at the peripheral site. And then we designed and synthesized compounds **9**–**14** by using different substitutions of R_1 group and changing the chain length of *N*-benzylpiperidine amines at the C-terminus of HE. And we also added the similar *N*-benzylpiperazine as the active group of AChE inhibitor for elementary study.

These compounds were evaluated the potencies against AChE and BACE-1 using donepezil and OM99–2 (one of the earliest inhibitors discovered by Ghosh et al.)[17,14d] as control, respectively. As shown in Table 1, through modifying the chain length of benzylpiperidine amines at the C-terminus of HE, except Y = N, all compounds maintained certain BACE-1 inhibitory activities. It is likely that the H atom of the C-terminal amide of HE may form effective hydrogen bond with enzyme that led to great effect on inhibitory activity against BACE-1. And the comparison among the active compounds (**10**, **11**, **12**, **14** demonstrated the C-terminal benzylpiperidine groups of HE cause slight effect on BACE-1 activity, which was consistent with our speculation that the S'_1—S'_2 pockets of BACE-1 were comparably 'tolerant'. Intriguingly, when *N*-benzylpiperidine groups were introduced into the ligands' P'_2 part, some compounds displayed weak activities against AChE (**10**, **11**, **12**, **14**), which encouraged us to further explore dual inhibitors that can increase the activity against AChE as well as maintain or elevate the BACE-1 inhibitory activity.

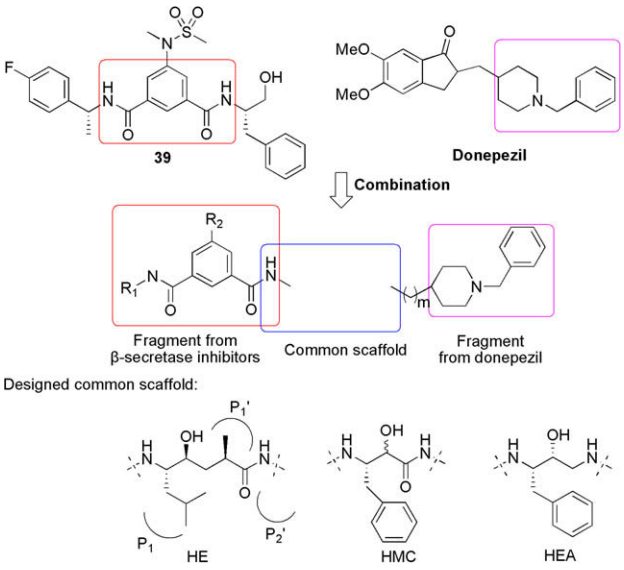
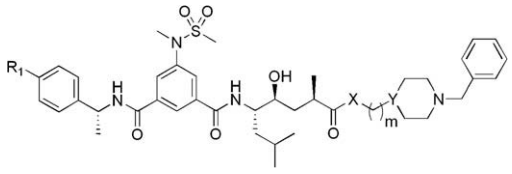


Figure 3. Structure of designed new hybrid dual inhibitors.

Table 1
Enzyme-inhibiting activities of compounds **9**–**14**



Compd	R_1	X	Y	*m*	BACE-1 IC_{50} (μM)	AChE[b] (inhibition%)
9	F	/	N	0	ND[a]	0.26
10	F	NH	C	0	1.504 ± 0.047	47.54
11	F	NH	C	1	0.616 ± 0.023	45.85
12	F	NH	C	2	1.339 ± 0.145	23.79
13	H	/	N	0	ND	5.18
14	H	NH	C	0	1.541 ± 0.256	48.70
39	/	/	/	/	0.462 ± 0.058	ND
donepezil	/	/	/	/	ND	7.86 ± 0.51 nM[c]
OM99-2	/	/	/	/	0.071 ± 0.009	ND

[a] 'ND'means the IC_{50} were not determined due to their inhibition activity <50% at 50 μg/mL.
[b] Inhibition of AChE produced by the tested compounds at 2.5×10^{-5} M.
[c] IC_{50} of donepezil.

Then we changed the HE scaffold to HMC, another Asp protease transition-mimic, for further exploration. As preliminary study, the hydroxyl of HMC was not stereospecifically synthesized, and the isobutyl at the P_1 position was substituted by the hydrophobic benzyl group (Fig. 3). As illustrated, our dual inhibitors were conceptually subdivided into three regions for discussion purposes: an N-terminus (An), a central chain (HMC isostere), and a C-terminus (BPn) (Fig. 4). The An and BPn were individually modified to probe the optimized activity. Thus, a small 3×3 library was designed and synthesized by choosing different acids (An) and modifying the chain length of benzylpiperidine amines (BPn). Based on the above study of HE-based dual inhibitors, it was anticipated that the introduction of the HMC scaffold to compounds would increase the activity towards AChE.

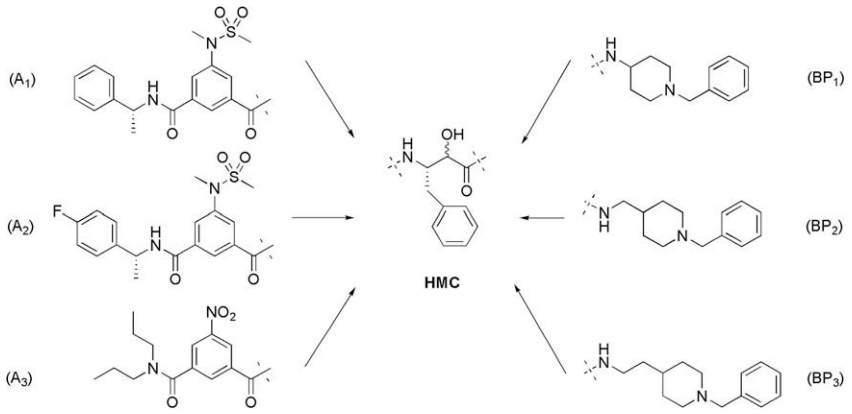
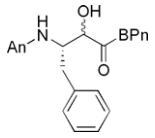


Figure 4. A small 3 × 3 library using HMC transition-state.

Table 2
Enzyme-inhibiting activities of HMC-based compounds **16**–**24**



Compd	AChE[a] (inhibition%)	AChE IC_{50} (μM)	BACE-1[b] (inhibition%)
16(A_1*BP_1)	6.38	ND[c]	48.99 ± 9.38
17(A_1*BP_2)	55.32	ND	27.78 ± 3.43
18(A_1*BP_3)	88.08	1.11 ± 0.12	42.41 ± 4.63
19(A_2*BP_1)	5.95	ND	33.30 ± 4.52
20(A_2*BP_2)	44.93	ND	34.00 ± 5.05
21(A_2*BP_3)	85.01	1.39 ± 0.07	57.04 ± 9.27
22(A_3*BP_1)	3.97	ND	18.56 ± 3.64
23(A_3*BP_2)	42.41	ND	19.68 ± 6.48
24(A_3*BP_3)	84.68	1.68 ± 0.27	22.79 ± 4.67

[a] Inhibition of AChE produced by the tested compounds at 2.5×10^{-5} M.
[b] Inhibition of BACE-1 produced by the tested compounds at 50 μg/mL.
[c] 'ND' means the IC_{50} were not determined.

Encouragingly, compounds bearing HMC scaffold, such as **18**, **21** and **24** (IC_{50} = 1.11, 1.39, 1.68 μM), showed greatly increase in potency towards AChE compared to the HE-bearing series (Table 2). And we also found that with elongation of the chain length of *N*-benzylpiperidine amines at C-terminus of HMC, the activities of inhibitors increased obviously (An-HMC-BP_3 > An-HMC-BP_2 > An-HMC-BP_1), and we have got the best inhibitors when $m = 2$. But the influence of groups at the 3- and 5-position of isophthalamides could not be observed. To our surprise, on the aspect of BACE-1, the inhibitory activities of compounds decreased dramatically compared to the HE-bearing series. The unexpected results pushed us to carefully study the reported work about the BACE-1 inhibitors bearing HMC transition-state mimic.[18] Through looking into the literature, we have got to know when using HMC as the transition-state mimic, Asn was not a proper amino acid residue at the P_2 position of BACE-1 inhibitors in peptide-scanning experiment.[16a] Isophthalamide was usually considered as the substitution for Asn of ligand's P_2 part[19,14a] and that may explain why we did not obtain the potent inhibitors when combined HMC and isophthalamides together. As the weak activities of these compounds towards BACE-1, actual contribution of individual diastereomers has not been determined.

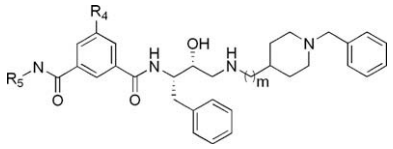
HEA is another important isostere which has been widely used in the aspartyl protease inhibitors and several HIV protease inhibitors adopting it as a scaffold have been approved to be on market several years ago.[20] And there are also multiple BACE-1 inhibitors bearing HEA, which show similar C-terminal and N-terminal moieties to the HE series, exhibiting good prosperities towards BACE-1 on both enzyme and cell in lots of recent literature.[10,6d–f] It was most likely that replacing HMC with HEA may increase the inhibitor's activity towards BACE-1 based on the reported results and our analysis (Fig. 3). But we were not sure if the deletion of the carbonyl group of HMC and choosing the stereospecific hydroxyl of HEA would cause influence to its AChE activity. Thus, we first synthesized compounds **26**–**28** to determine whether HEA was an appropriate common scaffold as we expected.

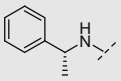
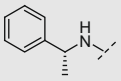
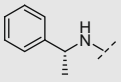
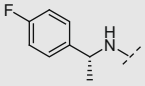
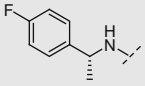
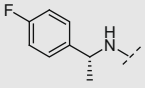
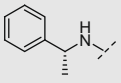
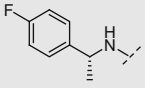
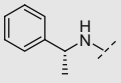
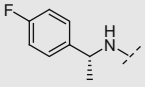
It was clear that the inhibitory activities of compounds against BACE-1 increased dramatically compared to the HMC series (Table 3). Interestingly, the activities of compounds towards AChE did not decrease when the carbonyl group of HMC was deleted and the requisite stereospecific hydroxyl of HEA was chosen, which demonstrated that HEA was a more suitable common scaffold than HE and HMC for the dual inhibitors design. Besides, we also observed that inhibitory activities of compounds against AChE increased with the elongation of chain length of *N*-benzylpiperidine amines (**26** < **27** < **28**), which was similar with the HMC series.

