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Benzenediol-berberine hybrids: Multifunctional agents for Alzheimer's disease

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ABSTRACT

We designed and synthesized a series of hybrid molecules, in an effort to identify novel multifunctional drug candidates for Alzheimer's disease (AD), by reacting berberine with benzenediol, melatonin, and ferulic acid. The products were evaluated for: (i) the ability to inhibit multiple cholinesterases (ChEs); (ii) the capacity to prevent amyloid β (Aβ) aggregation; and (iii) antioxidant activity. All of the derivatives were better antioxidants, and inhibited Aβ aggregation to a greater extent, than the lead compound, berberine. Two of the hybrids, in particular, have the potential to be excellent candidates for AD therapy: the berberine-pyrocatechol hybrid (compound **8**) was a much better inhibitor of acetylcholinesterase (AChE) than unconjugated berberine (IC_{50}: 0.123 vs 0.374 μM); and the berberine-hydroquinone hybrid (compound **12**) displayed high antioxidant activity, could inhibit AChE (IC_{50} of 0.460 μM), and had the greatest ability to inhibit Aβ aggregation.

1. Introduction

AD is a neurodegenerative disorder characterized by progressive cognitive decline, decreased cholinergic transmission, and the presence of senile plaques. Although the etiology of AD is not fully known, the presence of Aβ deposits, increased oxidative stress, and reduced levels of acetylcholine (ACh) are thought to play significant roles in the pathophysiology of the disease.[1]

There are two types of ChEs in the central nervous system (CNS), both of which can hydrolyze ACh: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Recent studies indicate that as AChE activity steadily decreases from mild to severe stages of AD (eventually reaching 10–15% of normal values), BuChE levels remain unchanged or may rise. In fact, evidence suggests that inhibiting BuChE raises ACh levels and improves cognition.[2,3] Dual-acting ChE inhibitors affect the activities of both AChE and BuChE.[4,5]

The formation of Aβ fibrils and their deposition into neurotoxic amyloid plaques appear to be the primary cause of AD.[6–8] Aβ deposits inflict oxidative injury to surrounding neurons and elicit an acute inflammatory response that contributes to the neurodegeneration.[9,10] Senile plaques release free radicals which are extremely toxic[11], and the accumulation of these reactive oxygen species (ROS) damages major cell components. Thus, oxidative stress is thought to play a major role in the pathogenesis of AD. Indeed, several antioxidant compounds have demonstrated efficacy in a number of recent studies.[12]

Because of the complex multifactorial etiology of AD, researchers have developed the *multi-target-directed ligand* (MTDL) strategy for drug discovery. It has become a valuable tool for developing drugs that target multiple factors implicated in AD pathogenesis.[13] In the MTDL strategy, distinct pharmacophores from different drugs are combined in a single molecule to produce multifunctional hybrids.[14,15] One of the most widely adopted approaches has been to modify the structure of AChE inhibitors to confer additional biological properties that are likely to be useful for treating AD.

In our previous work using berberine (**1**, Fig. 1) as the lead structure, we designed and synthesized a series of novel derivatives and found that most of them were capable of inhibiting both AChE and BuChE.[16] The most potent inhibitor, berberine linked with phenol by 4-carbon spacers (**2**, Fig. 1), strongly inhibited AChE (IC_{50} of 0.097 μM). We continued to pursue this promising strategy with the design, synthesis, and evaluation of numerous other compounds with therapeutic potential for treating AD.

Certain phenol and diphenol derivatives have previously been shown to possess both antioxidant activity[17,18] and the ability to inhibit Aβ aggregation.[19,20] For example, curcumin, which is produced by the rhizomes of *Curcuma longa*, is an effective scavenger of ROS. Ferulic acid (**4**, Fig. 1), one of the predominant phenolic acids in wheat (*Triticum aestivum*) and eucalyptus (*Eucalyptus globulus*), has been reported to impart resistance to Aβ toxicity in the brains of mice receiving long-term administration of this compound.[21,22] Melatonin (**3**, Fig. 1), a hormone produced by the pineal gland, is also a potent antioxidant, and is able to scavenge a variety of ROS.[23,24] We generated a series of hybrids of berberine, diphenol, ferulic acid, and melatonin; in effect combining two

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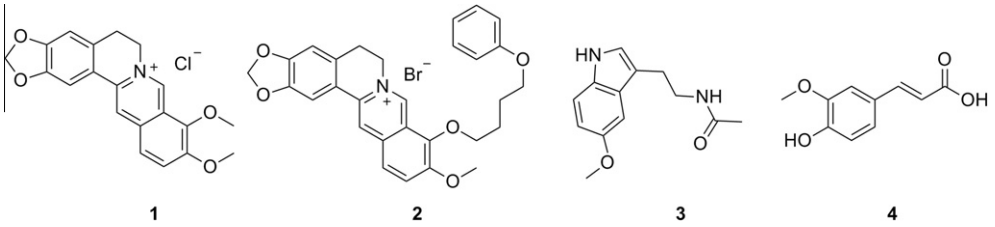


Figure 1. Structure of berberine (**1**), the phenol-berberine derivative (**2**), melatonin (**3**), and ferulic acid (**4**).

distinct pharmacophores to form hybrid compounds. In this report, we describe the synthesis of these hybrids and evaluate their potential, in vitro, as candidates for treating AD. We demonstrate that these compounds display antioxidant activity, and that some of them also have the ability to strongly inhibit cholinesterases and reduce Aβ aggregation.

2. Results and discussion

2.1. Chemistry

The synthesis of the berberine-benzenediol derivatives (**7**–**13**) is presented in Scheme 1. First, berberrubine (**5**) was obtained with a yield of 66% through selective demethylation of berberine (**1**) at 190 °C under vacuum,[25] and was then alkylated with dibromoalkanes in dimethylformamide (DMF)[26] to produce compounds **6a–c** with yields of 60–75%. Finally, **6a–c** were reacted with benzenediol in DMF for 4 h to produce **7**–**13** at high yields.

