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Synthesis and biological evaluation of a new series of berberine derivatives as dual inhibitors of acetylcholinesterase and butyrylcholinesterase

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ABSTRACT

A series of novel berberine derivatives were designed, synthesized, and biologically evaluated as inhibitors of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Among these derivatives, compound **48a**, berberine linked with 3-methylpyridinium by a 2-carbon spacer, was found to be a potent inhibitor of AChE, with an IC_{50} value of 0.048 μM and compound **40c**, berberine linked with 2-thionaphthol by a 4-carbon spacer, acted as the most potent inhibitor for BuChE with an IC_{50} value of 0.078 μM. Kinetic studies and molecular modeling simulations of the AChE-inhibitor complex indicated that a mixed-competitive binding mode existed for these berberine derivatives.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain that leads to a progressive decline in cognitive function, incapacitation and ultimately death. Current treatment approaches and major therapeutic strategies for this disease are focused on the cholinergic hypothesis.[1,2] The cholinergic hypothesis of AD suggests that low levels of acetylcholine (ACh) in specific regions of the brain results in learning and memory dysfunction,[3] and therefore, one of the most promising approaches is the design of new agents for the treatment of AD.

It is well known that there are two major forms of cholinesterases in mammalian tissues, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8).[4] Recent studies indicated that inhibition of brain BuChE may represent an important therapeutic target for AD as AChE activity in certain brain regions is already decreased, while BuChE levels are unchanged or even rise in advanced AD. This situation implies that the hydrolysis of ACh may occur more easily than that of BuChE.[5] In fact, some evidence suggests that the inhibition of BuChE can raise ACh levels and improve cognition in AD.[6–8] Consequently, dual AChE/BChE inhibitors may have better activity and show clinical efficacy without remarkable side effects.[9]

AChE consists of a narrow gorge with two separate ligand binding sites: an acylation site (ACS) at the bottom of the gorge and a peripheral anionic site (PAS) located at the gorge rim.[10,11] Some features of AChE suggest that the enzyme may have a potential noncatalytic function other than ACh hydrolysis.[12,13] Furthermore, it has been reported that the peripheral anionic site might be somehow related with aggregation and deposition of amyloid-β (Aβ) peptide.[14] In recent years, a discovery was made that the peripheral anionic site could be somehow related to aggregation and deposition of Aβ peptide, which is an early event in the neurodegenerative cascade of AD. This finding stimulated a great interest toward a bivalent ligand strategy for simultaneously inhibiting ACh hydrolysis and AChE-induced Aβ aggregation. Some hybrid compounds, such as tacrine-Melatonin hybrids (**A** in Fig. 1),[15] bis-galanthamine hybrids (**B** in Fig. 1),[16] and bis-(−)-nor-meptazinols (**C** in Fig. 1)[17] afford good effects with sub-nanomolar to picomolar IC_{50} values.

In our pursuit to design, synthesize and biologically evaluate dual inhibitors of AChE/BuChE, we have used berberine (**D** in Fig. 1), a representative protoberberine alkaloid, as the lead structure for the development of hybrid AChE inhibitors, which simultaneously interact with the ACS and the PAS. Molecular modeling showed that 9-O substituted berberine derivatives could interact by π–π stacking with the PAS of AChE, and the aromatic rings of the non-berberine moiety interacted with the catalytic center of AChE through a cation–π interaction. Biological evaluation showed that the berberine linked with phenol by 4-carbon spacers (**E** in Fig. 1), exhibited the most potent AChE inhibition with an IC_{50} of 0.097 μM.[18] In this paper, we report the design, synthesis and biological evaluation of a series of new berberine derivatives, in which the carbon spacers were retained (Fig. 2, **31**–**40**), linkage was replaced by an acyl alkyl group (Fig. 2, **41**–**45**), and the aromatic rings were also replaced with different substituted groups at the *para* position of phenyl or naphthol. The quaternary derivative (Fig. 2,

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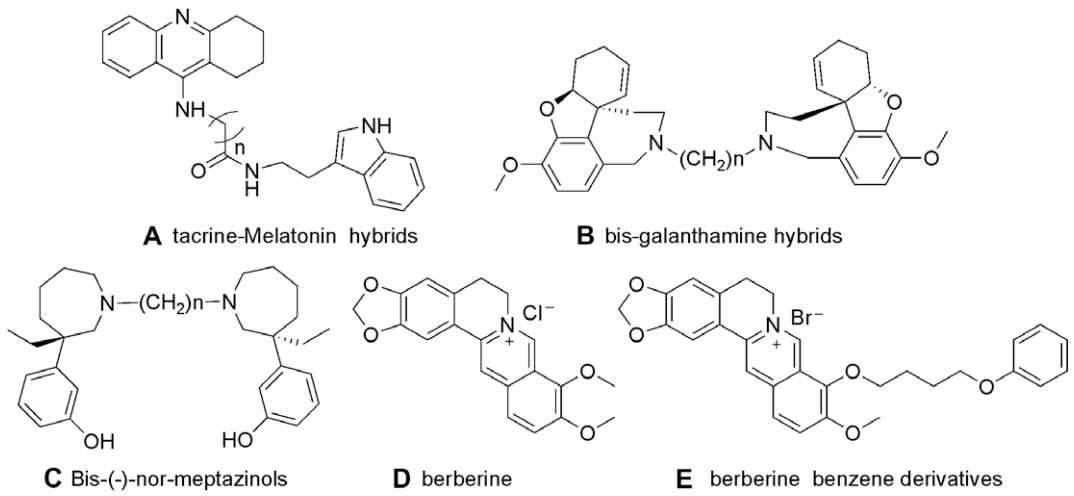


Figure 1. Stucture of reported homobivalent AChE inhibitors.

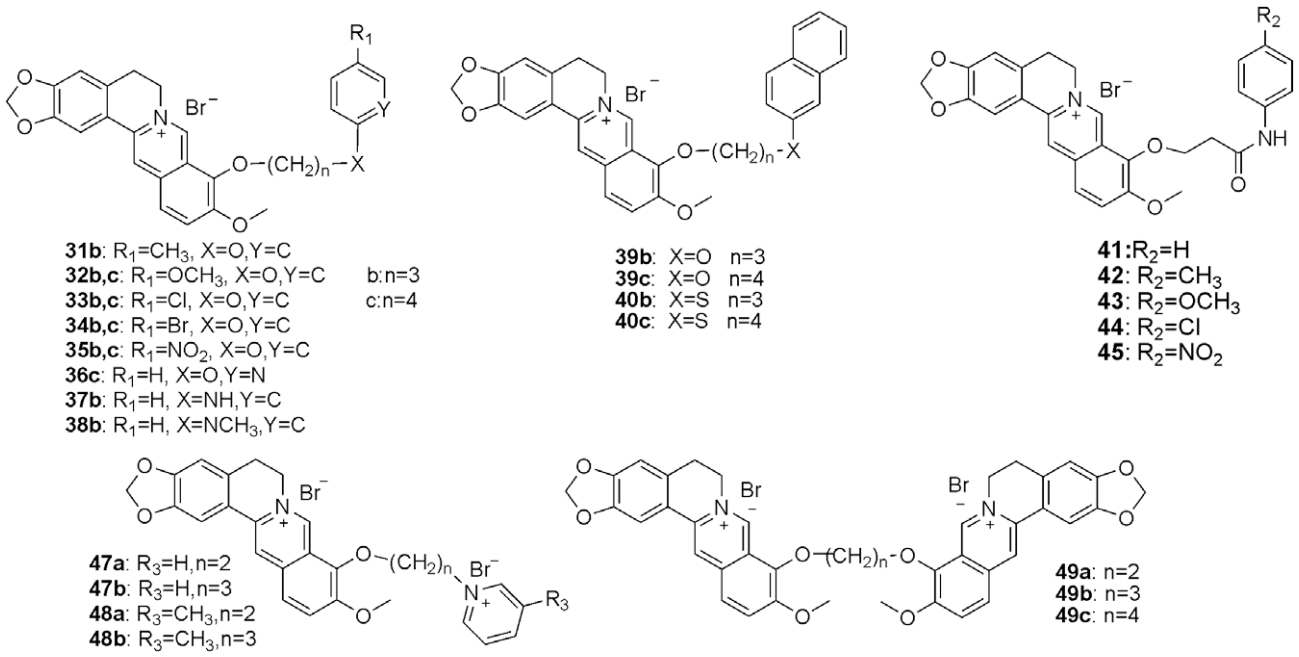


Figure 2. Stucture of 9-substituted berberine derivatives **31**–**45**, **47**–**49**.

47–**49**) was also included in this study to verify the role of a permanent positive charge on inhibition activity.