To further validate *N*-benzylpiperidine ethyl was the most suitable moiety at the C-terminus of HEA, compounds **29**–**31** were synthesized (Table 3). The results showed a similar trend with the conclusion discussed above, which was also consistent with the literature that compounds with two-carbon units distance between the nitrogen atom and the piperidine ring of benzylpiperidine showed the highest potencies against AChE.[16b,c,e] Moreover, we also found an interesting phenomena that compounds with $m = 1$ were obviously less potency towards BACE-1 than those with $m = 0, 2$ (**27** vs **26**, **28**; **30** vs **29**, **31**). Since the advantages of *N*-benzylpiperidine ethyl at C-terminus of HEA on both BACE-1 and AChE inhibitory activity, no further elaborations were made and *N*-benzylpiperidine ethyl moiety was adopted as the C-terminus of HEA in our study to explore further structure-activity relationships (SAR). The chosen N-terminal residues were a series of isophthalamide derivatives with various substituents at 3- and 5-position. The designed groups at 3- and 5-position were selected according to the previous studies of BACE-1 inhibitors reported by Merck's group[10] and ours.[14d] Methyl(methylsulfonyl)amino, nitro or hydrogen was incorporated into 5-position as R_4. R_5 at the 3-position was investigated using (*R*)-1-(4-fluorophenyl)ethyl, (*R*)-1-phenylethyl benzyl and *N*,*N*-dipropyl.

As it could be seen in Table 3, compounds bearing different substituents at the 3-position of isophthalamides showed closely enzymatic inhibition against AChE. Among these, compounds containing the *N*,*N*-dipropyl groups displayed a little more potency than those containing the other two groups (**32** vs **28**, **31**; **35** vs

Table 3
Enzyme-inhibiting activities of compounds **26**–**38**



Compd	*m*	R_4	R_5N	AChE[a] (inhibition%)	AChE IC_{50} (μM)	BACE-1 IC_{50} (μM)
26	0			11.16	ND[b]	0.511 ± 0.250
27	1			47.24	ND	3.999 ± 1.383
28	2			84.65	1.83 ± 0.12	0.567 ± 0.159
29	0			12.22	ND	0.771 ± 0.091
30	1			25.63	ND	3.233 ± 0.263
31	2			88.76	1.35 ± 0.06	0.222 ± 0.047
32	2			87.64	1.05 ± 0.09	2.392 ± 0.050
33	2	NO_2		67.30	2.53 ± 0.07	7.021 ± 2.817
34	2	NO_2		87.36	2.09 ± 0.18	2.682 ± 0.017
35	2	NO_2		90.02	1.27 ± 0.07	ND
36	2	H		73.98	3.06 ± 0.21	ND
37	2	H		62.50	11.70 ± 0.06	ND
38	2	H		89.04	2.28 ± 0.08	ND

[a] Inhibition of AChE produced by the tested compounds at 2.5×10^{-5} M.
[b] 'ND' means the IC_{50} of compounds were not obtained.

34, **33**; **38** vs **37**, **36**). Similarly, at the 5-position, methyl(methylsulfonyl)amino was a little superior substituent than the nitro and hydrogen groups (**28** vs **33**, **36**; **31** vs **34**, **37**; **32** vs **35**, **38**). On the contrary, substituents at the 3- and 5-position of isophthalamides played a significant role in inhibitory activity towards BACE-1. The compounds bearing (*R*)-1-(4-fluorophenyl)ethyl at the 3-position were about 3-fold and 10-fold more potent than those bearing (*R*)-1-phenylethyl benzyl (**28** vs **31**; **34** vs **33**) and *N*,*N*-dipropyl (**31** vs **32**), respectively. And more clearly, compounds with methyl(methylsulfonyl)amino at the 5-position of isophthalamides exhibited over 10 times potency than those with nitro and hydrogen. These results are in agreement with the previously reported SAR explorations of BACE-1 inhibitors.[10,14d]

To elucidate the binding modes of dual inhibitor in AChE and BACE-1, we performed a docking study on inhibitor **31** by utilizing the advanced AUTODOCK program. Due to many rotatable bonds contained in ligand, a two-step docking procedure was adopted to enable the AUTODOCK program to explore such large conformational space. So the ligand **31** was splitted into two parts. For BACE-1 docking study, we first docked the part containing isophthalamide (Fig. 5A, red part in bottom 2D structure diagram) of ligand **31** into BACE-1. Next four lowest conformations were selected and their torsion angles were fixed in the next docking studies. Then we connected this part with the remainder part (Fig. 5A, black part in bottom 2D structure diagram) together, and the whole ligand **31** was subjected to the docking study again. Finally the lowest energy conformation was selected for analysis. As demonstrated in Figure 5A, the isophthalamide moiety of inhibitor **31** adopts a very similar conformation compared with the ligand in original crystal structure 2B8L. And the hydroxyl of HEA scaffold can form effective hydrogen bonds with active site aspartic acid residue Asp228. But the benzylperidine group extends into the solvent and does not form any specific interactions with BACE-1. For AChE-ligand docking, a similar two-step procedure was used but with the part containing benzylpiperidine (Fig. 5B, red part in bottom 2D structure diagram) to perform docking first. Then the remainder part (Fig. 5B, black part in bottom 2D structure diagram) was connected, and after whole molecule docking study, the final lowest conformation was selected as the predicted bound conformation. Clearly, the benzylperidine group of inhibitor **31** properly binds with enzyme in a conformation similar to donepezil, whereas the isophthalamide moiety only extends to the outside of binding site and does not make any specific contact with the residues in AChE. These may shed light on why the inhibitors of this series only have moderate binding affinity towards AChE and BACE-1. However, these docking studies gave the rational explanations that how the dual inhibitors of BACE-1 and AChE interact with the target proteins, which may give us some directions to design more potent dual inhibitors.

According to the results described above, some potent dual or BACE-1 inhibitors (**26**, **28**, **29**, **31**, **32**, **33** and **34**) were selected to examine the effects on intracellular inhibition of endogenous BACE-1 activity in HEK293 cells transfected with human βAPP695wt. Whole cell Aβ lowering (sandwich Elisa) was used to detect inhibitory effect of the compounds on BACE-1 activity. Among these compounds, **26** (IC_{50} = 54.6 nM) and **28** (IC_{50} = 98.7 nM) displayed excellent inhibitory effects in cell-based assay on Aβ production (The IC_{50} of the other compounds were below 1 μM), which demonstrated *N*-benzylpiperidine might be a good group for compounds on cellular activities.

Then the protective effects against free radical of **26** and **28**, which might relate to a self propagating cascade of neurodegenerative events,[21] were assessed by measuring the ability of the compounds to protect against H_2O_2 injury according to the reported protocol with minor modification.[22] After 200 μM H_2O_2 exposure, cell viability as determined by MTT reduction was markedly decreased to 58% ($^{\#\#}P < 0.01$ vs control). However, compounds **26** and **28** showed mild protective effects against hydrogen peroxide-induced PC12 cell injury, which was statistically significant (Fig. 6).

Due to their favorable overall profiles, we decided to evaluate the in vivo efficacy. Encouragingly, intracerebroventricular administration of compounds **26** and **28** to APP transgenic mice, which contains a transgene encoding the human APP695 with Swedish double mutation, caused 23% and 29% decrease in endogenous $A\beta_{1-40}$ production compared to the vehicle-treated control mice, respectively (Fig. 7). However, further studies will be needed to verify the pharmacokinetic aspects of these compounds including the brain penetration.

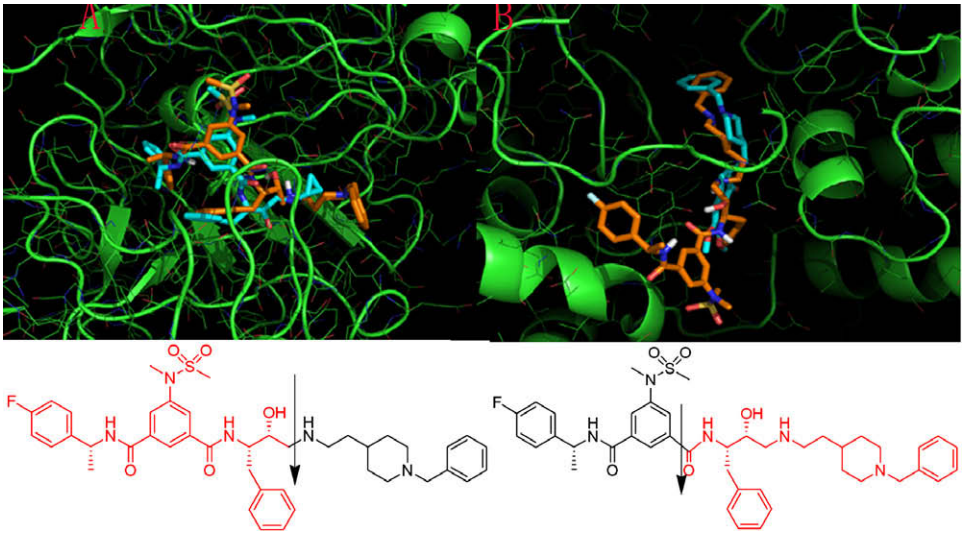


Figure 5. The docking studies of inhibitor **31.** Ligands were depicted as stick model. For BACE-1 docking study (A), the original ligand in crystal structure 2B8L was colored in cyan and inhibitor **31** was colored in orange. For AChE docking study (B), donepezil was colored in cyan and inhibitor **31** was colored in orange. The 2D skeleton of the inhibitor **31** at the bottom of the figure is to illustrate how the two-step docking studies were performed. The arrow is indicated the split point and the fragment used in the first step docking was colored in red. After the first step docking, the black part of molecule was connected and the torsion angle of the red part was fixed in the second step docking studies.