Berberine-melatonin hybrids (**17**–**19**) were synthesized as shown in Scheme 2. First, different β-bromo-*N*-arylpropiontryptamides (**14**–**16**) were obtained by reacting β-bromopropionyl chloride with the appropriate tryptamine in the presence of pyridine. Compounds **14**–**16** were then coupled with berberrubine (**5**) in DMF to produce the berberine-melatonin hybrids (**17**–**19**).

The synthesis of the berberine-ferulic acid hybrid (**21**) is shown in Scheme 3. The reaction of ferulic acid with 1,3-dibromopropane in the presence of Et_3N at 50 °C gave compound **20** with a yield of 67%. The reaction of **20** with berberrubine (**5**) in DMF produced the berberine-ferulic acid hybrid (**21**) with a yield of 43%.

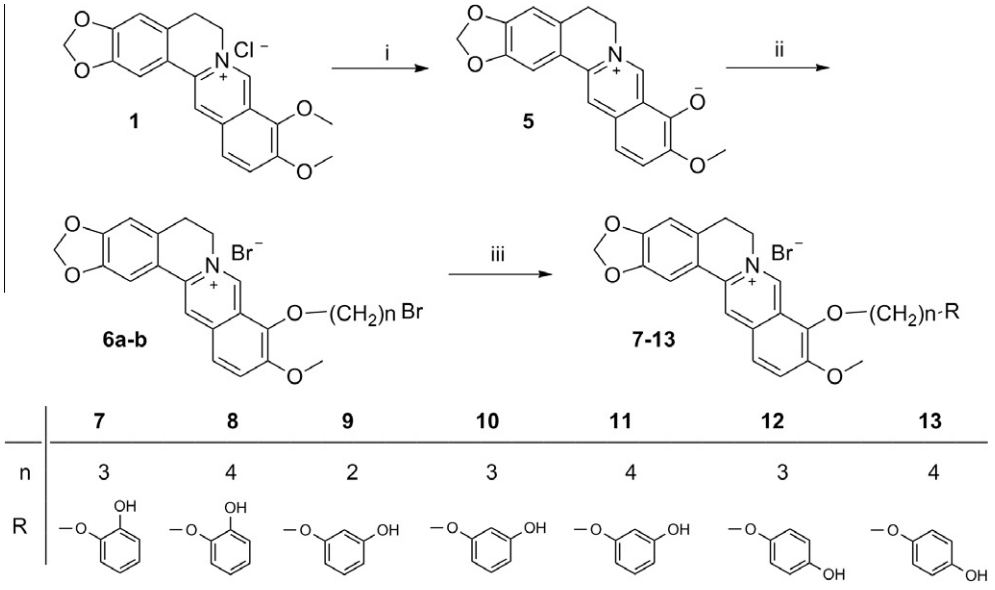
2.2. In vitro inhibition studies of AChE and BuChE

The ability of the derivatives to inhibit AChE activity was assessed by the method of Ellman,[27] using AChE from the electric eel and commercial galanthamine as the reference standard. The ability of these compounds to inhibit horse serum BuChE was determined using a similar method. The IC_{50} values for AChE and BuChE inhibition are summarized in Table 1. The berberine-benzenediol hybrids (**7**–**13**, IC_{50} values of 0.628–0.123 μM) were better inhibitors of AChE than the berberine-melatonin hybrids (**17**–**19**, IC_{50} values of 1.11–1.44 μM) or the berberine-ferulic acid hybrid (**21**, IC_{50} of 3.21 μM). However, the berberine-phenol derivative (**2**, IC_{50} of 0.097 μM) was the most potent inhibitor of AChE. The berberine-pyrocatechol hybrid (**8**) was also a strong AChE inhibitor (IC_{50} of 0.123 μM).

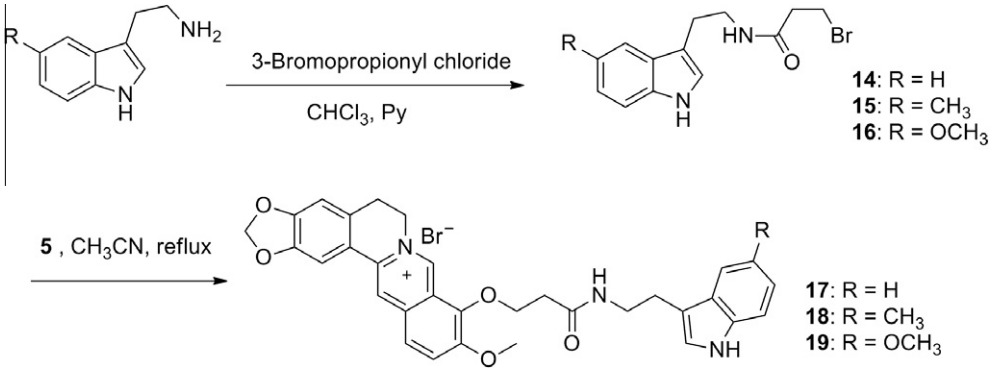
All of the berberine-benzenediol derivatives were potent inhibitors of BuChE with IC_{50} values of 1.04–2.72 μM, which was better than that of the leading compound berberine (Table 1, entry 1, 18.02 μM). As BuChE could also hydrolyze ACh, BuChE inhibition was also evaluated in the presence of ACh as the enzyme substrate. The results showed that most of these hybrids exhibited similar selectivity ratios of AChE/BuChE (Table 1), which indicated they could effectively inhibit both AChE and BuChE, and may be interesting as dual AChE/BuChE inhibitors for AD.