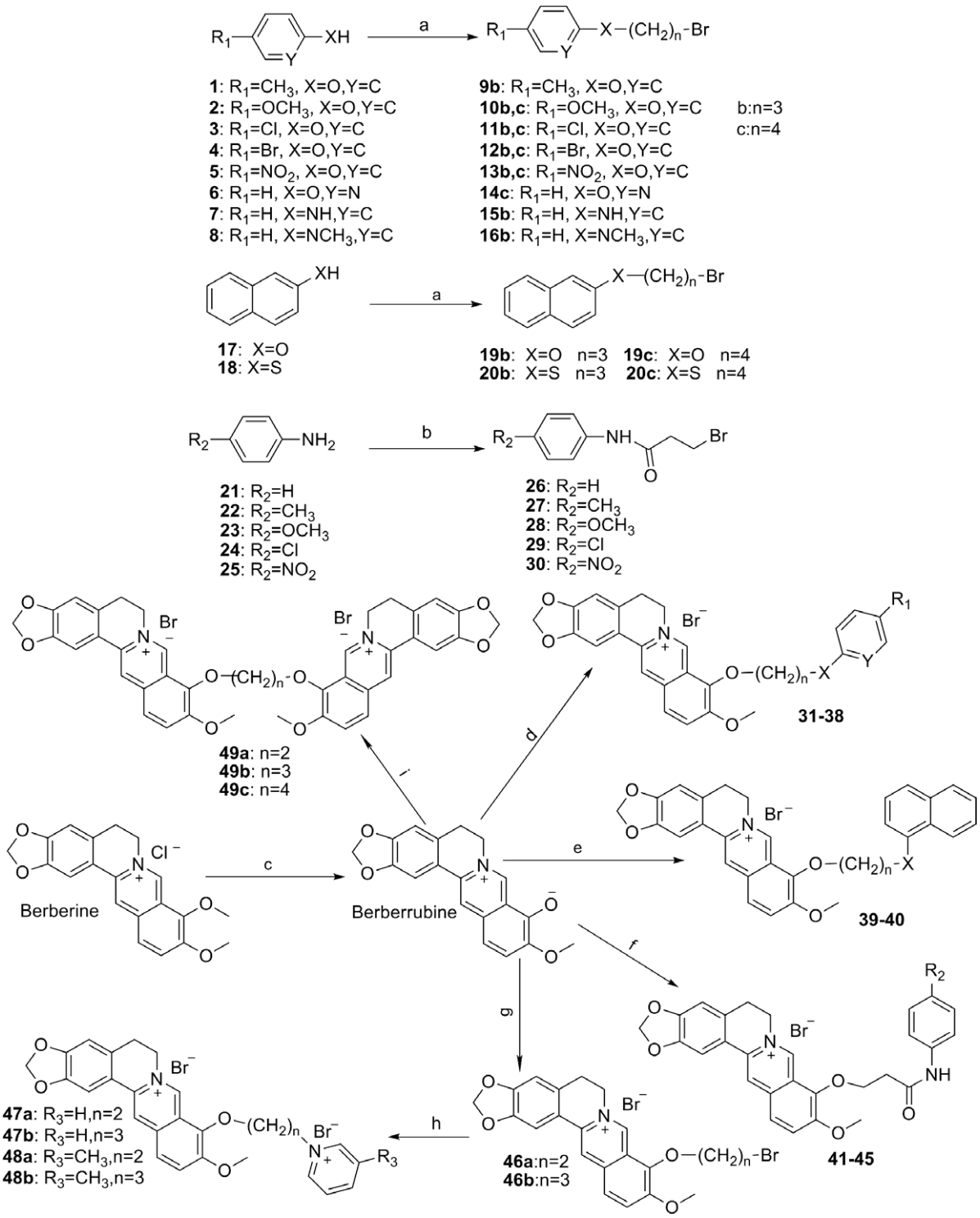
2. Results and discussion

2.1. Chemistry

The synthetic pathway of 9-substituted berberine derivatives **31**–**45**, **47**–**49** are shown in Scheme 1. First, ω-bromoalkyl ether derivatives (**9**–**16**) were synthesized from the commercially available 4-substitute phenol (**1**–**8**), which reacted with α,ω-dibromoalkanes in the presence of K_2CO_3 in butanone to give (**9**–**16**) a good yield. Compounds **19**–**20** were synthesized using the same method with 2-naphthol (**17**) and 2-naphthalenethiol as the starting material. Preparation of β-bromo-*N*-arylpropionamides (**26**–**30**) was carried out by reaction of β-bromopropionyl chloride with the appropriate arylamines (**21**–**25**) in the presence of pyridine. Then, the selective demethylation of berberine at 190 °C under vacuum gave a 68% berberrubine yield.[19] Finally, the target compounds **31**–**45** were obtained by reaction of berberrubine with **9**–**16**, **19**–**20** and **26**–**30** in CH_3CN for 12–24 h, respectively. 9-Bromoalkylberberine **46a**–**b** were prepared according to previous protocols[20] and the final products, **47**–**48,** were prepared by nucleophilic-aromatic displacement of **46a**–**b** with pyridine or 3-methylpyridine to give a 61–72% product yield. Berberine dimers, compounds **49a–c** were synthesized by coupling of berberrubine with different dibromoalkanes from two to four carbons in DMF to produce corresponding berberine dimers in 44–75% yield.

2.2. In vitro inhibition studies of AChE and BuChE

To determine the potential application of target compounds **31**–**45**, and **47**–**49** for the treatment of AD, their AChE inhibitory activity was determined by the method of Ellman et al.[21] on AChE from *electric eel,* using commercial galanthamine as the reference standard. BuChE inhibitory activity on equine serum BuChE was also examined using the same method.



Scheme 1. Reagents and conditions: (a) $Br(CH_2)nBr$, K_2CO_3, KI, butanone, 1–4 h; (b) 3-bromopropionyl chloride, pyridine, $CHCl_3$, 0 °C; (c) 190 °C, 20 mmHg, 15 min; (d) **9**–**16**, K_2CO_3, CH_3CN, 80 °C; (e) **19**–**20**, K_2CO_3, CH_3CN, 80 °C; (f) **26**–**30**, CH_3CN, 80 °C; (g) $Br(CH_2)nBr$, DMF; (h) pyridine or 3-methylpyridine, 105 °C; (i) $Br(CH_2)nBr$, DMF.

The IC_{50} values for AChE and BuChE inhibition are summarized in Table 1. The results reveal most of the berberine derivatives were potent inhibitors of AChE and BuChE, with IC_{50} values ranging from micromolar to sub-micromolar concentrations. Among these inhibitors, compound **48a** revealed the most potent inhibition for AChE (IC_{50} value = 0.048 μM). By contrast, compound **40c** gave the most potent inhibition of BuChE (IC_{50} = 0.078 μM). From the results in Table 1, we can see that the bulk of the scaffold in berberine derivatives at the end of the chain dramatically affected the AChE inhibitory activities. When the aromatic rings were replaced by naphthol (**39, 40**) or para substituents of phenyl (**31**–**35**), their AChE inhibitory activity were less potent than that of compound **E,** which had no substituent at the phenyl ring. The opposite observation was seen with BuChE, which was consistent with structure/activity relationships outlined in our previous reports.[18] IC_{50} values also revealed that the length of alkylene linkage has less effect on

Table 1
In vitro inhibition and selectivity of berberine compounds, **31**–**45**, **47**–**49**, and galanthamine on AChE and BuChE activities

No.[a]	R_1	R_2	R_3	X	Y	*n*	IC_{50} (μM) AChE ± SEM[b]	IC_{50} (μM) BuChE ± SEM[c]	Selectivity for AChE[d]
Berberine	—	—	—	—	—	—	0.374 ± 0.024	18.2 ± 0.683	48.6
Comp **E**[e]	H	—	—	O	C	4	0.097 ± 0.005	4.89 ± 0.035	50.4
31b	CH_3	—	—	O	C	3	0.878 ± 0.061	1.81 ± 0.134	2.1
32b	OCH_3	—	—	O	C	3	1.00 ± 0.075	2.86 ± 0.127	2.9
32c	OCH_3	—	—	O	C	4	0.748 ± 0.084	2.29 ± 0.076	3.1
33b	Cl	—	—	O	C	3	0.715 ± 0.109	1.80 ± 0. 141	2.5
33c	Cl	—	—	O	C	4	1.50 ± 0.020	2.20±0.170	1.5
34b	Br	—	—	O	C	3	0.265 ± 0.024	4.24 ± 0.148	16
34c	Br	—	—	O	C	4	0.743 ± 0.069	5.44 ± 0.500	7.3
35b	NO_2	—	—	O	C	3	0.400 ± 0.020	2.83 ± 0.136	7.1
35c	NO_2	—	—	O	C	4	0.490 ± 0.087	2.05 ± 0.121	4.2
36c	H	—	—	O	N	4	0.340 ± 0.013	4.16±0.05	12.2
37b	H	—	—	NH	C	3	0.138 ± 0.009	1.76 ± 0.114	13.0
38b	H	—	—	NCH_3	C	3	0.156 ± 0.013	2.17 ± 0.040	13.9
39b	—	—	—	O	—	3	0.359 ± 0.044	2.20 ± 0.127	6.1
39c	—	—	—	O	—	4	1.899 ± 0.894	1.10 ± 0.099	0.6
40b	—	—	—	S	—	3	0.500 ± 0.015	0.548 ± 0.014	1.1
40c	—	—	—	S	—	4	1.35 ± 0.092	0.078 ± 0.011	0.06
41	—	H	—	—	—	—	0.535 ± 0.008	2.18 ± 0.085	4.1
42	—	CH_3	—	—	—	—	1.46 ± 0.036	1.31 ± 0.148	0.9
43	—	OCH_3	—	—	—	—	2.80±0.027	1.01 ± 0.061	0.4
44	—	Cl	—	—	—	—	3.86 ± 0.014	1.68 ± 0.190	0.4
45	—	NO_2	—	—	—	—	6.50 ± 0.056	5.53 ± 0.090	0.8
47a	—	—	H	—	—	2	0.196 ± 0.013	2.82 ± 0.183	14.4
47b	—	—	H	—	—	3	0.359 ± 0.044	13.3 ± 0.749	37.0
48a	—	—	CH_3	—	—	2	0.048 ± 0.003	2.46 ± 0.141	51.2
48b	—	—	CH_3	—	—	3	0.152±0.016	15.8 ± 0.986	103.8
49a	—	—	—	—	—	2	0.320 ± 0.022	1.11 ± 0.050	3.5
49b	—	—	—	—	—	3	0.226 ± 0.006	0.531 ± 0.026	2.0
49c	—	—	—	—	—	4	0.176 ± 0.014	0.231 ± 0.023	1.3
Galanthamine	—	—	—	—	—	—	0.623 ± 0.099	15.7 ± 0.787	25.3