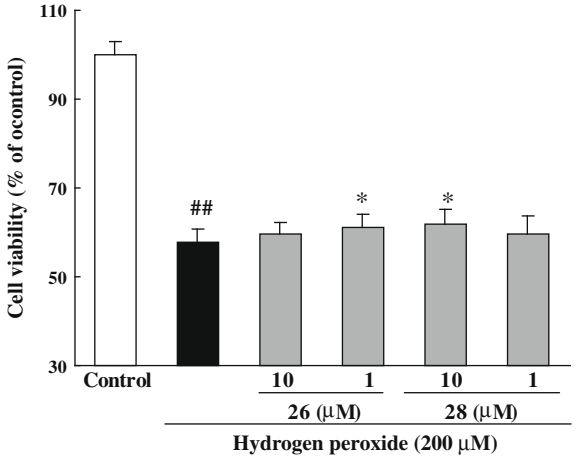


Figure 6. Protective effects of **26** and **28** on cell injury induced by hydrogen peroxide (200 μM) in PC12 cells. $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$ versus H_2O_2 group $^{**}P < 0.01$ versus H_2O_2 group.

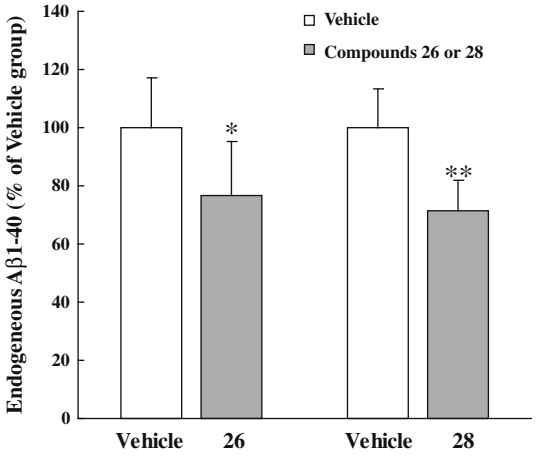


Figure 7. Compound **26** or **28** induced $A\beta_{1-40}$ level decreasing in APP transgenic mouse brain. $n = 6–8$. Data were MEAN ± SD expressed as percent of vehicle group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated mice.

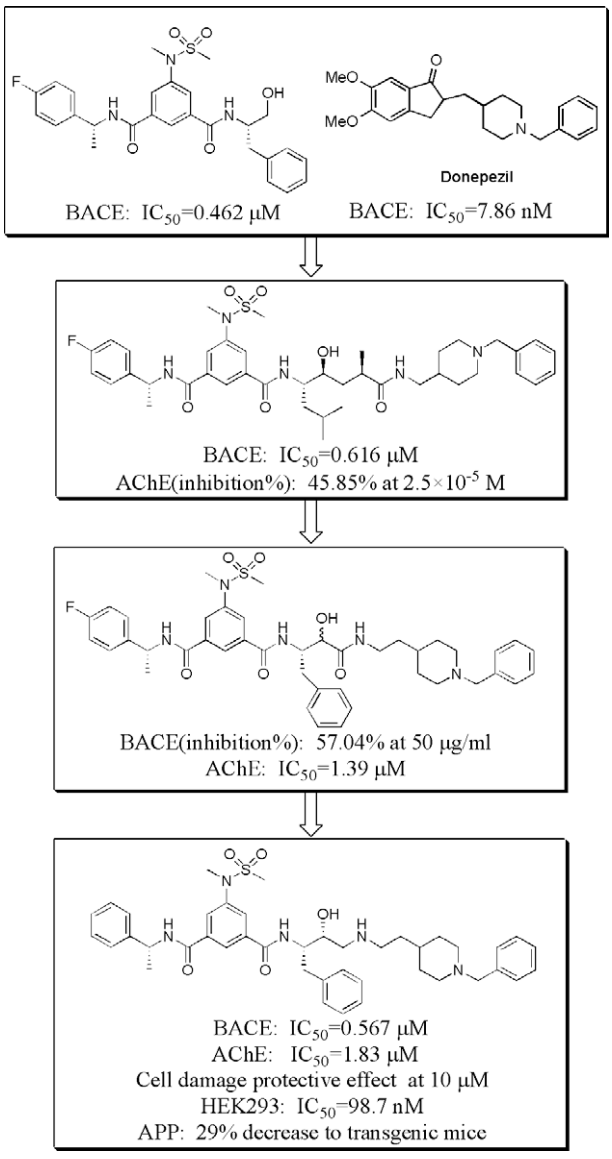


Figure 8. The route for discovering AChE and BACE-1 dual inhibitors.

4. Conclusion

To explore novel effective drugs for the treatment of Alzheimer's disease (AD), three series dual inhibiting molecules of AChE and BACE-1 were designed and investigated. Applying the fragment-based strategy, HE, HMC and HEA were chosen as the common scaffolds. These protease transition-mimics were combined with isophthalamide moiety and *N*-benzylpiperidine groups at the N-terminus and C-terminus, respectively. Compared with HE analogues which displayed strong BACE-1 inhibitory activities but weak AChE inhibitory activities, HMC derivatives showed greatly increase of AChE activities at the expense of dramatically loss of BACE-1 activities. Excitingly, HEA derivatives were obtained with good inhibiting activities on both AChE and BACE-1, which demonstrated HEA is a suitable common scaffold for dual inhibitors. Among this series, compounds **26** and **28** showed excellent activities in the cell-based assay. These two compounds were also proved to possess anti-oxidative effect against H_2O_2-induced cytotoxicity. More interestingly, our in vivo study further proved that intracerebroventricular administration of compounds **26** and **28** could markedly decrease the $A\beta_{1-40}$ production in APP transgenic mice. The present study indicated that hybrid **28** showed AChE inhibiting activity, as well as Aβ lowering and antioxidant function, which could be considered as a potential multi-target-directed agent and further studied for therapeutic application in the treatment of AD (Fig. 8). Further studies to evaluate **28** in vivo and to develop structural refinements are in progress in our laboratory and will be reported in due course.

5. Experimental

5.1. Chemistry

The ^{1}H NMR (300 MHz or 400 MHz) spectra were recorded on Varian Mercury-300 or 400 High Performance Digital FT-NMR using tetramethylsilane as an internal standard and the ^{13}C NMR (100 MHz) spectra were determined with Varian Mercury-400 High Performance Digital FT-NMR. The LC–MS were carried out on Thermo Finnigan LCQDECAXP and HRMS were performed with Finnigan MAT 95, EI: 70 eV, R: 10,000. The purity of the stereospecifical target compounds was recorded on HPLC system (HP 1100 series, Agilent Technologies, Palo Alto, USA) by two different gradient methods using an YMC ODS column (50 × 4.6 mm, 5 μm particle size). In addition, to detect the diastereomers **16**–**24**, an-

other three gradient methods were set. The optical rotation value was determined with PerkinElmer-341 (589 nm). All reagents are of analytical grade pure and used without further purification.

5.2. *N*-[(*R*)-1-(4-Fluorophenyl)ethyl]-*N*′-{(*S*)-3-methyl-1-[(2*S*,4*R*)-4-methyl-5-oxo-tetrahydrofuran-2-yl]butyl}-5-[methyl(methylsulfonyl)amino]isophthalamide (7)

To a solution of CF_3COOH (4 mL) in CH_2Cl_2 (10 mL) was added **6** (1.40 g, 4.91 mmol). The mixture was at room temperature for 0.5 h, and then it was concentrated under reduced pressure to afford the crude product used without further purification. To a solution of the product (0.23 g, 1.26 mmol) in DMF (8 mL) at 0 °C was added 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid (0.496 g, 8, 1.26 mmol), HOBt (0.179 g, 1.32 mmol), DIPEA (0.22 ml, 1.32 mmol) and stirred for 10 min. EDCI (0.254 g, 1.32 mmol) was added and the mixture was allowed to react overnight at room temperature. Then the resulting mixture was diluted by 100 mL EtOAc and washed with 1 N HCl aqueous solution three times (30 mL × 3), dilute aqueous $NaHCO_3$ three times (30 mL × 3) and saturated brine once (30 mL), sequentially. The organic layers were dried over Na_2SO_4, filtered and evaporated to give the residue, which was purified by silica gel chromatography to afford 0.66 g **7** (white foam solid, yield: 93%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.96 (m, 2H), 7.32 (m, 2H), 7.01 (m, 2H), 6.83 (br, 1H), 6.81 (br, 1H), 5.21 (m, 1H), 4.62 (m, 1H), 4.48 (m, 1H), 3.38 (s, 3H), 2.84 (s, 3H), 2.62 (m, 1H), 2.40 (m, 1H), 1.99 (m, 1H), 1.68 (m, 2H), 1.53 (d, *J* = 7.0 Hz, 3H), 1.44 (m, 1H), 1.25 (d, *J* = 7.2 Hz, 3H), 0.94 (d, *J* = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): 180.8, 166.0, 164.7, 163.1 (d, *J* = 244 Hz), 160.6, 142.2, 138.7, 135.6, 135.2, 128.1, 128.0, 127.8, 127.7, 124.1, 115.5, 115.3, 80.5, 50.8, 49.1, 40.9, 37.9, 35.6, 34.3, 32.5, 24.7, 23.1, 21.8, 21.7, 16.4; LC-MS: *m/z* 1123.1 $[2M+H]^+$; HRMS: calcd for $C_{28}H_{36}N_3O_6NaSF$ $[M+Na]^+$ 584.2207, found 584.2208.

5.3. *N*-{(*S*)-3-methyl-1-[(2*S*,4*R*)-4-methyl-5-oxo-tetrahydrofuran-2-yl]butyl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (8)

Compound **8** was obtained from **6** and 5-[methyl(methylsulfonyl)amino]-3-[(*R*)-1-phenylethylaminocarbonyl] benzoic acid according to the similar procedure used to prepare **7**, yield: 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.15 (s, 1H), 7.98 (m, 2H), 7.22–7.34 (m, 5H), 6.93 (br, 1H), 6.85 (br, 1H), 5.23 (m, 1H), 4.62 (m, 1H), 4.48 (m, 1H), 3.33 (s, 3H), 2.87 (s, 3H), 2.60 (m, 1H), 2.39 (m, 1H), 2.00 (m, 1H), 1.68 (m, 2H), 1.56 (d, *J* = 6.9 Hz, 3H), 1.44 (m, 1H), 1.25 (d, *J* = 7.4 Hz, 3H), 0.94 (d, *J* = 6.5 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): 180.8, 166.1, 164.8, 142.9, 142.1, 135.7, 135.1, 128.6, 128.2, 127.3, 126.0, 124.2, 80.5, 50.8, 49.7, 40.8, 37.8, 35.5, 34.3, 32.4, 24.7, 23.1, 21.8, 21.7, 16.4; LC–MS: *m/z* 1087.1 $[2M+H]^+$; HRMS: calcd for $C_{28}H_{37}N_3O_6NaS$ $[M+Na]^+$ 566.2301, found 566.2277.