2.3. Kinetics of AChE inhibition

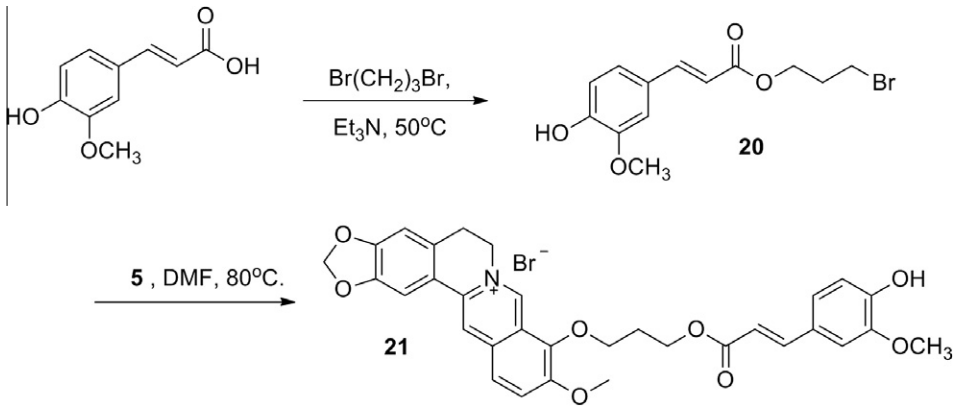
To investigate the kinetics of AChE inhibition, the most potent inhibitor, compound **8**, was chosen for further kinetic studies



Scheme 1. 9-Substituted berberine derivatives **7**–**13**. Reagents and conditions: (i) 190 °C, 20–30 mm Hg, 15 min; (ii) $Br(CH_2)_nBr$, DMF, 1–4 h; (iii) Benzenediol, DMF, −80 °C.



Scheme 2. Synthesis of the berberine-melatonin hybrids **17**–**19**.



Scheme 3. Synthesis of the berberine-ferulic acid hybrid **21**.

(Fig. 2.). The intersection point of Lineweaver–Burk reciprocal plot was in the third quadrant, which was considered as a mixed-type inhibition of uncompetitive inhibition and non-competitive inhibition.[29] Noncompetitive inhibition takes place when an inhibitor can bind to the enzyme at a site that is distinct from the active site. Uncompetitive inhibition, also known as anti-competitive inhibition, takes place when an enzyme inhibitor binds only to the complex formed between the enzyme and the substrate (the E–S complex). Both of them could only bind to the allosteric sites of enzyme. This phenomena may be explained as the hydroxyl group of phenol moiety in compound **8** hindered the penetration of inhibitor into the AChE gorge, which was consisted of hydrophobic amino acids, to reach the catalytic active site[33].

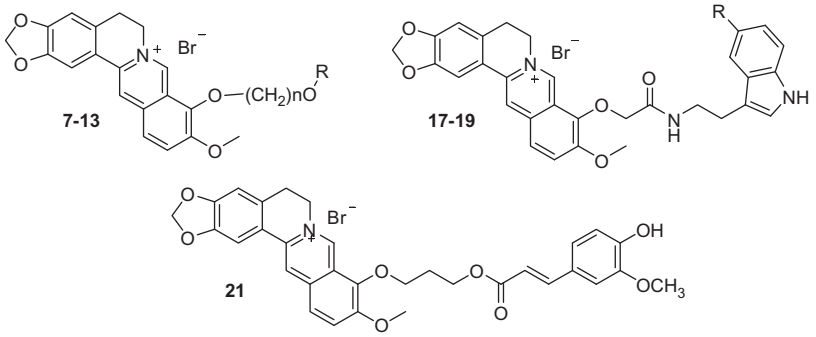
2.4. Antioxidant activity

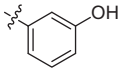
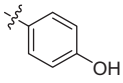
The antioxidant activity of the hybrids derived from berberine were determined using the oxygen radical absorbance capacity assay using fluorescein (ORAC-FL), according to the method originally described by Ou et al.[30] and modified by Daevalos[31] The vitamin E analog, Trolox, was used as a standard, and the antioxidant activity was expressed as Trolox equivalents (μmol of Trolox/μmol of tested compound). As shown in Table 1, berberine (**1**) and the berberine-phenol derivative (**2**) exhibited only mild antioxidant activity. All of the hybrids, including berberine-benzenediol (**7**–**13**), berberine-melatonin (**17**–**19**), and berberine-ferulic acid (**21**) demonstrated excellent antioxidant activity with ORAC-FL values of 3.1–9.53 Trolox equivalents. Moreover, most of the berberine-benzenediol derivatives (**9**–**13**) exhibited much better antioxidant activity than the berberine-melatonin hybrids or the berberine-ferulic acid hybrid, because of the existence of the phenolic hydroxyl. Compound **12**, which possessed a hydroxyl at the *para*-position on the benzene ring, was the strongest antioxidant (ORAC-FL value of 9.53). Comparing the ORAC-FL values of the berberine-melatonin hybrids (**17**–**19**) with that of melatonin (**3**), it is apparent that the berberine moiety does not significantly contribute to the radical scavenging ability of the hybrids. This is also evident when comparing the antioxidant activities of ferulic acid and the berberine-ferulic acid hybrid.

2.5. Inhibition of $A\beta_{1-42}$ aggregation

The ability of the novel berberine derivatives to inhibit $A\beta_{1-42}$ aggregation was assessed using the thioflavin T fluorescence assay, with a curcumin standard (Fig. 3). Significantly, all the berberine hybrids exhibited greater inhibitory activity than curcumin (52.1%), berberine-phenol hybrid **2** (57.4%), and berberine (36.3%) at a concentration of 20 μM. Among these, compound **12**, the hydroquinone-berberine hybrid, was the most potent inhibitor of Aβ aggregation (92.0%). Compound **12** also possessed the greatest antioxidant activity among the berberine derivatives (9.54 Trolox equivalents). The position of the phenolic hydroxyl on the benzene ring appears to influence the compound's ability to inhibit Aβ aggregation, paralleling its crucial role in the ability to inhibit AChE and BuChE. For example, compound **7**, with the phenolic hydroxyl at the ortho position, gave a lower inhibitory activity (83.5%) than the other isomers (compound **10**, resorcinol-berberine hybrid, 85.9%, and compound **12**, hydroquinone-berberine hybrid, 92%). Other hybrids, berberine-melatonin hybrids (**17**–**19**, 75.8–82.8%) and the berberine-ferulic acid hybrid (**21**, 75.8%) also exhibited