[a] Stucture of 9-substituted berberine derivatives **31**–**45**, **47**–**49** are shown in Figure 2.
[b] 50% inhibitory concentration (means ± SEM of three experiments) of AChE from *electric eel*.
[c] 50% inhibitory concentration (means ± SEM of three experiments) of BuChE from equine serum.
[d] Selectivity for AChE = IC_{50} (BuChE)/IC_{50} (AChE).
[e] Structure of **E** is shown in Figure 1 and data has been reported in Ref. 18.

the inhibition of both AChE and BuChE. Surprisingly when the alkylene chain was replaced with an acyl alkyl (**41**–**45**), the inhibitory activity decreased dramatically (with the IC_{50} values ranging from 0.535 to 6.50 μM). When oxygen or carbon atoms in the berberine derivatives (X and Y position in **31–38**) were replaced by nitrogen or sulfur atoms, a different inhibition of AChE and BuChE were observed. The replacement of the oxygen atom with a N atom (**37b**) or sulfur atom (**40c**) at the X position created a weak effect on AChE inhibitory activity. However, inhibition of BuChE was dramatically increased with sulfur atom substitution at the X position. Thus, compound **40c** displayed the most potent BuChE inhibitory activity with an IC_{50} value of 0.078 μM. Compound **48a**, where berberine was linked with 3-methylpyridine by 2-carbon spacers, provided the most inhibitory potency toward AChE (IC_{50} value = 0.048 μM). In reality, AChE and BuChE have different topologies. Compared with the larger Phe of the corresponding residues of AChE, Lys286 and Val288 lie in the gorge of BuChE, and this characteristic could allow bulky compounds to better fit inside the gorge of BuChE.[22] Therefore, berberine dimer **49** has 12–70-fold greater potency than compounds **47**–**48** towards BuChE. Almost all compounds showed potent activity towards BuChE, and several of them exhibited higher selectivity (**39c**, **40c**, **42**–**45**) for BuChE over AChE. Compound **48a**, the most potent for BuChE inhibition, gave an IC_{50} value of 0.078 μM, which was about 17-fold higher than that for AChE. These berberine derivatives may be interesting dual AChE/BuChE inhibitors for AD. For estimating the ability to penetrate blood–brain barrier of compound **48a** which has two positive charges, the log BBB value[23] was predicted with the ADMET program in Discovery studio 2.1. The result (log BBB value = 0.513) indicated that **48a** could penetrante blood–brain barrier easily.

2.3. Kinetic study of AChE

The mechanism of inhibition of AChE was investigated using the quaternary derivative **48a**, one of the most potent AChE inhibitors. The inhibitory behavior of **48a**, as illustrated in Figure 3, is very similar to that of some bis-tetrahydroaminoacridine inhibitors of AChE, a mixed-type of inhibitor which binds at both the catalytic and the peripheral sites of AChE.[24]

2.4. Molecular modeling studies

Molecular docking simulations have become important tools in recent years for gaining insight into the interaction mode and the structure–activity relationships of ligands with drones. We performed molecular docking simulations for 9-substituted berberine derivatives **48a** to TcAChE using the CDOCKER program in Discovery studio 2.1 software, based on the structure of the complex of T. californica enzyme (TcAChE; PDB entry 2CMF). The docking and subsequent scoring were performed using the default parameters of the CDOCKER program. The binding gorge of TcAChE composed of the central catalytic pocket and peripheral sites were taken as the binding site for docking. The position of compound **48a** with respect to the key residues in the binding site is shown in Figure 4. In the complex, the 3-methylpyridinium moiety binds to ACS (see Fig. 4, left), displaying a parallel π–π stacking interaction between Trp84 (3.89 Å) and Phe330 (4.49 Å) in a "sandwich" form. The B ring of the berberine moiety, linked at the top of the gorge (PAS), stacks against the indole ring of Trp279 (3.98 Å) through classical π–π interaction. In addition, the D and E rings of the berberine moiety, snaking along the gorge, making π–π interactions with aromatic rings in Tyr70, Tyr121 and Tyr334 (4.68–5.60 Å).

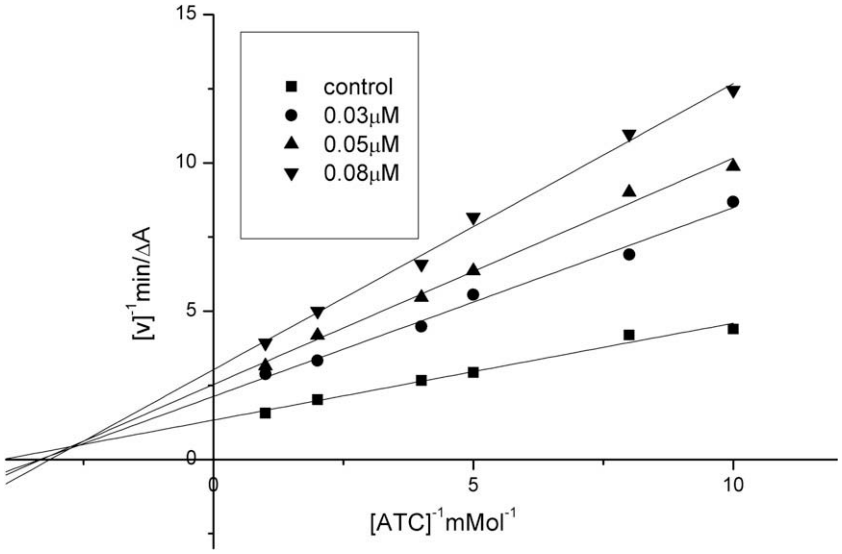


Figure 3. Steady state inhibition by **48a** of AChE hydrolysis of ATCh. Reciprocal plots of initial velocity and substrate concentration: the plots show mixed-type inhibition for **48a** on AChE.

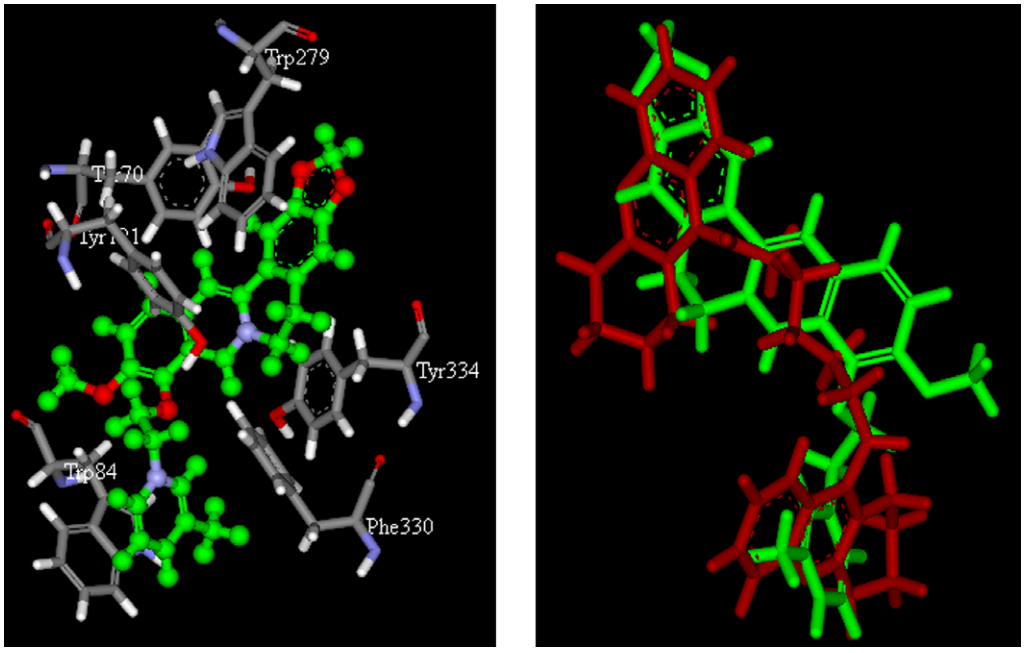


Figure 4. left: berberine derivatives **48a** (green) docked into the catalytic gorge of TcAChE, highlighting the protein residues belonging to ACS and PAS that establish the main interactions. Right: superposition of modeled structure of compound **48a** (colored in green) with X-ray crystal structure of TcAChE binding with bis(5)-tacrine (colored in red, PDB entry:2CMF).