5.4. General method for preparation of compounds 9–14 (exemplified by 9)

5.4.1. *N*-[(4*S*,5*S*,7*R*)-7-(4-Benzylpiperazin-1-yl)carbamoyl-5-hydroxy-2-methyloct-4-yl]-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (9)

Compound **8** (0.64 g, 1.14 mmol) was dissolved in 5 mL THF at 0 °C and 6 mL aqueous 1 N LiOH was added. The mixture was allowed to stir overnight at room temperature. Then the organic solvent was evaporated and 25% aqueous citric acid solution was added to adjust the pH to 3–4. The solution was extracted with EtOAc three times (20 mL × 3) and the combined organic layers were washed by saturated brine twice (10 mL × 2). Then the organic layers were dried over Na_2SO_4, filtered and evaporated to give white foamy solid acid, which was used without further purification (this product turns back slowly to **8** at room temperature). To a solution of the given acid (0.66 g, 1.14 mmol) in 9 mL DMF was added imidazole (1.17 g, 25.1 mmol), *tert*-butyldimethylsilyl chloride (1.89 g, 12.5 mmol) and stirred at room temperature for 26 h. Then 6 mL CH_3OH was added and the mixture stirred for another hour. After that, 30 mL 25% aqueous citric acid was added and the solution was extracted by EtOAc three times (30 mL × 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated to give the residue, which was purified by silica gel chromatography to afford 0.603 g (*2R,4S,5S*)-5-{3-[(*R*)-1-(4-fluorophenyl)ethylamido]-5-[methyl(methylsulfonyl)amino]benzamido}-4-(*tert*-butyldimethylsilyloxy)-2,7-dimethyloctanoic acid (white foam solid, yield: 76.3%). ^{1}H NMR (300 MHz, DMSO-*d*6): δ 9.01 (br d, *J* = 7.6 Hz, 1H), 8.32 (br d, *J* = 7.9 Hz, 1H), 8.26 (s, 1H), 7.98 (s, 1H), 7.96 (s, 1H), 7.42 (m, 2H), 7.16 (m, 2H), 5.16 (q, *J* = 7.2 Hz, 1H), 4.08 (m, 1H), 3.72 (m, 1H), 3.31 (s, 3H), 3.02 (s, 3H), 2.45 (m, 1H), 1.92 (m, 1H), 1.58 (m, 2H), 1.50 (d, *J* = 7.0 Hz, 3H), 1.22–1.40 (m, 2H), 1.11 (d, *J* = 7.1 Hz, 3H), 0.93 (d, *J* = 8.2 Hz, 3H), 0.86 (s, 9H), 0.82 (d, *J* = 6.3 Hz, 3H), 0.16 (s, 3H), 0.08 (s, 3H). To a solution of the above acid (0.080 g, 0.115 mmol) in 1 mL DMF at 0 °C was added *N*-benzylpiperazine (0.023 mL, 0.132 mmol), HOBt (0.018 g, 0.132 mmol), DIPEA (0.022 mL, 0.126 mmol) and stirred for 10 min. EDCI (0.025 g, 0.132 mmol) was added and the mixture was allowed to react overnight at room temperature. Then the resulting mixture was diluted by 30 mL EtOAc and washed with 1 N HCl aqueous three times (10 mL × 3), dilute aqueous $NaHCO_3$ three times (10 mL × 3) and saturated brine once (10 mL), sequentially. The organic layers were dried over Na_2SO_4, filtered and evaporated to give the crude product amide, which was used directly in the next step. To a solution of the given amide in 1 mL THF was added 0.3 mL 1.0 M tetrabutylammoniumfluoride in THF. The mixture was allowed to react for 24 h and concentrated in vacuum. The residue was purified by preparative thin layer chromatography or silica gel chromatography to afford the target compound **9**, yield: 64.2%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.22 (s, 1H), 8.01 (s, 2H), 7.40 (m, 2H), 7.28 (m, 5H), 7.04 (m, 2H), 5.23 (q, *J* = 7.0 Hz, 1H), 4.20 (m, 1H), 3.58 (m, 5H), 3.52 (s, 2H), 3.38 (s, 3H), 3.18 (m, 1H), 2.99 (s, 3H), 2.42 (m, 4H), 1.98 (m, 1H), 1.68 (m, 2H), 1.58 (d, *J* = 7.0 Hz, 3H), 1.42 (m, 2H), 1.12 (d, *J* = 7.0 Hz, 3H), 0.94 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): 175.6, 165.9, 164.9, 163.0, 160.6 (d, *J* = 244 Hz), 142.0, 138.9, 137.1, 135.8, 135.6, 129.0, 128.2, 128.1, 127.9, 127.8, 127.3, 124.1, 115.4, 115.2, 70.4, 62.6, 53.1, 52.9, 52.5, 48.9, 45.7, 41.8, 40.7, 37.9, 37.2, 35.4, 32.2, 24.8, 23.2, 21.9, 21.6, 17.2; LC-MS: *m/z* 738.7 $[M+H]^+$; HRMS: calcd for $C_{39}H_{53}N_5O_6FS$ $[M+H]^+$ 738.3701, found 738.3733; $[\alpha]_D^{22} = -36.0$ (*c* 1.11, acetone).

5.4.2. *N*-{(4*S*,5*S*,7*R*)-2-[7-(1-Benzylpiperidin-4-yl)]carbamoyl-5-hydroxy-2-methyloct-4-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (10)

Compound **10** was obtained from **8** and 4-amino-1-benzylpiperidine, yield: 51%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.25 (s, 1H), 8.02 (s, 2H), 7.45–7.32 (m, 7H), 7.06 (m, 2H), 5.22 (q, *J* = 6.9 Hz, 1H), 4.19 (m, 1H), 3.80 (s, 2H), 3.62 (m, 2H), 3.38 (s, 3H), 3.29 (m, 2H), 3.10 (m, 2H), 2.96 (s, 3H), 2.65 (m, 1H), 2.38 (m, 2H), 1.72–1.40 (m, 7H), 1.12 (d, *J* = 7.2 Hz, 3H), 1.01 (d, *J* = 7.3 Hz, 3H), 0.99 (d, *J* = 7.3 Hz, 3H), 0.94 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): 178.9, 169.1, 168.0, 164.9, 162.5 (d, *J* = 242 Hz), 144.2, 141.7, 138.0, 137.5, 132.0, 130.1, 129.9, 129.8, 129.7, 129.6, 126.3, 116.6, 116.4, 72.8, 63.2, 59.9, 54.5, 53.0, 50.8, 40.5, 39.5, 39.2, 38.8, 36.484, 26.6, 25.2, 24.3, 22.7, 22.6, 21.2, 19.6, 14.4; LC–MS: *m/z* 752.7 $[M+H]^+$; HRMS: calcd for

$C_{40}H_{55}N_5O_6SF$ $[M+H]^+$ 752.3857, found 752.3898; $[\alpha]_D^{22} = -31.3$ (*c* 1.64, acetone).

5.4.3. *N*-{(4*S*,5*S*,7*R*)-2-[7-(1-Benzylpiperidin-4-yl)]methylcarbamoyl-5-hydroxy-2-methyloct-4-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (11)

Compound **11** was obtained from **8** and (1-benzyl-4-piperidinyl)methylamine, yield: 43%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.31 (s, 1H), 8.02 (s, 2H), 7.58–7.35 (m, 7H), 7.02 (m, 2H), 5.22 (q, *J* = 7.1 Hz, 1H), 4.18 (m, 1H), 4.01 (m, 2H), 3.60 (m, 1H), 3.38 (s, 3H), 3.25 (m, 5H), 2.99 (s, 3H), 2.82 (m, 1H), 2.72–2.63 (m, 3H), 1.92 (m, 1H), 1.70–1.48 (m, 7H), 1.12 (d, *J* = 6.6 Hz, 3H), 1.02 (d, *J* = 7.2 Hz, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): 179.7, 169.1, 168.0, 164.9, 162.5 (d, *J* = 242 Hz), 144.2, 141.8, 138.1, 137.5, 132.6, 130.5, 130.3, 129.9, 129.8, 126.3, 116.6, 116.4, 72.7, 62.6, 59.9, 54.5, 54.1, 50.9, 45.2, 40.6, 39.8, 39.4, 38.9, 36.6, 26.6, 25.2, 24.4, 22.8, 22.7, 21.1, 19.7, 14.4; LC-MS: *m/z* 766.8 $[M+H]^+$; HRMS: calcd for $C_{41}H_{57}N_5O_6SF$ $[M+H]^+$ 766.4014, found 766.3995; $[\alpha]_D^{22} = -29.9$ (*c* 1.86, acetone).