Table 1
In vitro inhibition of AChE and BuChE and Oxygen Radical Absorbance Capacity (ORAC, Trolox equivalents) by berberine (**1**), melatonin (**3**), ferulic acid (**4**), galanthamine and the berberine derivatives **2**, **7**–**13**, **17**–**19**, **21**



Compound	R	*n*	IC_{50}[a] (μM)		Selectivity for AChE[d]	IC_{50} (μM) BuChE/ACh[e]	Trolox equiv[f]
			AChE[b]	BuChE[c]			
1	–		0.374 ± 0.024	18.2 ± 0.683	48.6	n.t.[g]	0.4 ± 0.01
2	–		0.097 ± 0.005	4.89 ± 0.035	50.4	n.t.	1.0 ± 0.02
7		3	0.490 ± 0.032	1.74 ± 0.028	3.55	1.30 ± 0.021	3.41 ± 0.25
8		4	0.123 ± 0.003	2.09 ± 0.14	17.0	1.74 ± 0.007	3.54 ± 0.27
9		2	0.422 ± 0.034	2.64 ± 0.063	6.26	2.26 ± 0.007	4.63 ± 0.01
10		3	0.628 ± 0.008	1.13 ± 0.014	1.80	0.898 ± 0.071	5.31 ± 0.33
11		4	0.547 ± 0.002	2.40 ± 0.021	4.39	1.92 ± 0.059	5.30 ± 0.41
12		3	0.460 ± 0.013	2.06 ± 0.035	4.48	1.66 ± 0.106	9.54 ± 0.62
13		4	0.304 ± 0.005	1.04 ± 0.077	3.42	0.95 ± 0.098	6.45 ± 0.39
17	H	–	1.11 ± 0.021	1.64 ± 0.206	7.96	1.46 ± 0.015	3.78 ± 0.01
18	CH_3	–	1.42 ± 0.042	1.96 ± 0.014	1.39	1.79 ± 0.212	3.11 ± 0.21
19	OCH_3	–	1.44 ± 0.054	2.72 ± 0.035	1.88	2.49 ± 0.024	3.61 ± 0.30
21		–	3.21 ± 0.155	2.40 ± 0.042	0.75	2.25 ± 0.113	3.43 ± 0.11
3			n.t.	n.t.	–	n.t.	2.3 ± 0.1[h]
4		–	>100	n.t.	–	n.t.	3.13 ± 0.12
Curcumin		–	>100	n.t.	–	n.t.	n.t.
Galanthamine		–	0.623 ± 0.099	15.7 ± 0.787	25.3	n.t.	n.t.

[a] Mean ± SD of at least three independent measurements.
[b] Acetylcholine substrate for evaluation of antiacetylcholinesterase activity. AChE from electric eel.
[c] Butyrylcholine substrate for evaluation of antibutyrylcholinesterase activity. BuChE from equine serum.
[d] Selectivity for AChE = IC_{50} (BuChE)/IC_{50} (AChE).
[e] Acetylcholine substrate for evaluation of antibutyrylcholinesterase activity.
[f] Data are expressed as μ mol of trolox equivalent/μ mol of tested compound and are the mean (n = 3) ±SD.
[g] n.t. = not tested.
[h] Data from Ref. [28]

better inhibitory activity than that of curcumin, berberine and compound **2** which without the hydroxyl group on the phenyl ring.

3. Conclusion

In summary, we designed and synthesized a series of novel berberine conjugates, including berberine-benzenediol derivatives (**7–13**), berberine-melatonin hybrids (**17–19**), and a berberine-ferulic acid hybrid (**21**). We evaluated their potential as multifunctional drug candidates for AD. All of these compounds exhibited antioxidant activity and inhibited Aβ aggregation better than unconjugated berberine, although some of them had a reduced ability to inhibit AChE. The hydroquinone-berberine hybrid, compound **12**, had the greatest ability to suppress Aβ aggregation. It was also an excellent antioxidant and a reasonable AChE and BuChE inhibitor. Compound **8** was a potent AChE inhibitor and a strong antioxidant. It also inhibited Aβ aggregation. A study of enzyme kinetics revealed that the inhibition of **8** was a mixed-type inhibition of uncompetitive inhibition and non-competitive inhibition. Combining antioxidant activity with the ability to inhibit ChEs and impede Aβ aggregation, these hybrid compounds are excellent multifunctional drug candidates for AD. Further investigation into these potential therapeutic agents is in progress.

4. Material and methods

4.1. Chemistry

The ^{1}H NMR and ^{13}C NMR spectra were recorded using TMS as the internal standard on a Bruker BioSpin GmbH spectrometer at 400.132 and 100.614 MHz, respectively. Coupling constants are given in Hz. High-resolution mass spectra were obtained using a Shimadzu LCMS-IT-TOF mass spectrometer. Flash column chromatography was performed using 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 using silica gel. Berberine chloride was isolated from Chinese herbal medicine *C. chinensis* Franch and recrystallized from

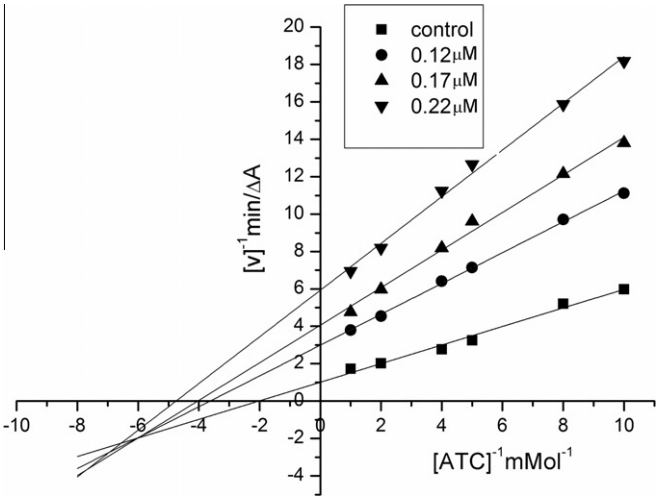


Figure 2. Steady state inhibition by compound **8** of AChE hydrolysis of ACh; the plots show mixed type inhibition for compound **8** on AChE.

hot water. Compound **5** and intermediates **6a**, **6b** and **6c** were synthesized as previously reported.[26]

4.2. General procedure for the preparation of 7–13

To a solution of compounds **6a**, **6b** or **6c** (1 mmol) in DMF (15 ml), benzenediol (2 mmol) was added at room temperature. After stirring at 80 °C for 4 h, the mixture was cooled to room temperature and evaporated under vacuum. The crude product was chromatographed on an Al_2O_3 column and eluted with $CHCl_3$/MeOH (100:1–50:1) to obtain purified compound.