By aligning the conformation of **48a** to the original ligand of the X-ray structure bis(5)-tacrine (Fig. 4, right), we found that both molecules could regulate themselves in order to establish the best interactions with both the acylation site and PAS residues. The number of atoms between nitrogen of 3-methylpyridinium and the B ring of berberine moiety in **48a** was equal to the length of tether of two tacrine moieties in bis(5)-tacrine. This indicated that the length between two functional groups in berberine derivatives was important to its activity.

Berberine derivatives **E**, **32C**, **33C**, **34C**, **35C** and **39c** were also docked into the *Tc*AChE by the same method and the most probable conformation of the derivatives were chosen based on the X-ray structure of AchE binding with bis(5)-tacrine (Fig. 5). The cdocker interaction energy of these berberine derivatives in docking simulations was found to correlated well with inhibition activity (Table 2). The results implied that steric effect plays a key role in inhibitory activity for these berberine derivatives, compounds *E*, with a phenyl linked with the leading compound, gave a low cdocker interaction energy and potent inhibitory activity. In a contrary, its para substituents and naphthol, as their steric effect, provided higher cdocker interaction energy and less potent activity.

On the other hand, we docked compounds **39c** and **40c** into the catalytic gorge of *Hu*BuChE (code ID:2PM8) and chose the most probable conformation of the compounds based on the lowest cdocker interaction energy value (−59.26 and −55.82 Kcal/mol, respectively). The results (Fig. 6) show that although behaved dif-

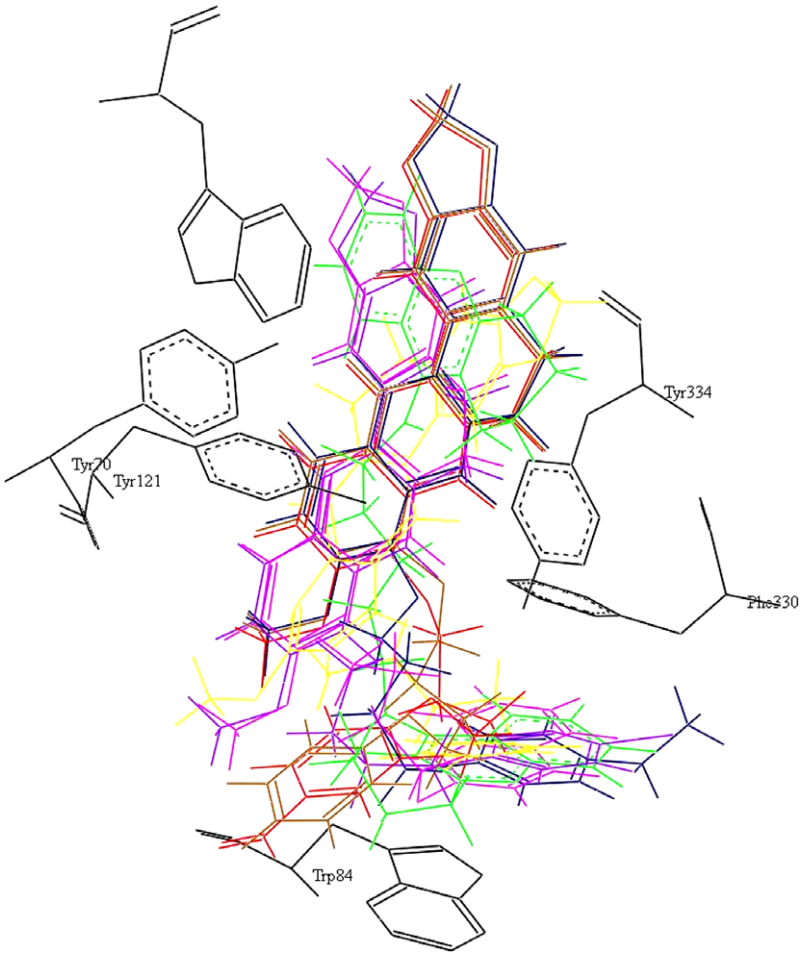


Figure 5. Docking model of compound–enzyme complex. Berberine derivatives **E** (brown), **32c** (blue), **33c** (yellow), **34c** (mauve), **35c** (red) and **39c** (pink) docked into the catalytic gorge of *Tc*AChE (code ID:2CMF) and superposition with original ligand of the X-ray structure bis(5)-tacrine (green).

Table 2
The cdocker interaction energy of derivatives in molecular docking

Compound	IC_{50} (μM)	PIC_{50}	cdocker interaction energy (Kcal/mol)
E	0.097	7.013	−49.28
32c	0.748	6.126	−39.01
33c	1.500	5.823	−19.48
34c	0.743	6.129	−31.51
35c	0.490	6.310	−47.86
39c	1.899	5.721	−30.73

ferent binding model, compounds **39c** and **40c** could interact with both PAS and CAS residue. In order to deep insert the CAS, the linkages of **39c** and **40c** change their orientation, which results in **39c** a higher cdoker interaction energy (the angle of C–S–C is 104.0° and the angle of C–O–C is 117.2°) and lower inhibition activity to BuChE.

3. Conclusion

In summary, a series of new berberine derivatives were designed, synthesized, and evaluated for their inhibitory activity against AChE and BuChE. Among all derivatives prepared, compound **48a** was found to be the best, potent inhibitor with an IC_{50} value of 0.048 μM on AChE. By contrast, compound **40c** gave the most potent inhibition for BuChE with an IC_{50} value of 0.078 μM. The preliminary structure–activity relationship studies showed that the bulk of the scaffold of berberine derivatives at the end of

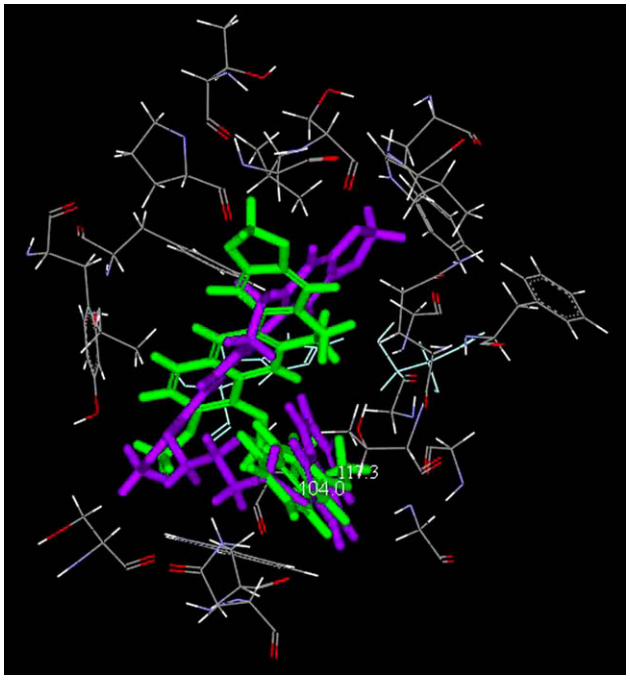


Figure 6. Docking model of compound–enzyme complex. Berberine derivatives **39c** (green) and **40c** (purple) docked into the catalytic gorge of *Hu*BuChE (code ID:2PM8).

the chain affected the AChE inhibitory activities dramatically. The inhibitory activity obviously decreased when the alkylene chain was replaced with an acyl alkyl group (with the IC_{50} values ranging from 0.535 to 6.50 μM). Moreover, the replacement of an oxygen atom with nitrogen (**37b**) or sulfur atom (**40c**) at the X position afforded a weak effect on AChE inhibitory activity. It is interesting that almost all the compounds showed potent activity to BuChE. Kinetic studies and molecular modeling simulations of the AChE-inhibitor complex indicated that a mix-competitive binding mode existed for these berberine derivatives. Further investigations into AD candidates based on these results is in progress.

4. Experimental section

4.1. Chemistry

The NMR spectra were recorded with TMS as the internal standard on a Varian 400 MHz spectrometer. Coupling constants were given in Hz. MS spectra were recorded on a Agilent LC-MS 6120 instrument with an ESI mass selective detector. Flash column chromatography was performed with silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd or alumina from Sinopharm Chemical Reagent Co. Ltd. All the reactions were monitored by thin layer chromatography on silica gel.