5.4.4. *N*-{(4*S*,5*S*,7*R*)-2-[7-(1-Benzylpiperidin-4-yl)]ethylc arbamoyl-5-hydroxy-2-methyloctan-4-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (12)

Compound **12** was obtained from **8** and 2-(1-benzylpiperidin-4-yl)ethylamine, yield: 53%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.25 (s, 1H), 8.03 (s, 1H), 8.01 (s, 1H), 7.43 (m, 2H), 7.32 (m, 5H), 7.02 (m, 2H), 5.23 (q, *J* = 7.1 Hz, 1H), 4.21 (m, 1H), 3.63 (s, 2H), 3.58 (m, 1H), 3.36 (s, 3H), 3.16 (m, 2H), 2.98 (s, 3H), 2.92 (m, 2H), 2.61 (m, 1H), 2.18 (m, 2H), 1.90 (m, 2H), 1.62 (m, 4H), 1.58 (d, *J* = 7.0 Hz, 3H), 1.45–1.23 (m, 6H), 1.13 (d, *J* = 7.3 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): 176.9, 166.0, 164.7, 163.0, 160.6 (d, *J* = 244 Hz), 142.1, 139.2, 136.0, 135.8, 135.6, 129.6, 128.3, 128.0, 127.9, 127.5, 123.9, 115.3, 115.1, 70.9, 62.7, 53.2, 52.4, 49.1, 40.6, 38.4, 37.6, 37.0, 35.6, 35.5, 33.0, 31.1, 24.8, 23.2, 22.0, 21.8, 17.7; LC-MS: *m/z* 780.7 $[M+H]^+$; HRMS: calcd for $C_{42}H_{59}N_5O_6SF$ $[M+H]^+$ 780.4170, found 780.4177; $[\alpha]_D^{22} = -33.8$ (*c* 0.62, acetone).

5.4.5. *N*-{(4*S*,5*S*,7*R*)-2-[7-(4-Benzylpiperazin-1-yl)]carbamoyl-5-hydroxy-2-methyloct-4-yl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (13)

Compound **13** was obtained from **7** and *N*-benzylpiperazine, yield: 47%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.23 (s, 1H), 8.01 (s, 2H), 7.19–7.41 (m, 10H), 5.25 (q, *J* = 7.0 Hz, 1H), 4.22 (m, 1H), 3.58 (m, 5H), 3.52 (s, 2H), 3.38 (s, 3H), 3.20 (m, 1H), 2.99 (s, 3H), 2.40 (m, 4H), 1.98 (m, 1H), 1.67 (m, 2H), 1.59 (d, *J* = 7.0 Hz, 3H), 1.42 (m, 2H), 1.11 (d, *J* = 6.9 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): 175.5, 165.8, 164.7, 142.9, 142.0, 137.3, 135.8, 135.7, 129.0, 128.5, 128.2, 127.9, 127.3, 127.2, 126.2, 123.9, 70.3, 62.6, 53.1, 52.9, 52.5, 49.6, 45.7, 41.8, 40.9, 37.9, 37.3, 35.4, 32.3, 24.8, 23.2, 22.0. 21.6, 17.2; LC-MS: *m/z* 720.6 $[M+H]^+$; HRMS: calcd for $C_{39}H_{53}N_5O_6SNa$ $[M+Na]^+$ 742.3614, found 742.3659; $[\alpha]_D^{22} = -38.3$ (*c* 0.36, acetone).

5.4.6. *N*-{(4*S*,5*S*,7*R*)-2-[7-(1-Benzylpiperidin-4-yl)]carbamoyl-5-hydroxy-2-methyloct-4-yl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenyl ethyl]isophthalamide (14)

Compound **14** was obtained from **7** and 4-amino-1-benzylpiperidine, yield: 41%. ^{1}H NMR (300 MHz, CD_3OD): δ 8.26 (s, 1H), 8.03 (s, 1H), 7.99 (s, 1H), 7.21–7.42 (m, 10H), 5.27 (q, *J* = 7.1 Hz, 1H), 4.20 (m, 1H), 3.79 (s, 2H), 3.58 (m, 2H), 3.38 (s, 3H), 3.26 (m, 2H), 3.09 (m, 2H), 2.98 (s, 3H), 2.62 (m, 1H), 2.44 (m, 2H), 1.90 (m, 2H), 1.45–1.75 (m, 5H), 1.13 (d, *J* = 7.1 Hz, 3H), 1.01 (d, *J* = 7.6 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 3H), 0.94 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): 178.9, 169.2, 168.0, 145.5, 144.2, 138.0, 137.6, 131.9, 130.2, 130.0, 129.7, 128.6, 127.8, 126.4, 72.8, 63.2, 59.9, 54.3, 51.4, 50.1, 40.1, 39.2, 38.8, 36.4, 31.4, 26.6, 25.2, 24.3, 22.7, 22.6, 21.2, 19.4, 14.4; LC-MS: *m/z* 734.6 $[M+H]^+$; HRMS: calcd for $C_{40}H_{56}N_5O_6S$ $[M+H]^+$ 734.3951, found 734.3958; $[\alpha]_D^{22} = -39.0$ (*c* 0.86, acetone).

5.5. General method for preparation of compounds 16–24 (exemplified by 16)

5.5.1. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]carbamoyl-3-hydroxy-1-phenylbut-2-yl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (16)

To a solution of **15** (0.030 g, 0.102 mmol) in 10 mL DMF at 0 °C was added 4-amino-1-benzylpiperidine (0.020 g, 0.104 mmol), HOBt (0.014 g, 0.104 mmol), DIPEA (0.018 mL, 0.104 mmol) and stirred for 10 min. EDCI (0.020 g, 0.104 mmol) was added and the mixture was allowed to react overnight at room temperature. Then the resulting mixture was diluted by 30 mL EtOAc and washed with 1 N HCl aqueous three times (10 mL × 3), dilute aqueous $NaHCO_3$ three times (10 mL × 3) and saturated brine once (20 mL), sequentially. The organic layers were dried over Na_2SO_4, filtered and concentrated. The crude product was dissolved in 1 mL 4 N HCl/1,4-dioxane and stirred for 2 h. The organic solvent was evaporated to give the amine hydrochloride. To a solution of the resulting product in 2 mL DMF was added 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid (0.035 g, 0.093 mmol), HOB*t* (0.014 g, 0.104 mmol), DIPEA (0.018 mL, 0.104 mmol) and stirred for 10 min. EDCI (0.020 g, 0.104 mmol) was added and the mixture was allowed to react overnight at room temperature. Then the resulting mixture was diluted by 30 mL EtOAc and washed with 1 N HCl aqueous three times (10 mL × 3), dilute aqueous $NaHCO_3$ three times (10 mL × 3) and saturated brine once (20 mL), sequentially. The organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel chromatography to give **16** as yellow foam solid, yield: 49%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55–8.13 (m, 5H), 7.09–7.34 (m, 13H), 5.22 (m, 1H), 4.56 (m, 1H), 4.36 (m, 0.5H), 4.12 (m, 0.5H), 3.72 (m, 1H), 3.58 (m, 2H), 2.68–3.22 (m, 10H), 2.20 (m, 2H), 1.79 (m, 1H), 1.58 (m, 4H), 1.25 (m, 2H); LC-MS: *m/z* 726.5 $[M+H]^+$; HRMS: calcd for $C_{40}H_{48}N_5O_6S$ $[M+H]^+$ 726.3325, found 726.3318.

5.5.2. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]methylcarbamoyl-3-hydroxy-1-phenylbut-2-yl}-5-[methyl-(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (17)

Compound **17** was obtained from **15**, (1-benzyl-4-piperidinyl)methylamine and 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 39%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (m, 1H), 7.78–8.02 (m, 3H), 7.07–7.46 (m, 14H), 5.26 (m, 1H), 4.66 (m, 1H), 4.36 (m, 0.5H), 4.11 (m, 0.5H), 3.62 (m, 2H), 2.69–3.29 (m, 12H), 1.88–2.14 (m, 2H), 1.16–1.60 (m, 8H); LC-MS: *m/z* 741.0 $[M+H]^+$; HRMS: calcd for $C_{41}H_{50}N_5O_6S$ $[M+H]^+$ 740.3482, found 740.3448.

5.5.3. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]ethyl- carbamoyl-3-hydroxy-1-phenylbut-2-yl}-5-[methyl- (meth ylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (18)

Compound **18** was obtained from **15**, (1-benzyl-4-piperidinyl)ethylamine and 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 43%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46–8.04 (m, 4H), 6.89–7.03 (m, 14H), 5.20 (m, 1H), 4.44 (m, 1H), 4.36 (m, 0.6H), 4.09 (m, 0.4H), 3.46 (m, 2H), 2.70–3.29 (m, 12H), 1.89–2.20 (m, 3H), 1.09–1.61 (m, 9H);

LC-MS: *m/z* 754.6 $[M+H]^+$; HRMS: calcd for $C_{42}H_{52}N_5O_6S$ $[M+H]^+$ 754.3638, found 754.3611.

5.5.4. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]carbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (19)

Compound **19** was obtained from **15**, 4-amino-1-benzylpiperidine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 26%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (m, 1H), 7.54–7.96 (m, 3H), 7.10–7.38 (m, 11H), 6.92 (m, 2H), 5.21 (m, 1H), 4.54 (br, 1H), 4.34 (m, 0.5H), 4.14 (m, 0.5H), 3.70 (m, 4H), 2.61–3.24 (m, 9H), 2.24 (m, 2H), 1.81 (m, 1H), 1.53 (m, 4H), 1.25 (m, 2H); LC-MS: *m/z* 744.8 $[M+H]^+$; HRMS: calcd for $C_{40}H_{46}N_5O_6SFNa$ $[M+Na]^+$ 766.3051, found 766.3029.

5.5.5. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]methylcarbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (20)

Compound **20** was obtained from **15**, (1-benzyl-4-piperidinyl)methylamine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 53%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (m, 1H), 7.76–7.95 (m, 3H), 6.86–7.40 (m, 11H), 6.86 (m, 2H), 5.23 (m, 1H), 4.62 (m, 1H), 4.35 (m, 0.5H), 4.12 (m, 0.5H), 4.00 (m, 2H), 3.61 (m, 2H), 2.74–3.23 (m, 10H), 1.88–2.14 (m, 2H), 1.18–1.57 (m, 8H); LC-MS: *m/z* 758.8 $[M+H]^+$; HRMS: calcd for $C_{41}H_{49}N_5O_6SF$ $[M+H]^+$ 758.3388, found 758.3400.

5.5.6. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]ethylcarbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]iso phthal amide (21)

Compound **21** was obtained from **15**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 38%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74–8.22 (m, 4H), 6.89–7.30 (m, 13H), 5.16 (m, 1H), 4.62 (m, 1H), 4.09 (m, 1H), 3.59 (m, 2H), 2.70–3.21 (m, 12H), 2.03 (m, 3H), 1.14–1.60 (m, 9H); LC-MS: *m/z* 772.8 $[M+H]^+$; HRMS: calcd for $C_{42}H_{51}N_5O_6SF$ $[M+H]^+$ 772.3544, found 772.3553.