4.2.1. 9-*O*-[3-(Phenylol-2-yloxy) propyl] berberine bromide (7)

Compound **6b** was treated with 1,2-benzenediol according to a general protocol to obtain compound **7** as a yellow solid (37% yield). mp 213.1–215.7 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.77 (s, 1H), 8.94 (s, 1H), 8.88 (s, 1H), 8.19 (d, J = 9.1 Hz, 1H), 8.00 (d, J = 9.0 Hz, 1H), 7.80 (s, 1H), 7.09 (s, 1H), 6.98 (d, J = 7.2 Hz, 1H), 6.78 (d, J = 15.1 Hz, 3H), 6.18 (s, 2H), 4.92–4.79 (m, 2H), 4.51 (t, J = 6.2 Hz, 2H), 4.25 (t, J = 6.1 Hz, 2H), 4.02 (s, 3H), 3.18 (s, 2H), 2.38–2.23 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 150.37, 149.84, 147.69, 146.91, 146.77, 145.20, 142.82, 137.46, 133.06, 130.61, 126.79, 123.36, 121.59, 121.24, 120.39, 120.22, 119.23, 115.74, 114.11, 108.38, 105.41, 102.05, 71.45, 65.28, 57.01, 55.29, 29.52, 26.33.HRMS m/z $[M-Br]^+$ Calcd for $C_{28}H_{25}NO_6$ 472.1760, found 472.1781.

4.2.2. 9-*O*-[4-(Phenylol-2-yloxy)butyl] berberine bromide (8)

Compound **6c** was treated with 1,2-benzenediol according to a general protocol to obtain compound **8** as a yellow solid (31% yield). mp 197.2–198.9 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.70 (s, 1H), 8.70 (s, 1H), 8.12 (d, J = 9.1 Hz, 1H), 8.02 (s, 1H), 7.68 (s, 1H), 6.98 (s, 1H), 6.93 (dd, J = 10.6, 6.8 Hz, 1H), 6.77 (t, J = 2.8 Hz, 2H), 6.13 (s, 2H), 4.90 (d, J = 6.6 Hz, 2H), 4.55 (s, 2H), 4.20 (d, J = 5.9 Hz, 2H), 4.11 (s, 3H), 3.24 (s, 2H), 2.14 (dd, J = 11.3, 6.0 Hz, 4H). ^{13}C NMR (101 MHz, MeOD) δ 161.50, 159.24, 155.67, 150.61, 147.82, 145.90, 144.98, 144.42, 135.45, 130.6, 126.9, 125.20, 124.93, 123.29, 120.70, 120.23, 118.8, 112.6, 110.5, 106.8, 103.9, 102.1, 71.62, 65.37, 56.93, 53.13, 30.175, 28.46, 26.38. HRMS m/z $[M-Br]^+$ Calcd for $C_{29}H_{27}NO_6$ 486.1917, found 486.1899.

4.2.3. 9-*O*-[2-(Phenylol-3-yloxy)ethyl] berberine bromide (9)

Compound **6a** was treated with 1,3-benzenediol according to general protocol to obtain compound **9** as a yellow solid (33% yield). mp 217.4–218.8 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.63 (s, 1H), 8.68 (s, 1H), 8.09 (dd, J = 41.2, 9.1 Hz, 2H), 7.60 (d, J = 27.5 Hz, 1H), 7.05 (t, J = 8.2 Hz, 1H), 6.94 (s, 1H), 6.38 (dd, J = 8.0, 1.9 Hz, 1H), 6.29 (dd, J = 8.1, 2.1 Hz, 1H), 6.18 (d, J = 2.1 Hz, 1H), 6.12 (s, 2H), 4.81–4.73 (m, 2H), 4.72–4.61 (m, 2H), 4.40–4.27 (m, 2H), 4.14 (s, 3H), 3.19–3.05 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 160.93, 159.95, 152.17, 152.13, 149.90, 146.31, 144.53, 139.48, 135.02, 131.71, 131.27, 127.73, 124.92, 123.94, 121.73, 121.51, 109.39, 109.34, 106.54, 106.24, 103.68, 102.88, 73.82, 68.29, 57.60, 57.32, 28.13. HRMS m/z $[M-Br]^+$ Calcd for $C_{27}H_{23}NO_6$ 458.1604, found 458.1624.