Berberine chloride was isolated from Chinese herbal medicine *Coptis chinensis* Franch and recrystallized from hot water. Compounds **9**–**16**, **19**–**20**, **26**–**49** were synthesized as the following procedures.

4.2. General procedures for the preparation of 9–16, 19–20

To a stirred suspension of selective compounds (**1**–**8**, **17**–**18**) (10 mmol) and K_2CO_3 (15 mmol) in butanone (50 mL), dibromoalkanes (12 mmol) and KI (1 mmol) were added. After stirred at room temperature for 1–4 h, the mixture was filtered and then evaporated under vacuum to afford the crude product, which was purified by chromatography on an silica gel column with EtOAc/petroleum ether as eluent to give the products (**9**–**16**, **19**–**20**).

4.3. General procedures for the preparation of 31–40

Intermediates **9**–**16**, **19**–**20** (2.4 mmol) was added respectively to a suspension of berberrubine (2 mmol), K_2CO_3 (6 mmol) in CH_3CN (15 mL) and the mixture was stirred in reflux for 12–24 h. When the reaction was finished (monitored by TLC), the mixture was cooled to room temperature, filtered, and then evaporated under vacuum. The crude product was purified by chromatography on an Al_2O_3 column with $CHCl_3$/MeOH (100:1–50:1) as eluent to afford the target compound.

4.3.1. 9-*O*-[3-(4-Methyl-phenoxyl) propyl]-berberine bromide (31b)

Berberrubine was treated with **9b** according to general procedure to give the desired product **31b** as a yellow solid (47% yield). Mp 201.1–213.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.76 (s, 1H), 8.93 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.99 (d, J = 9.1 Hz, 1H), 7.79 (s, 1H), 7.10–7.08 (m, 3H), 6.85 (d, J = 8.2 Hz, 2H), 6.17 (s, 2H), 4.86 (t, J = 6.0 Hz, 2H), 4.47 (t, J = 6.3 Hz, 2H), 4.21 (t, J = 6.2 Hz, 2H), 4.01 (s, 3H), 3.18 (t, J = 6.1 Hz, 2H), 2.32 (p, J = 6.3 Hz, 2H), 2.23 (s, 3H). LC/MS (ESI) m/z: $[M-Br]^+$ 470.2. Anal. Calcd for $C_{29}H_{28}BrNO_5$: C, 63.28; H, 5.13; N, 2.54. Found: C, 63.00; H, 5.39; N, 2.67.

4.3.2. 9-*O*-[3-(4-Methoxy-phenoxyl)-propyl]-berberine bromide (32b)

Berberrubine was treated with **10b** according to general procedure to give the desired product **32b** as a yellow solid (55% yield). Mp 208.7–210.4 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.93 (s, 1H), 8.19 (d, J = 9.1 Hz, 1H), 7.99 (d, J = 9.1 Hz, 1H), 7.79 (s, 1H), 7.09 (s, 1H), 6.88 (q, J = 9.2 Hz, 4H), 6.17 (s, 2H), 4.87 (t, J = 6.3 Hz, 2H), 4.47 (t, J = 6.3 Hz, 2H), 4.19 (t, J = 6.2 Hz, 2H), 4.02 (s, 3H), 3.69 (s, 3H), 3.19 (t, J = 6.0 Hz, 2H), 2.31 (p, J = 6.1 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 486.2. Anal. Calcd for $C_{29}H_{28}BrNO_6$: C, 61.49; H, 4.98; N, 2.47. Found: C, 61.40; H, 5.01; N, 2.49.

4.3.3. 9-*O*-[3-(4-Methoxy-phenoxyl)butyl]-berberine bromide (32c)

Berberrubine was treated with **10c** according to general procedure to give the desired product **32c** as a yellow solid (51% yield). Mp 188.3–190.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.76 (s, 1H), 8.92 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 7.97 (d, J = 9.1 Hz, 1H), 7.77 (s, 1H), 7.07 (s, 1H), 6.82 (dd, J = 9.0, 2.7 Hz, 4H), 6.15 (s, 2H), 4.92 (t, J = 5.9 Hz, 2H), 4.34 (t, J = 6.4 Hz, 2H), 4.02 (s, 3H), 3.99 (t, J = 6.2 Hz, 2H), 3.66 (s, 3H), 3.18 (t, J = 5.9 Hz, 2H), 2.06–1.96 (m, 2H), 1.96–1.86 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 500.2. Anal. Calcd for $C_{30}H_{30}BrNO_6$: C, 62.07; H, 5.21; N, 2.41. Found: C, 62.10; H, 5.11; N, 2.33.

4.3.4. 9-*O*-[3-(4-Chloro-phenoxyl)propyl]-berberine bromide (33b)

Berberrubine was treated with **11b** according to general procedure to give the desired product **33b** as a yellow solid (64% yield). Mp 214.5–216.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.84 (s, 1H), 8.99 (s, 1H), 8.25 (d, J = 9.2 Hz, 1H), 8.06 (d, J = 9.1 Hz, 1H), 7.85 (s, 1H), 7.40 (d, J = 9.0 Hz, 2H), 7.15 (s, 1H), 7.06 (d, J = 9.0 Hz, 2H), 6.23 (s, 2H), 4.96 (t, J = 6.1 Hz, 2H), 4.53 (t, J = 6.3 Hz, 2H), 4.32 (t, J = 6.2 Hz, 2H), 4.06 (s, 3H), 3.29–3.22 (m, 2H), 2.39 (p, J = 6.1 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 531.6. Anal. Calcd for $C_{28}H_{25}BrClNO_5$: C, 58.91; H, 4.41; N, 2.45. Found: C, 58.80; H, 4.39; N, 2.48.

4.3.5. 9-*O*-[3-(4-Chloro-phenoxyl) butyl]-berberine bromide (33c)

Berberrubine was treated with **11c** according to general procedure to give the desired product **33c** as a yellow solid (70% yield). Mp 196.7–199.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.93 (s, 1H), 8.20 (d, J = 9.1 Hz, 1H), 7.99 (d, J = 9.1 Hz, 1H), 7.80 (s, 1H), 7.32 (d, J = 8.9 Hz, 2H), 7.09 (s, 1H), 6.96 (d, J = 8.9 Hz, 2H), 6.17 (s, 2H), 4.93 (t, J = 6.0 Hz, 2H), 4.35 (t, J = 6.2 Hz, 2H), 4.08 (t, J = 6.0 Hz, 2H), 4.04 (s, 3H), 3.20 (t, J = 5.5 Hz, 2H), 2.08–1.91 (m, 4H). LC/MS (ESI) m/z: $[M-Br]^+$ 504.2. Anal. Calcd for $C_{29}H_{27}BrClNO_5$: C, 59.55; H, 4.65; N, 2.39. Found: C, 59.67; H, 4.50; N, 2.47.

4.3.6. 9-*O*-[3-(4-Bromo-phenoxyl)propyl]-berberine bromide (34b)

Berberrubine was treated with **12b** according to general procedure to give the desired product **34b** as a yellow solid (72% yield). Mp 216.2–218.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H), 8.90 (s, 1H), 8.15 (d, J = 9.1 Hz, 1H), 7.95 (d, J = 9.1 Hz, 1H), 7.76 (s, 1H), 7.42 (d, J = 8.7 Hz, 2H), 7.05 (s, 1H), 6.91 (d, J = 8.7 Hz, 2H), 6.13 (s, 2H), 4.85 (t, J = 5.7 Hz, 2H), 4.41 (t, J = 6.2 Hz, 2H), 4.21 (t, J = 6.1 Hz, 2H), 3.96 (s, 3H), 3.15 (t, J = 5.7 Hz, 2H), 2.29 (t, J = 6.1 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$534.1. Anal. Calcd for $C_{28}H_{25}Br_2NO_5$: C, 54.66; H, 4.10; N, 2.28. Found: C, 54.63; H, 4.12; N, 2.29.

4.3.7. 9-*O*-[3-(4-Bromo-phenoxyl)butyl]-berberine bromide (34c)

Berberrubine was treated with **12c** according to general procedure to give the desired product **34c** as a yellow solid (73% yield). Mp 200.3–202.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 1H), 8.99 (s, 1H), 8.25 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 9.1 Hz, 1H), 7.85

(s, 1H), 7.50 (d, J = 8.7 Hz, 2H), 7.15 (s, 1H), 6.98 (d, J = 8.7 Hz, 2H), 6.23 (s, 2H), 5.00 (t, J = 6.1 Hz, 2H), 4.41 (t, J = 6.1 Hz, 2H), 4.15 (d, J = 6.0 Hz, 2H), 4.11 (d, J = 6.1 Hz, 3H), 3.27 (s, 2H), 2.11–2.01 (m, 4H). LC/MS (ESI) m/z: $[M-Br]^+$ 548.1. Anal. Calcd for $C_{29}H_{27}Br_2NO_5$: C, 55.35; H, 4.32; N, 2.23. Found: C, 55.33; H, 4.35; N, 2.24.