5.5.7. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]carbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′,*N*′-dipropyl-5-nitroisophthalamide (22)

Compound **22** was obtained from **15**, 4-amino-1-benzylpiperidine and 3-(dipropylcarbamoyl)-5-nitrobenzoic acid, yield: 36%. ^{1}H NMR (300 MHz MHz, $CDCl_3$): δ 8.54 (m, 1H), 8.25 (m, 1H), 8.03 (m, 1H), 7.88 (m, 1H), 7.08–7.39 (m, 9H), 5.30 (m, 1H), 4.52 (m, 1H), 3.76 (m, 1H), 3.56 (s, 2H), 3.44 (t, *J* = 7.5 Hz, 2H), 2.86–3.16 (m, 6H), 2.31 (m, 2H), 1.45–1.94 (m, 8H), 0.66–0.99 (m, 6H); LC-MS: *m/z* 644.7 $[M+H]^+$; HRMS: calcd for $C_{36}H_{45}N_5O_6$ Na $[M+Na]^+$ 666.3268, found 666.3272.

5.5.8. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]methylcarbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′,*N*′-dipropyl-5-nitroisophthalamide (23)

Compound **23** was obtained from **15**, (1-benzyl-4-piperidinyl)methylamine and 3-(dipropylcarbamoyl)-5-nitrobenzoic acid, yield: 61%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.58 (t, *J* = 1.7 Hz, 0.5H), 8.50 (t, *J* = 1.7 Hz, 0.5H), 8.32 (d, *J* = 9.0 Hz, 0.5H), 8.20 (m, 1H), 8.16 (m, 0.5H), 8.03 (m, 0.5H), 7.97 (d, *J* = 8.0 Hz, 0.5H), 7.56 (br, 0.5H),7.09–7.46 (m, 8.5H), 4.61–4.85 (m, 2H), 4.37 (m, 0.5H), 4.21 (m, 0.5H), 3.75–3.92 (m, 1H), 3.70 (s, 1H), 3.42 (t, *J* = 6.9 Hz, 2H), 2.77–3.50 (m, 8H), 2.17 (m, 2H), 1.43–1.73 (m, 9H), 0.96 (m, 3H), 0.74 (m, 3H); LC-MS: *m/z* 658.7 $[M+H]^+$; HRMS: calcd for $C_{37}H_{47}N_5O_6Na$ $[M+Na]^+$ 680.3424, found 680.3443.

5.5.9. *N*-{[2*S*,3(*R*,*S*)]-2-[4-(1-Benzylpiperidin-4-yl)]ethylcarbamoyl-3-hydroxy-1-phenylbut-2-yl}-*N*′,*N*′-dipropyl-5-nitroisophthalamide (24)

Compound **24** was obtained from **15**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-(dipropylcarbamoyl)-5-nitrobenzoic acid, yield: 45%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48 (s, 0.5H), 8.41 (s, 0.5H), 8.25 (s, 0.5H), 8.18 (m, 1H), 8.02 (s, 0.5H), 7.96 (m, 1H), 7.10–7.29 (m, 9H), 5.28 (m, 1H), 4.57 (m, 1H), 4.33 (m, 0.5H), 4.19 (m, 0.5H), 3.44 (m, 4H), 3.27 (m,2H), 3.10 (m, 3H), 2.88 (m, 3H), 2.16 (m, 1H), 1.86 (m, 2H), 1.27–1.78 (m, 6H), 1.20 (m, 4H), 0.95 (m, 3H), 0.73 (m, 3H); LC-MS: *m/z* 672.8 $[M+H]^+$; HRMS: calcd for $C_{38}H_{50}N_5O_6$ $[M+H]^+$ 672.3761; found 672.3748.

5.6. General method for preparation of compounds 26–38 (exemplified by 26)

5.6.1. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[(1-benzylpiperidin-4-yl)amino]-propyl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (26)

To a solution of **25** (0.026 g, 0.100 mmol) in 1 mL isopropylalcohol was added 4-amino-1-benzylpiperidine (0.060 g, 0.312 mmol) and stirred at 90 °C for 24 h. Then the resulting mixture was directly isolated by simply preparative thin layer chromatography to offer the pure product. The product was dissolved in 1 mL 4 N HCl/1,4-dioxane and stirred for 2 h. To a solution of the resulting product in 1 mL DMF was added 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid (0.010 g, 0.050 mmol), HOBt (0.007 g, 0.052 mmol), DIPEA (0.009 mL, 0.104 mmol) and stirred for 10 min. EDCI (0.010 g, 0.052 mmol) was added and the mixture was allowed to react overnight at room temperature. Then the resulting mixture was diluted by 30 mL EtOAc and washed with 1 N HCl aqueous three times (10 mL × 3), dilute aqueous $NaHCO_3$ three times (10 mL × 3) and saturated brine once (20 mL), sequentially. The organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was purified by preparative thin layer chromatography to give **26** as yellow foam solid, yield: 33%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.00 (m, 1H), 7.96 (m, 1H), 7.78 (m, 1H), 7.08–7.40 (m, 15H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.27 (m, 1H), 3.79 (m, 1H), 3.53 (s, 2H), 3.32 (s, 3H), 3.22 (m, 1H), 2.78–2.98 (m, 8H), 2..65 (m, 1H), 2..07 (m, 2H), 1.91 (m, 2H), 1.56 (d, *J* = 7.0 Hz, 3H), 1.48 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): 166.3, 164.7, 143.3, 141.9, 137.8, 137.7, 135.7, 135.4, 129.0, 128.4, 128.4, 128.1, 127.6, 127.1, 127.0, 126.4, 126.2, 123.9, 70.0, 62.7, 55.3, 51.8, 49.7, 48.0, 37.6, 36.3, 35.2, 31.3, 21.8; LC-MS: *m/z* 712.6 $[M+H]^+$; HRMS: calcd for $C_{40}H_{50}N_5O_5S$ 712.3533, found 712.3576; $[\alpha]_D^{20} = -56.5$ (*c* 1.10, CH_2Cl_2).

5.6.2. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[(1-benzylpiperidin-4-yl)methylamino]-propyl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (27)

Compound **27** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)methylamine and 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 54%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.03 (t, *J* = 1.3 Hz, 1H), 7.96 (t, *J* = 1.3 Hz, 1H), 7.78 (t, *J* = 2.0 Hz, 1H), 7.18–7.40 (m, 14H), 7.11 (m, 1H), 5.48 (s, 1H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.27 (m, 1H), 3.81 (m, 1H), 3.52 (s, 2H), 3.30 (s, 3H), 3.23 (m, 1H), 2.75–2.95 (m, 8H), 2.58 (m, 2H), 2.02 (m, 2H), 1.75 (m, 2H), 1.57 (d, *J* = 7.0 Hz, 4H), 1.27–1.55 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): 166.4, 164.7, 143.4, 142.0, 137.7, 137.6, 135.8, 135.0, 129.2, 129.1, 128.5, 128.5, 128.2, 127.8, 127.2, 127.2, 126.5, 126.3, 124.1, 69.9, 63.0, 54.8, 54.8, 52.8, 51.3, 49.9, 37.7, 36.1, 35.6, 34.2, 29.7, 22.0; LC-MS:

m/z 726.6 $[M+H]^+$; HRMS: calcd for $C_{41}H_{51}N_5O_5SNa$ $[M+Na]^+$, 748.3509, found 748.3543; $[\alpha]_D^{20} = -42.4$ (*c* 0.90, CH_2Cl_2).

5.6.3. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-5-[methyl(methylsulfonyl)amino]-*N*′-[(*R*)-1-phenylethyl]isophthalamide (28)

Compound **28** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-[(*R*)-1-phenylethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 50%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.02 (m, 1H), 7.97 (m, 1H), 7.78(m, 1H), 7.15–7.40 (m, 15H), 5.48 (m, 1H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.26 (m, 1H), 3.79 (m, 1H), 3.50 (s, 2H), 3.31 (s, 3 H), 3.18 (m, 1 H), 2.75–2.95 (m, 10H), 2.00 (m, 2H), 1.77 (m, 2H), 1.57 (d, *J* = 7.0 Hz, 5H), 1.20–1.50 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 166.4, 164.8, 143.3, 141.9, 137.9, 137.8, 135.7, 135.2, 129.2, 129.0, 128.5, 128.4, 128.1, 127.6, 127.2, 127.0, 126.4, 126.3, 124.1, 70.1, 63.2, 55.1, 53.4, 51.1, 49.8, 47.1, 37.7, 36.0, 35.3, 34.8 33.4, 31.8, 21.9; LC-MS: *m/z* 740.5 $[2M+H]^+$; HRMS: calcd for $C_{42}H_{54}N_5O_5S$ $[M+H]^+$, 740.3846, found 740.3862;$[\alpha]_D^{22} = -34$ (*c* 0.30, CH_2Cl_2).

5.6.4. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[(1-benzylpiperidin-4-yl)amino]-propyl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (29)

Compound **29** was obtained from **25**, 4-amino-1-benzylpiperidine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 33%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.05 (m, 1H), 7.96 (m, 1H), 7.80 (m, 1H), 7.41 (m, 2H), 7.18–7.35 (m, 12H), 5.48 (s, 1H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.27 (m, 1H), 3.81 (m, 1H), 3.51 (s, 2H), 3.31 (s, 3H), 3.23 (m, 1H), 2.79–2.99 (m, 8H), 2.67 (m, 1H), 2.09 (m, 2H), 1.92 (m, 2H), 1.54 (d, *J* = 7.0 Hz, 3H), 1.48 (m, 2H); ^{13}C NMR (100 MHz MHz, CD_3OD): 159.1, 158.1, 155.0, 152.5 (d, *J* = 242 Hz), 134.1, 131.6, 130.3, 128.7, 127.8, 127.5, 121.2, 120.8, 119.9, 119.8, 119.7, 119.6, 119.3, 119.0, 117.8, 116.4, 62.8, 54.1, 47.0, 46.5, 45.3, 43.4, 40.8, 40.4, 28.7, 27.9, 26.3, 22.2, 21.9, 12.6; LC-MS: *m/z* 730.8 $[M+H]^+$; HRMS: calcd for $C_{40}H_{49}N_5O_5FS$ $[M+H]^+$, 730.3438, found 730.3458; $[\alpha]_D^{22} = -56.3$ (*c* 0.95, CH_2Cl_2).