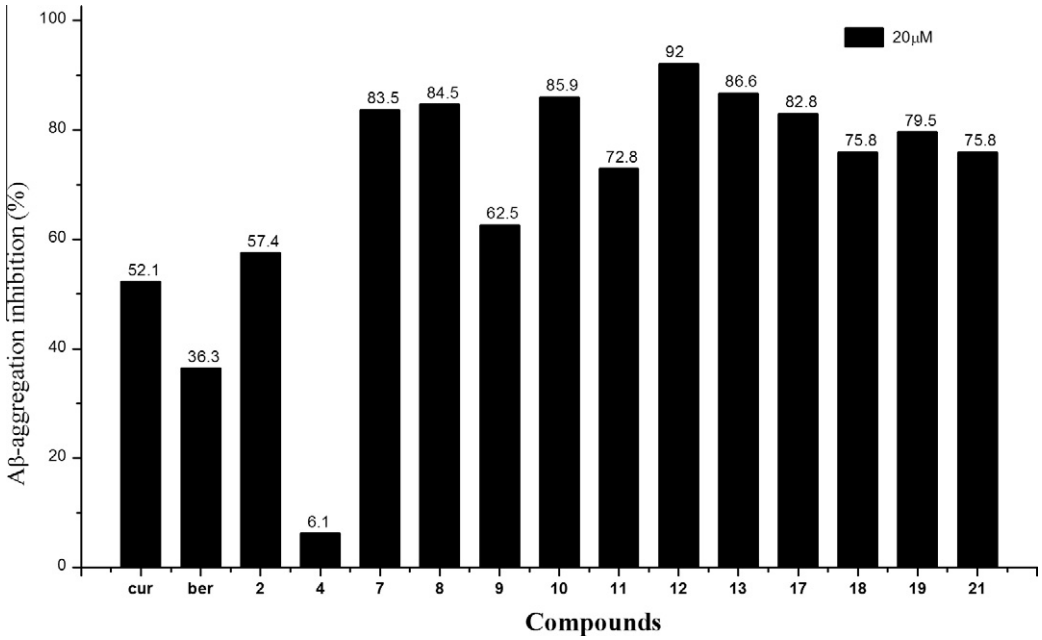


Figure 3. Effects on the $A\beta_{1-42}$ peptide aggregation inhibition at 20 μM of berberine derivatives.

4.2.4. 9-*O*-[3-(Phenylol-3-yloxy)propyl] berberine bromide (10)

Compound **6b** was treated with 1,3-benzenediol according to general protocol to obtain compound **10** as a yellow solid (40% yield). mp 211.5–214.0 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.43 (s, 1H), 8.60 (s, 1H), 8.03 (d, J = 9.1 Hz, 1H), 7.93 (d, J = 9.1 Hz, 1H), 7.56 (s, 1H), 7.02 (t, J = 8.1 Hz, 1H), 6.87 (s, 1H), 6.42–6.28 (m, 3H), 6.04 (s, 2H), 4.53 (dd, J = 12.6, 6.6 Hz, 4H), 4.25 (s, 2H), 3.99 (s, 3H), 3.12–3.03 (m, 2H), 2.30 (p, J = 5.9 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 159.43, 158.74, 150.72, 150.20, 147.28, 146.59, 143.12, 139.90, 134.04, 133.16, 132.01, 126.17, 124.95, 124.05, 122.35, 122.07, 109.21, 107.34, 105.94, 104.25, 102.67, 101.03, 72.53, 69.29, 58.47, 56.88, 29.91, 26.73. HRMS m/z [M−Br]$^+$ Calcd for $C_{28}H_{25}NO_6$ 472.1760, found 472.1779.

4.2.5. 9-*O*-[4-(Phenylol-3-yloxy)butyl] berberine bromide (11)

Compound **6c** was treated with 1,3-benzenediol according to general protocol to obtain compound **11** as a yellow solid (42% yield). mp 205.1–207.3 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.53 (s, 1H), 8.56 (s, 1H), 7.98 (d, J = 9.1 Hz, 1H), 7.88 (s, 1H), 7.53 (s, 1H), 6.83 (s, 1H), 6.62 (d, J = 9.1 Hz, 2H), 6.56 (d, J = 9.1 Hz, 2H), 5.99 (s, 2H), 4.79–4.74 (m,2H), 4.39 (s, 2H), 3.97 (s, 3H), 3.93 (t, J = 6.0 Hz, 2H), 3.14–3.08 (m, 2H), 2.03–1.95 (m, 2H), 1.91 (dd, J = 7.5, 5.8 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 160.83, 156,57, 151.92, 148.73, 147.17, 146.50, 146.45, 144.73, 141.28, 131.64, 130.88, 130.75, 123.84, 120.64, 111.75, 116.79, 110.06, 106.87, 106.72, 103.56, 101.25, 101.06, 72.37, 69.46, 57.70, 56.04, 29.11, 27.79, 27.65 HRMS m/z [M−Br]$^+$ Calcd for $C_{29}H_{27}NO_6$ 486.1917, found 486.1928.

4.2.6. 9-*O*-[3-(Phenylol-4-yloxy)propyl] berberine bromide (12)

Compound **6b** was treated with 1,4-benzenediol according to a general protocol to obtain compound **12** as a yellow solid (55% yield). mp 218.5–220.8 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.52 (s, 1H), 8.68 (s, 1H), 8.10 (d, J = 9.0 Hz, 1H), 8.00 (d, J = 9.0 Hz, 1H), 7.63 (s, 1H), 6.94 (s, 1H), 6.81 (d, J = 8.9 Hz, 2H), 6.73 (d, J = 8.9 Hz, 2H), 6.61 (s, 1H), 6.11 (s, 2H), 4.60 (dt, J = 11.6, 5.9 Hz, 4H), 4.26 (t, J = 5.7 Hz, 2H), 4.06 (s, 3H), 3.19–3.11 (m, 2H), 2.41–2.30 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 160.27, 158.30, 151.27, 151.03, 147.94, 144.08, 143.37, 139.55, 134.52, 130.73, 130.26, 126.50, 123.71, 122.67, 120.55, 120.36, 108.39, 108.27, 105.68, 105.50, 101.90, 101.07, 73.10, 64.95, 57.47, 56.75, 29.14, 28.08. HRMS m/z [M−Br]$^+$ Calcd for $C_{28}H_{25}NO_6$ 472.1760, found 472.1751.

4.2.7. 9-*O*-[4-(Phenylol-4-yloxy)butyl] berberine bromide (13)

Compound **6c** was treated with 1,4-benzenediol according to general protocol to obtain compound **13** as a yellow solid (55% yield). mp 200.3–202.1 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.40 (s, 1H), 8.57 (s, 1H), 8.00 (d, J = 9.1 Hz, 1H), 7.90 (d, J = 9.1 Hz, 1H), 7.53 (s, 1H), 6.99 (t, J = 8.1 Hz, 1H), 6.84 (s, 1H), 6.38–6.27 (m, 3H), 6.01 (s, 2H), 4.50 (dd, J = 13.3, 7.2 Hz, 5H), 4.20 (t, J = 5.8 Hz, 2H), 3.97 (s, 3H), 3.11–3.01 (m, 2H), 2.28 (p, J = 5.9 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 161.68, 159.96, 152.31, 152.18, 149.91, 145.92, 144.68, 139.60, 135.18, 131.80, 131.20, 127.86, 124.69, 123.55, 121.79, 121.61, 109.35, 109.16, 106.71, 106.53, 103.68, 103.00, 72.30, 65.25, 57.58, 57.18, 30.89, 28.22.HRMS m/z [M−Br]$^+$ Calcd for $C_{29}H_{27}NO_6$ 486.1917, found 486.1932.