4.3.8. 9-*O*-[3-(4-Nitro-phenoxyl)propyl]-berberine bromide (35b)

Berberrubine was treated with **13b** according to general procedure to give the desired product **35b** as a yellow solid (67% yield). Mp 226.2–227.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.86 (s, 1H), 8.98 (s, 1H), 8.29 (d, J = 9.2 Hz, 2H), 8.24 (d, J = 9.2 Hz, 1H), 8.05 (d, J = 9.1 Hz, 1H), 7.85 (s, 1H), 7.26 (d, J = 9.3 Hz, 2H), 7.15 (s, 1H), 6.23 (s, 2H), 4.97 (t, J = 6.1 Hz, 2H), 4.54 (t, J = 6.2 Hz, 2H), 4.49 (t, J = 6.2 Hz, 2H), 4.05 (s, 3H), 3.31–3.21 (m, 2H), 2.44 (p, J = 6.2 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 501.2. Anal. Calcd for $C_{28}H_{25}BrN_2O_7$: C, 57.84; H, 4.33; N, 4.82. Found: C, 57.80; H, 4.31; N, 4.82.

4.3.9. 9-*O*-[3-(4-Nitro-phenoxyl)butyl]-berberine bromide (35c)

Berberrubine was treated with **13c** according to general procedure to give the desired product **35c** as a yellow solid (70% yield). Mp 215.5–217.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.93 (s, 1H), 8.20 (dd, J = 9.3, 3.0 Hz, 3H), 8.00 (d, J = 9.1 Hz, 1H), 7.79 (s, 1H), 7.16 (d, J = 9.2 Hz, 2H), 7.09 (s, 1H), 6.18 (s, 2H), 4.94 (t, J = 5.9 Hz, 2H), 4.37 (t, J = 5.7 Hz, 2H), 4.26 (t, J = 5.5 Hz, 2H), 4.05 (s, 3H), 3.21 (t, J = 6.0 Hz, 2H), 2.04 (m, 4H). LC/MS (ESI) m/z: $[M-Br]^+$ 515.2. Anal. Calcd for $C_{29}H_{27}BrN_2O_7$: C, 58.50; H, 4.57; N, 4.70. Found: C, 58.51; H, 4.58; N, 4.67.

4.3.10. 9-*O*-[3-(2-Pyridinoxyl)butyl]-berberine bromide (36c)

Berberrubine was treated with **14c** according to general procedure to give the desired product **36c** as a yellow solid (40% yield). Mp 197.7–199.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.12 (d, J = 9.1 Hz, 1H), 7.93 (d, J = 9.1 Hz, 1H), 7.72 (s, 1H), 7.68 (d, J = 5.9 Hz, 1H), 7.39–7.34 (m, 1H), 7.03 (s, 1H), 6.33 (d, J = 9.1 Hz, 1H), 6.19 (t, J = 6.6 Hz, 1H), 6.12 (s, 2H), 4.93 (t, J = 5.7 Hz, 2H), 4.27 (t, J = 5.2 Hz, 2H), 3.99 (s, 3H), 3.94 (t, J = 6.2 Hz, 2H), 3.20–3.14 (m, 2H), 1.83 (m, 5H). LC/MS (ESI) m/z: $[M-Br]^+$ 471.2. Anal. Calcd for $C_{28}H_{27}BrN_2O_5$: C, 60.99; H, 4.94; N, 5.08. Found: C, 61.09; H, 5.10; N, 5.20.

4.3.11. 9-*O*-[3-(Phenylamino)propyl]-berberine bromide (37b)

Berberrubine was treated with **15b** according to general procedure to give the desired product **37b** as a yellow solid (38% yield). Mp 193.5–196.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H), 8.92 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 8.00 (d, J = 9.1 Hz, 1H), 7.79 (s, 1H), 7.09 (t, J = 7.9 Hz, 3H), 6.61 (d, J = 7.8 Hz, 2H), 6.54 (t, J = 7.3 Hz, 1H), 6.17 (s, 2H), 4.85 (t, J = 6.2 Hz, 2H), 4.42 (t, J = 6.3 Hz, 2H), 4.04 (s, 3H), 3.29 (t, J = 6.7 Hz, 2H), 3.18 (t, J = 6.4 Hz, 2H), 2.20–2.10 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 455.2. Anal. Calcd for $C_{28}H_{27}BrN_2O_4$: C, 62.81; H, 5.08; N, 5.23. Found: C, 62.51; H, 4.98; N, 5.29.

4.3.12. 9-*O*-[3-(*N*-Methyl-phenylamino)propyl]-berberine bromide (38b)

Berberrubine was treated with **16b** according to general procedure to give the desired product **38b** as a yellow solid (43% yield). Mp 207.3–210.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.71 (s, 1H), 8.89 (s, 1H), 8.15 (d, J = 9.2 Hz, 1H), 7.96 (d, J = 9.1 Hz, 1H), 7.75 (s, 1H), 7.13 (t, J = 7.9 Hz, 2H), 7.04 (s, 1H), 6.71 (d, J = 8.1 Hz, 2H), 6.57 (t, J = 7.1 Hz, 1H), 6.13 (s, 2H), 4.87 (t, J = 6.4 Hz, 2H), 4.33 (t, J = 6.2 Hz, 2H), 3.99 (s, 3H), 3.57 (t, J = 6.3 Hz, 2H), 3.17 (t, J = 6.0 Hz, 2H), 2.89 (s, 3H), 2.14–2.03 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 469.2. Anal. Calcd for $C_{29}H_{29}BrN_2O_4$: C, 63.39; H, 5.32; N, 5.10. Found: C, 63.46; H, 5.50; N, 5.00.

4.3.13. 9-*O*-[3-(Naphthalen-2-yloxy)propyl]-berberine bromide (39b)

Berberrubine was treated with **19b** according to general procedure to give the desired product **39b** as a yellow solid (55% yield). Mp 201.0–212.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.80 (s, 1H), 8.92 (s, 1H), 8.15 (d, J = 9.1 Hz, 1H), 7.95 (d, J = 9.1 Hz, 1H), 7.85–7.80 (m, 4H), 7.41 (t, J = 7.5 Hz, 1H), 7.35 (s, 1H), 7.30 (t, J = 7.6 Hz, 1H), 7.13 (d, J = 9.0, Hz, 1H), 7.03 (s, 1H), 6.13 (s, 2H), 4.85 (t, J = 5.8 Hz, 2H), 4.48 (t, J = 6.2 Hz, 2H), 4.35 (t, J = 6.1 Hz, 2H), 4.00 (s, 3H), 3.11 (t, J = 6.2 Hz, 2H), 2.41–2.33 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 506.2. Anal. Calcd for $C_{32}H_{28}BrNO_5$: C, 65.53; H, 4.81; N, 2.39. Found: C, 65.52; H, 4.83; N, 2.37.

4.3.14. 9-*O*-[3-(Naphthalen-2-yloxy)butyl]-berberine bromide (39c)

Berberrubine was treated with **19c** according to general procedure to give the desired product **39c** as a yellow solid (47% yield). Mp 182.5–183.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.90 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.81–7.77 (m, 4H), 7.43 (t, J = 7.6 Hz, 1H), 7.41–7.32 (m, 2H), 7.14 (d, J = 9.0, Hz, 1H), 7.01 (s, 1H), 6.16 (s, 2H), 4.92 (t, J = 6.0 Hz, 2H), 4.39 (t, J = 6.1 Hz, 2H), 4.21 (t, J = 6.1 Hz, 2H), 4.01 (s, 3H), 3.18 (t, J = 6.1 Hz, 2H), 2.06–1.97 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 520.2. Anal. Calcd for $C_{33}H_{30}BrNO_5$: C, 66.00; H, 5.04; N, 2.33. Found: C, 65.97; H, 5.07; N, 2.34.

4.3.15. 9-*O*-[3-(Naphthalene-2-ylthio)propyl]-berberine bromide (40b)

Berberrubine was treated with **20b** according to general procedure to give the desired product **40b** as a yellow solid (27% yield). Mp 193.7–195.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.81 (s, 1H), 8.93 (s, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.00 (d, J = 9.0 Hz, 1H), 7.88–7.85 (m, 4H), 7.79 (s, 1H), 7.50–7.48 (m, 3H), 7.09 (s, 1H), 6.18 (s, 2H), 4.92 (t, J = 6.0 Hz, 2H), 4.46 (t, J = 6.1 Hz, 2H), 4.03 (s, 3H), 3.19 (m, 4H), 2.26–2.23 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 522.2. Anal. Calcd for $C_{32}H_{28}BrNO_4S$: C, 63.79; H, 4.68; N, 2.32. Found: C, 63.55; H, 4.60; N, 2.23.