5.6.5. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[(1-benzylpiperidin-4-yl)methylamino]-propyl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-[methyl(methylsulfonyl)amino]isophthalamide (30)

Compound **30** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)methylamine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 66%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.01 (m, 1H), 7.95 (m, 1H), 7.78 (m, 1H), 7.41 (m, 2H), 7.00–7.35 (m, 12H), 5.48 (s, 0.5H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.27 (m, 1H), 3.77 (m, 1H), 3.49 (s, 2H), 3.31 (s, 3H), 3.24 (m, 1H), 2.65–2.95 (m, 8H), 2..49 (m, 2H), 1.99 (m, 2H), 1.71 (m, 2H), 1.57 (d, *J* = 7.0 Hz, 4H), 1.20–1.35 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): 165.8, 164.5, 163.1, 160.7 (d, *J* = 244 Hz), 142.0, 138.6, 138.3, 137.6, 135.7, 135.6, 129.1, 128.5, 128.1, 127.9, 127.8, 127.4, 127.0, 126.8, 126.5, 123.8, 69.9, 63.4, 55.9, 55.0, 53.4, 51.3, 48.9, 37.7, 36.4, 35.7, 35.2, 30.3, 21.6; LC-MS: *m/z* 744.3 $[M+H]^+$; HRMS: calcd for $C_{41}H_{51}N_5O_5FS$ $[M+H]^+$, 744.3595, found 744.3660; $[\alpha]_D^{20} = -27.8$ (*c* 0.40, CH_2Cl_2).

5.6.6. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-5-[methyl(methylsulfonyl)-amino]-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]isophthalamide (31)

Compound **31** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-[(*R*)-1-(4-fluorophenyl)ethylaminocarbonyl]-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 53%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.05 (m, 1H), 7.97 (m, 1H), 7.80 (m, 1H), 7.42 (m, 2H), 7.00–7.35 (m, 12H), 5.48 (s, 1H), 5.22 (q, *J* = 7.0 Hz, 1H), 4.26 (m, 1H), 3.87 (m, 1H), 3.52 (s, 2H), 3.31 (s, 3H), 3.30 (m, 1H), 2.80–3.02 (m, 10H), 2.03 (m, 2H), 1.71 (m, 2H), 1.57(d, *J* = 7.0 Hz, 5H), 1.20–1.50 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 166.3, 164.6, 162.9, 160.5 (d, *J* = 244 Hz), 141.9, 139.2, 138.1, 137.8, 135.6, 135.3, 129.1, 129.0, 128.4, 128.0, 128.0, 127.9, 127.5, 126.9, 126.4, 124.0, 115.3, 115.1, 70.1, 63.2, 55.3, 53.3, 51.2, 49.1, 47.2, 37.7, 36.1, 35.3, 35.2, 33.5, 31.9, 21.9; LC-MS: *m/z* 758.6 $[M+H]^+$; HRMS: calcd for $C_{42}H_{52}N_5O_5FS$ 758.3751, found 758.3774; $[\alpha]_D^{22} = -53.1$ (*c* 0.55, CH_2Cl_2).

5.6.7. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′,*N*′-dipropyl-5-[methyl(methylsulfonyl)amino]isophthalamide (32)

Compound **32** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-(dipropylcarbamoyl)-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 39%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.69 (m, 1H), 7.56 (m, 1H), 7.49 (m, 1H), 7.23–7.31 (m, 10H), 5.30 (m, 1H), 4.35 (m, 1H), 3.69 (m, 1H), 3.47 (m, 4H), 3.30 (s, 3H), 3.13 (m, 2H), 3.01 (m, 2H), 2.79–2.88 (m, 7H), 2.68 (m, 2H), 1.93 (m, 2H), 1.64 (m, 4H), 1.48 (m, 3H), 1.28 (m, 4 H), 0.97 (s, 3H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 169.8, 166.1, 141.6, 138.2, 138.1, 137.9, 135.9, 129.3, 129.2, 128.4, 128.1, 127.7, 126.9, 126.4, 124.7, 123.9, 70.2, 63.3, 54.4, 53.6, 51.1, 50.8, 47.2, 46.7, 37.8, 36.1, 35.5, 35.0, 33.5, 32.0, 21.8, 20.6, 11.4, 10.9; LC-MS: *m/z* 720.3 $[M+H]^+$; HRMS: calcd for $C_{40}H_{58}N_5O_5S$ $[M+H]^+$, 720.4159, found 720.4142; $[\alpha]_D^{22} = -15.6$ (*c* 0.45, CH_2Cl_2).

5.6.8. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-5-nitro-*N*′-[(*R*)-1-phenylethyl]isophthalamide (33)

Compound **33** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-[(*R*)-1-phenylethyl]carbamoyl-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 41%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65 (m, 1H), 8.49 (m, 1H), 8.43 (m, 1H), 8.34 (d, *J* = 8.3 Hz, 1H), 7.25–7.40 (m, 13H), 7.03 (d, *J* = 7.5 Hz, 1H), 5.29 (m, 1H), 4.42 (m, 1H), 3.76 (m, 1H), 3.46 (s, 2H), 3.02 (m, 1H), 2.78–2.95 (m, 5H), 2.64 (m, 2H), 1.88 (m, 2H), 1.59 (m, 5H), 1.41 (m, 2H), 1.26 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 164.5, 163.3, 148.1, 142.4, 138.2, 137.5, 136.5, 136.3, 131.1, 129.2, 129.1, 128.7, 128.6, 128.1, 127.6, 126.9, 126.7, 126.2, 124.7, 123.9, 69.4, 63.4, 55.8, 53.6, 51.1, 49.8, 47.5, 36.7, 36.3, 33.7, 32.2, 21.5; LC-MS: *m/z* 678.3 $[M+H]^+$; HRMS: calcd for $C_{40}H_{48}N_5O_5$ $[M+H]^+$, 678.3655, found 678.3639; $[\alpha]_D^{22} = -45.6$ (*c* 0.55, CH_3OH).

5.6.9. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-5-nitroisophthalamide (34)

Compound **34** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethanamine and 3-[(*R*)-1-(4-fluorophenyl)ethyl]carbamoyl-5-[methyl(methylsulfonyl)amino]benzoic acid, yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 8.53 (s, 1H), 8.47 (s, 1H), 7.54 (m, 1H), 7.10–7.40 (m, 11H), 6.95 (m, 2H), 5.30 (m, 1H), 4.39 (br, 1H), 3.90 (br, 1H), 3.46 (s, 2H), 2.60–3.20 (m, 8H), 1.89 (m, 2H), 1.47–1.59 (m, 7H), 1.25 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 164.8, 163.4, 163.1, 160.7 (d, *J* = 245 Hz), 148.1, 138.5, 137.8, 137.3, 136.3, 135.8, 131.0, 139.2, 129.0, 128.6, 128.1, 128.0, 127.9, 127.0, 126.8, 125.1, 124.3, 115.5, 115.3, 69.5, 63.2, 55.4, 53.0, 50.8, 49.3, 47.2, 36.2, 34.9, 33.5, 31.9, 21.7; LC-MS: *m/z* 696.7 $[M+H]^+$; HRMS: calcd for $C_{40}H_{47}N_5O_5F$ $[M+H]^+$, 696.3561, found 696.3572; $[\alpha]_D^{22} = -40.2$ (*c* 0.45, CH_3OH).

5.6.10. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′,*N*′-dipropyl-5-nitroisophthalamide (35)

Compound **35** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-(dipropylcarbamoyl)-5-[methyl(methylsulfo-

nyl)amino]benzoic acid, yield: 49%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.61 (m, 1H), 8.29 (m, 1H), 8.14 (m, 1H), 7.10–7.28 (m, 10H), 4.38 (m, 1H), 3.83 (m, 1H), 3.46 (m, 4H), 3.20 (m, 3 H), 2.78 (m, 7H), 1.90 (m, 2H), 1.22–1.80 (m, 11H), 0.95 (m, 3H), 0.72 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 168.1, 164.7, 147.8, 138.7, 137.8, 136.2, 131.4, 129.5, 129.2, 128.5, 128.3, 127.5, 126.5, 123.7, 123.0, 69.6, 62.8, 54.8, 53.2, 51.2, 50.9, 47.0, 46.8, 35.9, 33.0, 31.2, 29.6, 21.8, 20.6, 11.4, 11.0; LC-MS: *m/z* 658.6 $[M+H]^+$; HRMS: calcd for $C_{38}H52N_5O_5$ $[M+H]^+$, 658.3968, found 696.3951; $[\alpha]_D^{22} = -27.1$ (*c* 0.35, CH_2Cl_2).

5.6.11. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′-[(*R*)-1-phenylethyl]isophthalamide (36)

Compound **36** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-{[(*R*)-1-phenylethyl]carbamoyl}benzoic acid, yield: 44%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.86 (d, *J* = 7.8 Hz, 1H), 7.70 (d, *J* = 10.8 Hz, 1H), 7.47 (d, *J* = 4.6 Hz, 1H), 7.16–7.40 (m, 14H), 6.85 (d, *J* = 7.3Hz, 1H), 5.30 (m, 1H), 4.39 (m, 1H), 3.78 (m, 1H), 3.46 (s, 2H), 2.98 (d, *J* = 6.9 Hz, 2H), 2.81 (m, 4H), 2.64 (m, 2H), 1.87 (m, 2H), 1.58 (d, *J* = 7.0 Hz, 5H), 1.44 (m, 2H), 1.25 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 167.2, 165.6, 143.2, 138.2, 138.0, 137.5, 134.6, 133.9, 130.7, 129.9, 130.7, 129.9, 129.2, 129.1, 128.8, 128.6, 128.5, 128.1, 127.3, 127.0, 126.6, 126.3, 125.3, 69.9, 63.2, 54.4, 53.5, 51.0, 49.4, 47.0, 36.1, 33.4, 31.9, 29.6, 21.8; LC-MS: *m/z* 651.3 $[M+H]^+$; HRMS: calcd for $C_{40}H_{48}N_4O_3F$ $[M+H]^+$, 651.3710, found 651.3701; LC-MS: *m/z* 633.3 $[M+H]^+$; HRMS: calcd for $C_{40}H_{49}N_4O_3$ $[M+H]^+$, 633.3805, found 633.3818; $[\alpha]_D^{22} = -34.0$ (*c* 0.75, CH_2Cl_2).