4.3. General protocol for the preparation of compounds 14–16

To a solution of substituted tryptamine (10 mmol) in chloroform (re-distilled, 50 ml), β-bromopropionyl chloride (11 mmol) was slowly added at 0 °C, followed by pyridine (11 mmol). After stirring 1 h at room temperature, the reaction mixture was washed with $NaHCO_3$ solution (2 × 50 ml), water (2 × 50 ml), and brine (1 × 50 ml), consecutively, then dried with anhydrous Na_2SO_4 and concentrated under vacuum to generate compounds **14–16**, which were used for the next step without purification.

4.4. General protocol for the preparation of 17–19

Compounds **14–16** (2.4 mmol) were added to a magnetically-stirred suspension of berberrubine (**5**; 2 mmol) in CH_3CN (15 ml) and the mixture was stirred in a reflux apparatus for 12–24 h. The mixture was cooled to room temperature, filtered, and then evaporated under vacuum. The crude product was chromatographed on an Al_2O_3 column and eluted with $CHCl_3$/MeOH (100:1–50:1) to obtain the purified compound.

4.4.1. 9-*O*-[(3-Oxo-tryptamino)propyl]-berberine bromide (17)

Berberrubine was treated with compound **14** according to a general protocol to obtain compound **17** as a yellow solid (27% yield). mp 156.4–158.7 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.44 (s, 1H), 8.62 (s, 1H), 8.09 (s, 1H), 8.02 (s, 1H), 7.63 (s, 1H), 7.55 (dt, J = 6.7, 2.7 Hz, 1H), 7.08 (d, J = 1.8 Hz, 2H), 6.98–6.87 (m, 3H), 6.15 (s, 2H), 4.64–4.54 (m, 4H), 4.12 (s, 3H), 3.64 (t, J = 6.8 Hz, 2H), 3.18–3.09 (m, 2H), 2.99 (t, J = 6.8 Hz, 2H), 2.81 (t, J = 5.6 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 169.55, 160.75, 158.57, 152.44, 149.35, 146.73, 146.11, 141.56, 136.44, 132.59, 131.56, 127.30, 124.01, 122.27, 122.18, 121.46, 119.56, 118.69, 117.09, 112.80, 111.30, 109.22, 104.22, 100.87,100.57, 73.26, 59.80, 57.94, 57.20, 39.91, 39.76, 27.53. HRMS m/z [M−Br]$^+$ Calcd for $C_{32}H_{29}N_3O_5$ 536.2185, found 536.2171.

4.4.2. 9-*O*-[(3-Oxo-5-methyltryptamino)propyl]-berberine bromide (18)

Berberrubine was treated with compound **15** according to a general protocol to obtain compound **18** as a yellow solid (23% yield). mp 150.9–153.2 °C; ^{1}H NMR (400 MHz, MeOD) δ 9.33 (s, 1H), 8.50 (s, 1H), 8.02 (s, 1H), 7.95 (s, 1H), 7.54 (s, 1H), 7.27 (s, 1H), 6.99 (s, 1H), 6.96–6.83 (m, 2H), 6.73–6.63 (m, 1H), 6.12 (s, 2H), 4.61–4.47 (m, 4H), 4.09 (s, 3H), 3.60 (t, J = 6.7 Hz, 2H), 3.08 (s, 2H), 2.92 (t, J = 6.6 Hz, 2H), 2.80 (t, J = 5.3 Hz, 2H), 2.26 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 168.99, 161.27, 157.24, 150.89, 150.03, 147.13, 146.56, 139.34, 135.72, 133.87, 130.15, 128.37, 123.93, 122.35, 121.98, 121.69, 120.27, 119.36, 118.36, 113.57, 110.97, 109.72, 106.48, 103.81,101.04, 72.45, 59.07, 56.98, 40.93, 39.88, 27.52, 25.28, 22.41. HRMS m/z [M−Br]$^+$ Calcd for $C_{33}H_{31}N_3O_5$ 550.2342, found 550.2338.

4.4.3. 9-*O*-[(3-Oxo-5-methoxytryptamino)propyl]-berberine bromide (19)

Berberrubine was treated with compound **16** according to general protocol to obtain compound **19** as a yellow solid (33% yield). mp 189.1–191.3 °C; ^{1}H NMR (400 MHz, DMSO) δ 10.60 (s, 1H), 9.80 (s, 1H), 8.91 (s, 1H), 8.29 (t, J = 5.7 Hz, 1H), 8.19 (d, J = 9.2 Hz, 1H), 8.00 (d, J = 9.1 Hz, 1H), 7.78 (s, 1H), 7.24 (d, J = 7.4 Hz, 1H), 7.16 (t, J = 8.5 Hz, 2H), 7.12–7.06 (m, 2H), 6.99 (d, J = 2.4 Hz, 1H), 6.66 (dd, J = 8.7, 2.4 Hz, 1H), 6.18 (s, 2H), 4.90–4.78 (m, 2H), 4.48 (t, J = 6.0 Hz, 2H), 4.06 (s, 3H), 3.72 (s, 3H), 3.25–3.13 (m, 3H), 2.78 (t, J = 7.3 Hz, 2H), 2.71 (t, J = 6.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 165.53, 154.06, 151.17, 148.33, 147.15, 146.99, 146.30, 145.74, 141.48, 131.60, 131.72, 131.63, 127.55, 123.21, 123.0 121.54, 117.82, 116.20, 112.44, 112.19, 112.00, 109.47, 103.59, 101.26, 100.45, 71.59, 59.23, 57.83, 55.98, 40.32, 38.87, 29.30, 28.59. HRMS m/z [M−Br]$^+$ Calcd for $C_{33}H_{31}N_3O_6$ 566.2291, found 566.2319.