4.3.16. 9-*O*-[3-(Naphthalene-2-ylthio)butyl]-berberine bromide (40c)

Berberrubine was treated with **20c** according to general procedure to give the desired product **40c** as a yellow solid (32% yield). Mp 173.1–175.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H), 8.90 (s, 1H), 8.18 (d, J = 9.2 Hz, 1H), 7.98 (d, J = 9.1 Hz, 1H), 7.84–7.81 (m, 4H), 7.78 (s, 1H), 7.52–7.41 (m, 3H), 7.08 (s, 1H), 6.18 (s, 2H), 4.89 (t, J = 6.2 Hz, 2H), 4.34 (t, J = 6.4 Hz, 2H), 4.03 (s, 3H), 3.22 (t, J = 7.2 Hz, 2H), 3.19–3.15 (m, 2H), 2.08–2.03 (m, 2H), 1.94–1.86 (m, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 536.2. Anal. Calcd for $C_{33}H_{30}BrNO_4S$: C, 64.28; H, 4.90; N, 2.27. Found: C, 64.20; H, 4.71; N, 2.20.

4.4. General procedures for the preparation of 26–30

To the substituted anilines (**21**–**25**) (10 mmol) solution in chloroform (re-distilled, 50 ml), β-bromopropionyl chloride (11 mmol) was slowly added at 0 °C, and then pyridine (11 mmol). After stirred for 1 h at room temperature, the reaction mixture was washed with $NaHCO_3$ solution (2 × 50 ml), water (2 × 50 ml), brine (1 × 50 ml), and then dried with anhydrous Na_2SO_4, concentrated in vacuo to afford the resulting compound (**26**–**30**) which was used for the next step without purification.

4.5. General procedures for the preparation of 41–45

Compounds **26**–**30** (2.4 mmol) was added to a magnetically stirred suspension of berberrubine (2 mmol) in CH_3CN (15 mL)

and the mixture was stirred in reflux for 12–24 h. When the mixture was cooled to room temperature, filtered, and then evaporated under vacuum. The crude product was chromatographed on an Al_2O_3 column, eluted with $CHCl_3$/MeOH (100:1–50:1) as eluent to afford the proposed compound.

4.5.1. 9-*O*-[(3-Oxo-3-phenylamino)propyl]-berberine bromide (41)

Berberrubine was treated with **26** according to general procedure to give the desired product **41** as a yellow solid (58% yield). Mp 175.0–177.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.48 (s, 1H), 9.84 (s, 1H), 8.90 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.98 (d, J = 9.1 Hz, 1H), 7.76 (s, 1H), 7.61 (d, J = 7.7 Hz, 2H), 7.27 (t, J = 7.9 Hz, 2H), 7.07 (s, 1H), 7.02 (t, J = 7.4 Hz, 1H), 6.17 (s, 2H), 4.82 (t, J = 6.1 Hz, 2H), 4.63 (t, J = 5.9 Hz, 2H), 4.07 (s, 3H), 3.12 (t, J = 6.1 Hz, 2H), 2.98 (t, J = 5.9 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 469.2. Anal. Calcd for $C_{28}H_{25}BrN_2O_5$: C, 61.21; H, 4.59; N, 5.10. Found: C, 61.11; H, 4.80; N, 4.98.

4.5.2. 9-*O*-[(3-Oxo-3-*p*-tolylamino)propyl]-berberine bromide (42)

The preparation of compound Berberrubine was treated with **27** according to general procedure to give the desired product **42** as a yellow solid (62% yield). Mp 189.1–191.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.15 (s, 1H), 9.75 (s, 1H), 8.87 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.97 (d, J = 9.1 Hz, 1H), 7.74 (s, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.06–7.04 (m, 3H), 6.15 (s, 2H), 4.73 (t, J = 6.1 Hz, 2H), 4.60 (t, J = 5.8 Hz, 2H), 4.05 (s, 3H), 3.11 (t, J = 6.0 Hz, 2H), 2.90 (t, J = 5.9 Hz, 2H), 2.20 (s, 3H). LC/MS (ESI) m/z: $[M-Br]^+$ 503.1. Anal. Calcd for $C_{28}H_{24}BrN_2O_5$: C, 66.73; H, 4.80; N, 5.56. Found: C, 66.89; H, 4.98; N, 5.67.

4.5.3. 9-*O*-[(3-Oxo-3-*p*-methoxyamino)propyl]-berberine bromide (43)

Berberrubine was treated with **28** according to general procedure to give the desired product **43** as a yellow solid (70% yield). Mp 194.9–197.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H), 9.80 (s, 1H), 8.88 (s, 1H), 8.18 (d, J = 9.2 Hz, 1H), 7.97 (d, J = 9.1 Hz, 1H), 7.75 (s, 1H), 7.46 (d, J = 9.0 Hz, 2H), 7.06 (s, 1H), 6.82 (d, J = 9.0 Hz, 2H), 6.16 (s, 2H), 4.78 (t, J = 6.0 Hz, 2H), 4.60 (t, J = 5.8 Hz, 2H), 4.06 (s, 3H), 3.67 (s, 3H), 3.15–3.07 (m, 2H), 2.90 (t, J = 5.8 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 499.2. Anal. Calcd for $C_{29}H_{27}BrN_2O_6$: C, 69.73; H, 5.45; N, 5.61. Found: C, 69.44; H, 5.81; N, 5.88.

4.5.4. 9-*O*-[(3-Oxo-3-*p*-chlorophenylamino)propyl]-berberine bromide (44)

Berberrubine was treated with **29** according to general procedure to give the desired product **44** as a yellow solid (55% yield). Mp 202.8–205.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.58 (s, 1H), 9.81 (s, 1H), 8.87 (s, 1H), 8.16 (d, J = 9.2 Hz, 1H), 7.95 (d, J = 9.1 Hz, 1H), 7.73 (s, 1H), 7.61 (d, J = 8.8 Hz, 2H), 7.30 (d, J = 8.9 Hz, 2H), 7.05 (s, 1H), 6.14 (s, 2H), 4.83 (t, J = 5.6 Hz, 2H), 4.59 (t, J = 5.7 Hz, 2H), 4.03 (s, 3H), 3.13 (t, J = 5.8 Hz, 2H), 2.95 (t, J = 5.7 Hz, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 483.2. Anal. Calcd for $C_{28}H_{24}BrClN_2O_5$: C, 57.60; H, 4.14; N, 4.80. Found: C, 57.61; H, 4.15; N, 4.82.

4.5.5. 9-*O*-[(3-Oxo-3-*p*-nitrophenylamino)propyl]-berberine bromide (45)

Berberrubine was treated with **30** according to general procedure to give the desired product **45** as a yellow solid (72% yield). Mp 213.4–215.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.06 (s, 1H), 9.85 (s, 1H), 8.89 (s, 1H), 8.20 (d, J = 8.8 Hz, 3H), 7.98 (d, J = 9.1 Hz, 1H), 7.87 (d, J = 9.0 Hz, 2H), 7.76 (s, 1H), 7.09 (s, 1H), 6.17 (s, 2H), 4.89 (s, 2H), 4.65 (s, 2H), 4.07 (s, 3H), 3.18 (s, 2H), 3.06 (s, 2H). LC/MS (ESI) m/z: $[M-Br]^+$ 514.2. Anal. Calcd for $C_{28}H_{24}BrN_3O_7$: C, 56.58; H, 4.07; N, 7.07. Found: C, 56.60; H, 4.09; N, 7.06.

4.6. General procedures for the preparation of 47–48

A suspension of **46a** or **46b** (1 mmol) in pyridine or 3-methylpyridine (10 mL) was heated at 105 °C for 6 h , The reaction mixture was concentrated in vacuo, and then cooled to room temperature. The resulting solid was chromatographed on a Al_2O_3 column, eluted with $CHCl_3$/MeOH (50/1, v/v) to afford the proposed compound **47**–**48** as a light yellow solid.

4.6.1. 9-O-[(2-Pyridinium)bromide ethyl]-berberine bromide (47a)

Compound **46a** was treated with pyridine according to general procedure to give the desired product **47a** as a yellow solid (52% yield). Mp 224.3–226.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.07 (s, 1H), 9.40 (d, J = 5.8 Hz, 2H), 8.96 (s, 1H), 8.72 (t, J = 7.8 Hz, 1H), 8.28 (t, J = 7.0 Hz, 2H), 8.12 (d, J = 9.2 Hz, 1H), 8.01 (d, J = 9.2 Hz, 1H), 7.79 (s, 1H), 7.11 (s, 1H), 6.17 (s, 2H), 5.32 (t, J = 6.0 Hz, 2H), 5.02 (t, J = 5.9 Hz, 2H), 4.79 (t, J = 5.9 Hz, 2H), 3.84 (s, 3H), 3.23 (t, J = 6.0 Hz, 2H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 214.1. Anal. Calcd for $C_{26}H_{24}Br_2N_2O_4$: C, 53.08; H, 4.11; N, 4.76. Found: C, 53.17; H, 4.35; N, 4.51.