5.6.12. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′-[(*R*)-1-(4-fluorophenyl)ethyl]-isophthalamide (37)

Compound **37** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-{[(*R*)-1-(4-fluorophenyl)ethyl]carbamoyl}-benzoic acid, yield: 46%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (s, 1H), 7.80 (d, *J* = 7.7 Hz, 1H), 7.65 (d, *J* = 7.7 Hz, 1H), 7.55 (d, *J* = 8.9 Hz, 1H), 7.10–7.42 (m, 11H), 6.91 (m, 3H), 5.23(m, 1H), 4.31 (m, 1H), 3.72 (m, 1H), 3.39 (s, 2H), 2.89 (d, *J* = 6.9 Hz, 2H), 2.78 (m, 4H), 2.58 (m, 2H), 1.81 (m, 2H), 1.50 (d, *J* = 7.0 Hz, 5H), 1.36 (m, 2H), 1.17 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 167.5, 166.4, 162.9, 160.5 (d, *J* = 245 Hz), 139.4, 137.6, 137.4, 137.2, 134.6, 134.3, 133.6, 131.1, 130.3, 129.4, 129.3, 129.1, 128.8, 128.5, 128.2, 128.1, 128.0, 127.9, 127.8, 127.2, 127.0, 126.6, 125.4, 115.3, 115.1, 69.9, 63.1, 54.4, 53.2, 50.9, 48.9, 46.8, 35.6, 33.6, 33.2, 32.1, 22.0; LC-MS: *m/z* 651.3 $[M+H]^+$; HRMS: calcd for $C_{40}H_{48}N_4O_3F$ $[M+H]^+$, 651.3710, found 651.3701; $[\alpha]_D^{22} = -52.9$ (*c* 0.85, CH_2Cl_2).

5.6.13. (1*S*,2*R*)-*N*-{1-Benzyl-2-hydroxy-3-(*S*)-[2-(1-benzylpiperidin-4-yl)ethylamino]-propyl}-*N*′,*N*′-dipropylisophthalamide (38)

The title compound **38** was obtained from **25**, 2-(1-benzylpiperidin-4-yl)ethylamine and 3-(dipropylcarbamoyl)benzoic acid, yield: 46%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.70 (m, 1H), 7.56 (m, 1H), 7.49 (m, 2H), 7.10–7.45 (m, 10H), 4.26 (m, 1H), 3.84 (m, 1H), 3.51 (s, 2H), 3.46 (m, 2H), 3.27 (m, 1H), 3.13 (m, 2H), 2.86 (m, 7H), 2.02 (m, 2H), 1.72 (m, 4H), 1.51 (m, 4H), 1.28 (m, 3H), 0.99 (t, *J* = 7.7 Hz, 3H), 0.69 (t, *J* = 7.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 170.9, 167.2, 138.2, 137.8, 137.4, 134.4, 129.3, 129.2, 128.6, 128.4, 128.1, 127.9, 126.9, 126.4, 125.2, 70.1, 63.3, 54.0, 53.5, 51.2, 50.7, 47.0, 46.4, 36.2, 35.0, 33.3, 31.9, 21.8, 20.6, 11.4, 11.0; LC-MS: *m/z* 613.6 $[M+H]^+$; HRMS: calcd for $C_{38}H_{53}N_4O_3$ $[M+H]^+$, 613.4188, found 613.4133.

5.7. Biology

This section describes the various assays used to assess the biological profiles of the compounds reported in this paper.

5.8. Enzyme-based assay of AChE

AChE is from rat cortex homogenate. The AChE activity was evaluated by studying the hydrolysis of acetylthiocholine (ATCh) following the method of Ellman as reported in detail elsewhere. Five different concentrations of each compound were used in order to obtain inhibition of AChE activity comprised between 20% and 80%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.035 unit/mL of human recombinant AChE derived from human serum (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide). Test compounds were added to the assay solution and preincubated at 37 °C with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE in order to account for nonenzymatic reaction. The reaction rates were compared, and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration–inhibition curves.

5.9. Enzyme-based assay of BACE-1

Recombinant human β-secretase ectodomain (amino acid residues 1–460) was expressed as a secreted protein with a C-terminal His tag in insect cells using baculovirus infection. The BACE activity was determined at room temperature by monitoring the hydrolysis of FRET substrate DABCYL-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-EDANS (SynPep Corp, USA). In a typical 100 μL assay mixture containing 100 mM ammonium acetate, pH 4.0, 20 μM substrate, and 50 nM purified recombinant human BACE-1/Fc, the enzyme activity was continuously monitored with excitation 355 nm/emission 460 nm filter set for 20 min and the initial rate of the hydrolysis was determined using the early linear region of the enzymatic reaction kinetic curve.

5.10. Cellular Aβ lowering assay in APP transfected HEK293 cells

Human embryonic kidney 293 cell transfected with APP695 cDNA containing the Swedish double mutation (HEK293sw), a line known for its tendency to generate high level of aggregating Aβ, was used to examine the effects of the compounds on BACE-1 activity. HEK293sw cells were seeded into 6-well plates with a total volume of 1.5 mL of the cell suspension in Dulbecco's modified Eagle medium (DMEM, Gibco), supplemented with 10% (v/v) heat-inactivated Fetal bovine serum. In the meantime, different concentrations of the compounds were added to the cultures and incubated for 24 h at 37 °C and 5% CO_2. The conditioned medium was removed from the culture wells and $A\beta_{1-40}$ peptide levels in the media were analyzed by Human β Amyloid 1–40 Colormetric Elisa kit (BioSource International, Inc. California, USA) after 24 h.

5.11. Cell protective test in PC12 cells

The antioxidative activity against H_2O_2-induced cytotoxicity was tested according to the reported protocol.[22] Briefly, PC12 cells were seeded into 96-well plates at a density of 1×10^5 cells per mL in DMEM medium (Gibco), supplemented with 10% heat-inactivated bovine calf serum. Experiments were carried out 24 h after cells were seeded. The compounds were dissolved with 2% DMSO

first, and then diluted with phosphate-buffered saline (PBS). After pretreatment with the compounds for 2 h, H_2O_2 was added to PC12 cell cultures for 30 min, and then replaced with fresh DMEM medium (without phenol red). Assays for cell viability were performed 24 h after cultured in fresh medium. Cell survival was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) reduction. The amount of MTT formazan was quantified by determining the absorbance at 570 and 630 nm using Universal Microplate Reader (Bio-Tek). Data were expressed as means ± SD and evaluated for statistical significance with one-way ANOVA followed by Duncan's multiple range tests.

5.12. Animal-based experiment

Experiments were carried out under principles of Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China on November 14, 1988 and followed the guidelines of the Animal Care and Use Committee of Shanghai Institute of Materia Medica. APP transgenic mice, 3–4 months old were generously provided by Prof. Stephen Brimijoin (Mayo Clinic, Rochester), and used for both vehicle-treated and compound-treated groups ($n = 8$ per group). Compound **26** (10 nmol/mice) and compound **28** (8 nmol/mice) was stereotaxically administered into the cerebral ventricle (Bregma −0.2 mm, 1.0 mm lateral, 2.5 mm depth). For control, distilled H_2O was injected. After 4 h, the hemispheres were isolated quickly on ice, and the samples were processed and analyzed for the $A\beta_{1-40}$ level following the instruction of Human β Amyloid 1–40 Colormetric Elisa kit (BioSource International, Inc. California, USA). Data were expressed as means ± SD and evaluated for statistical significance Independent T-test.

5.13. Docking

5.13.1. General AUTODOCK parameter setting

The AUTODOCK Tool software was utilized to prepare the proteins and ligands for docking studies. The hydrogens were added to protein structure and the protein atomic partial charges were assigned with Amber force field. While the partial charges of ligands in docking study were calculated with Gasteiger method. The software AUTODOCK4[23] was adopted to dock the ligand **31** into the binding site of AChE/BACE-1. The Lamarckian genetic algorithm (LGA)[24] was applied to deal with the protein–ligand interactions. A Solis and Wets local search was performed for the energy minimization on a user-specified proportion of the population. To explore the conformational space of ligands, the overall translation steps was set to 0.2 Å, and the overall rotation and torsion rotation step were set to 5° in the docking studies. The number of GA generations, energy evaluations, and docking runs were set to 370,000, 8,000,000, and 50, respectively.

5.13.2. BACE-1

The 3-dimensional crystal structure of BACE-1 was retrieved from the PDB database (the access PDB code: 2B8L). A two-step procedure was adopted to enable AUTODOCK program to explore large conformation space in inhibitors. For BACE-1 docking studies, the ligand was separated into two parts (see Fig. 5A), namely isophthalamide containing part (red colour) and the remainder part (black colour). We first docked the isophthalamide containing part into the BACE-1 binding site, and four lowest energy conformations were selected and the torsion angles in this part will be fixed in the second docking step. Then we connected this part with the remainder part to make the complete ligand, which were docked into the BACE-1 binding site again. Finally the lowest energy conformations were selected for analyzing the interactions between the BACE-1 and inhibitor.

5.13.3. AChE

The 3-dimensional crystal structure of AChE was retrieved from the PDB database (the access PDB code: 1EVE). Similar to docking studies for BACE-1, a two-step procedure was utilized for docking inhibitors **31** into the AChE binding site. The ligand was separated into two parts (see Fig. 5B), namely benzylpiperidine containing part (red colour) and the remainder part (black colour). In AChE docking studies, we first docked the benzylpiperidine containing part into the binding site. Four conformations were selected and the torsion angles were fixed in the second docking step. The remainder part was then connected with the pre-docked part to make complete ligands, which were subjected to the second docking study. Finally the lowest conformations for three inhibitors were selected to investigate the interaction mechanism.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.067.

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