4.4.4. (*E*)-3-Bromopropyl 3-(4-hydroxy-3-methoxyphenyl) acrylate (20)

1,3-Dibromopropane (4.0 mmol) was added to a magnetically-stirred suspension of ferulic acid (2 mmol) in acetone (30 ml) in

the presence of Et_3N and the mixture was stirred in a reflux apparatus for 4 h. Then the mixture was cooled to room temperature and evaporated under vacuum. The crude product was chromatographed on a silica gel column to obtain compound **20** as an oil (82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, J = 15.9 Hz, 1H), 7.08 (dd, J = 8.2, 1.9 Hz, 1H), 7.03 (d, J = 1.8 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.28 (d, J = 15.9 Hz, 1H), 5.90 (s, 1H), 4.34 (t, J = 6.0 Hz, 2H), 3.93 (s, 3H), 3.52 (t, J = 6.6 Hz, 2H), 2.33–2.18 (m, 2H).

4.4.5. 9-*O*-[3-(Ferulic acid)propyl] berberine bromide (21)

Compound **20** (2.4 mmol) was added to a magnetically-stirred suspension of berberrubine (**5**; 2 mmol) in CH_3CN (15 ml) and the mixture was stirred in a reflux apparatus for 12 h. The mixture was cooled to room temperature, filtered, and then evaporated under vacuum. The crude product was chromatographed on an Al_2O_3 column and eluted with $CHCl_3$/MeOH (100:1–50:1) to generate compound **21** as a yellow solid (43% yield). mp 202.4–203.7 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.82 (s, 1H), 8.93 (s, 1H), 8.20 (d, J = 9.2 Hz, 1H), 8.01 (d, J = 9.1 Hz, 1H), 7.78 (s, 1H), 7.56 (d, J = 15.9 Hz, 1H), 7.30 (d, J = 1.5 Hz, 1H), 7.09 (s, 2H), 6.81 (d, J = 8.1 Hz, 1H), 6.48 (d, J = 15.9 Hz, 1H), 6.18 (s, 2H), 5.02–4.89 (m, 2H), 4.43 (dd, J = 11.5, 6.0 Hz, 4H), 4.05 (s, 3H), 3.81 (s, 3H), 3.21 (s, 2H), 2.27 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 166.71, 150.27, 149.83, 149.27, 147.87, 147.68, 145.25, 145.13, 142.63, 137.50, 133.05, 130.63, 126.74, 125.49, 123.44, 123.05, 121.56, 120.39, 120.19, 115.45, 114.29, 111.33, 108.37, 105.40, 102.04, 71.08, 60.76, 57.07, 55.71, 55.30, 29.13, 26.32. HRMS m/z $[M-Br]^+$ Calcd for $C_{32}H_{29}NO_8$ 556.1971, found 556.1977.

4.5. Biological activity

4.5.1. In vitro inhibition of AChE and BuChE

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from the electric eel), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), acetylthiocholine chloride (ATC), and butylthiocholine chloride (BTC) 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 the final concentration.

All the in vitro AChE assays were carried out in 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 preincubated at 37 °C with the enzyme for 15 min, followed by the addition of substrate. 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 Ellman et al.[27] Each concentration was assayed in triplicate.

The in vitro BuChE assay (BuChE or ACh as the enzyme substrate) was performed according to a method similar to that described above.

4.5.2. Kinetic characterization of AChE inhibition

The kinetic characterization of AChE inhibitory activity was performed according to a published protocol.[32] Briefly, test compound was added to the assay solution and incubated with the enzyme at 37 °C for 15 min, then substrate was added. Kinetic characterization of the hydrolysis of ATC catalyzed by AChE was performed spectrophotometrically at 412 nm. A parallel control, with no inhibitor in the mixture, allowed activities to be measured at various times. The plots were analyzed using 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.

4.5.3. Inhibition of $A\beta_{1-42}$ peptide aggregation

HFIP pretreated $A\beta_{1-42}$ samples (AnaSpec) were resolubilized with a 50 mM phosphate buffer (pH 7.4) in order to have a stable stock solution ([Aβ] = 200 μM). The peptide was incubated in 50 mM phosphate buffer (pH 7.4) at 37 °C for 48 h (final Aβ concentration 50 μM) with and without the tested compound at 20 μM. After incubation, the samples were diluted to a final volume of 200 μL with 50 mM glycine-NaOH buffer (pH 8.0) containing thioflavin T. Then, a 300-seconds-time scan of fluorescence intensity was performed (λ_{exc} = 450 nm; λ_{em} = 485 nm), and values at plateau were averaged after subtracting the background fluorescence of thioflavin T solution.

4.5.4. The antioxidant activity assay

The antioxidant activity was determined by the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay.[30,31,34] All the assays were under 75 mM phosphate buffer (pH 7.4) and the final reaction mixture was 200 μL. Antioxidant (20 μL) and fluorescein (120 μL, 300 nM final concentration) were placed in the wells of a black 96-well plate and the mixture was incubated for 10 min at 37 °C. Then, AAPH (Aldrich) solution (60 μL; 12 mM final concentration) was added rapidly. The plate was immediately placed into a Spectrafluor Plus plate reader (Tecan, Crailsheim, Germany) and the fluorescence was measured every 60 s for 4 h with exitation at 485 nm and emission at 535 nm. Trolox was used as standard (1–10 μM, final concentration). A blank (FL+AAPH) using phosphate buffer instead of antioxidant and Trolox calibration were carried out in each assay. The samples were measured at different concentrations (0.5–10 μM). All reaction mixtures were prepared fourfold and at least four independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (without antioxidant). The ORAC-FL values were calculated as described in the reference[34] and the final results were in μM of Trolox equivalent/μM of pure compound.

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