4.6.2. 9-*O*-[(3-Pyridinium bromide)propyl]-berberine dibromide (47b)

Compound **46b** was treated with pyridine according to general procedure to give the desired product **47b** as a yellow solid (47% yield). Mp 267.1–268.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.95 (s, 1H), 9.32 (d, J = 6.1 Hz, 2H), 8.96 (s, 1H), 8.64 (t, J = 7.8 Hz, 1H), 8.21 (m, 3H), 8.02 (d, J = 9.1 Hz, 1H), 7.80 (s, 1H), 7.09 (s, 1H), 6.16 (s, 2H), 5.08–4.96 (m, 4H), 4.37 (t, J = 5.7 Hz, 2H), 4.04 (s, 3H), 3.22 (t, J = 5.9 Hz, 2H), 2.67–2.59 (m, 2H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 221.1. Anal. Calcd for $C_{27}H_{26}Br_2N_2O_4$: C, 53.84; H, 4.35; N, 4.65. Found: C, 54.01; H, 4.27; N, 4.33.

4.6.3. 9-*O*-[(2-(3-Methyl)pyridinium bromide)ethyl]-berberine bromide (48a)

Compound **46a** was treated with 3-methylpyridine according to general procedure to give the desired product **48a** as a yellow solid (49% yield). Mp 242.1–244.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.94 (s, 1H), 9.23 (s, 1H), 9.10 (d, J = 6.0 Hz, 1H), 8.96 (s, 1H), 8.46 (d, J = 8.0 Hz, 1H), 8.19 (d, J = 9.2 Hz, 1H), 8.10 (m, 2H), 8.00 (d, J = 9.1 Hz, 1H), 7.79 (s, 1H), 7.09 (s, 1H), 6.16 (s, 2H), 5.02 (t, J = 6.0 Hz, 2H), 4.92 (t, J = 5.9 Hz, 2H), 4.34 (t, J = 5.7 Hz, 2H), 4.03 (s, 3H), 3.20 (t, J = 6.0 Hz, 2H), 2.65–2.56 (m, 2H), 2.51 (s, 3H). LC/MS (ESI) m/z: $[M/2-Br]/2^+$ 221.1. Anal. Calcd for $C_{27}H_{26}Br_2N_2O_4$: C, 53.84; H, 4.35; N, 4.65. Found: C, 53.77; H, 4.19; N, 4.80.

4.6.4. 9-*O*-[3-(3-Methyl pyridinium bromide)propyl]-berberine bromide (48b)

Compound **46b** was treated with 3-methylpyridine according to general procedure to give the desired product **48b** as a yellow solid (42% yield). Mp 278.5–279.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.13 (s, 1H), 9.39 (s, 1H), 9.27 (d, J = 6.0 Hz, 1H), 8.94 (s, 1H), 8.52 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 8.12 (dd, J = 7.7, 6.3 Hz, 1H), 7.98 (d, J = 9.2 Hz, 1H), 7.76 (s, 1H), 7.07 (s, 1H), 6.14 (s, 2H), 5.26 (t, J = 6.1 Hz, 2H), 5.03 (t, J = 5.9 Hz, 2H), 4.77 (t, J = 6.0 Hz, 2H), 3.83 (s, 3H), 3.21 (t, J = 6.0 Hz, 2H), 2.64–2.60 (m, 2H), 2.53 (s, 3H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 228.1. Anal. Calcd for $C_{28}H_{28}Br_2N_2O_4$: C, 54.56; H, 4.58; N, 4.55. Found: C, 54.34; H, 4.77; N, 4.65.

4.7. General procedures for the preparation of 49a–c

To a solution of beberrubine (2.2 mmol) in dry DMF (5 mL), dibromoethane (1 mmol) was added and the mixture was heated at 60 °C for 6 h. When the mixture was cooled to room temperature, Et_2O was added and the resulting solid was filtered which was purified by chromatography on a Al_2O_3 column with $CHCl_3$/MeOH (50/1, v/v) as eluent to give light yellow solids of **49a–c**.

4.7.1. 1,2-Di(berberine-9-*O*-yl)ethane dibromide (49a)

Berberrubine was treated with 1,2-dibromoethane according to general procedure to give the desired product **49a** as a yellow solid (34% yield). Mp 309.8–311.4 °C; ^{1}H NMR (400 MHz, DMSO-d_6) 9.83 (s, 2H), 8.92 (s, 2H), 7.86 (d, J = 8.9 Hz, 2H), 7.81 (s, 2H), 7.75 (d, J = 8.9 Hz, 2H), 7.09 (s, 2H), 6.18 (s, 4H), 4.92 (d, J = 5.7 Hz, 4H), 4.61 (d, J = 5.9 Hz, 4H), 4.53 (d, J = 6.0 Hz, 4H), 4.07 (s, 6H), 3.19 (d, J = 5.9 Hz, 4H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 335.1. Anal. Calcd for $C_{40}H_{34}Br_2N_2O_8$: C, 57.85; H, 4.13; N, 3.37. Found: 57.55; H, 4.02; N, 3.55.

4.7.2. 1,3-Di(berberine-9-*O*-yl)ethane dibromide (49b)

Berberrubine was treated with 1,3-dibromopropane according to general procedure to give the desired product **49a** as a yellow solid (40% yield). Mp 301.2–303.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.89 (s, 2H), 8.96 (s, 2H), 8.21 (d, J = 9.2 Hz, 2H), 8.00 (d, J = 9.1 Hz, 2H), 7.81 (s, 2H), 7.10 (s, 2H), 6.18 (s, 4H), 4.96 (t, J = 6.1 Hz, 4H), 4.57 (t, J = 6.4 Hz, 4H), 4.03 (s, 6H), 3.16 (t, J = 6.2 Hz, 4H), 2.58–2.54 (m, 2H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 342.1. Anal. Calcd for $C_{41}H_{36}Br_2N_2O_8$: C, 58.31; H, 4.30; N, 3.32. Found: C, 59.01; H, 4.61; N, 3.15.

4.7.3. 1,4-Di(berberine-9-*O*-yl)ethane dibromide (49c)

Berberrubine was treated with 1,2-dibromobutane according to general procedure to give the desired product 49a as a yellow solid (31% yield). Mp 282.9–285.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 2H), 8.97 (s, 2H), 8.22 (d, J = 9.2 Hz, 2H), 8.01 (d, J = 9.1 Hz, 2H), 7.81 (s, 2H), 7.10 (s, 2H), 6.18 (s, 4H), 4.97 (t, J = 6.1 Hz, 4H), 4.42 (t, J = 6.0 Hz, 4H), 4.06 (s, 6H), 3.21 (t, J = 6.0 Hz, 4H), 2.15 (m, 4H). LC/MS (ESI) m/z: $[M/2-Br]^+$ 349.1. Anal. Calcd for $C_{42}H_{38}Br_2N_2O_8$: C, 58.75; H, 4.46; N, 3.26. Found: C, 58.89; H, 4.32; N, 3.11.

4.8. Biological activity

4.8.1. In vitro inhibition studies on AChE and BuChE

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from *electric eel*), butylcholinesterase (BuChE, E.C. 3.1.1.8, from *equine serum*), 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), acetylthiocholine chloride (ATC), butylthiocholine chloride (BTC), and tarcine hydrochloride were purchased from Sigma Aldrich. Berberine derivatives were dissolved in DMSO and then diluted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0) to provide a final concentration range.

In vitro AChE assay: All the assays were carried out under 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 8.0, using a Shimadzu UV-2450 Spectrophotometer. AChE and BuChE solutions were prepared to give 2.0 units/mL in 2 mL aliquots. The assay medium (1 mL) consisted of phosphate buffer (pH 8.0), 50 μl of 0.01 M DTNB, 10 μl of enzyme, and 50 μl of 0.01 M substrate (ACh chloride solution). Test compounds were added to the assay solution and pre-incubated at 37 °C with the enzyme for 15 min followed by the addition of substrate. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals at 37 °C. Calculations were performed according to the method of the equation in Ellman et al.[21] Each concentration was assayed in triplicate.

In vitro BuChE assay was similar with the method described above.

4.8.2. Kinetic characterization of AChE inhibition

Kinetic characterization of AChE was performed using a reported method.[25] Test compound was added into the assay solution and pre-incubated with the enzyme at 37 °C for 15 min, followed by the addition of substrate. Kinetic characterization of the hydrolysis of ATC catalyzed by AChE was carried out spectrometrically at 412 nm. A parallel control was made with the assay solution of no inhibitor for each times. 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 the inhibitors in a weighted analysis and K_i was determined as the ratio of the replot intercept to the replot slope.

4.8.3. Molecular modeling

The simulation system was built based on the X-ray crystal structure of the bistacrine–AChE complex which was obtained from the Protein Data Bank (PDB entry 2CMF). The original ligand was removed while water molecules present in the PDB file were maintained in their position. 3D structures of the 9-substituted berberine derivatives were generated and optimized by Discovery studio 2.1 package (Accelrys Inc., San Diego, CA). The CDOCKER program in Discovery studio 2.1 software was used to perform docking simulations, which allows full flexibility of the ligand.

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