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Synthesis and anticancer activity of a novel series of 9-*O*-substituted berberine derivatives: A lipophilic substitute role

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ABSTRACT

To alter its hydrophobicity, a series of compounds bearing 9-*O*-alkyl- or 9-*O*-terpenyl- substituted berberine were synthesized and evaluated for anticancer activity against human cancer HepG2 and HT29 cell lines. We found that the lipophilic substitute of 9-*O*-alkyl- and 9-*O*-terpenyl berberine derivatives plays a role in inhibiting the human cancer cell growth and its activity could be maximized with the optimized substitute type and chain length. Most strikingly, nonetheless, of the six compounds prepared, sample **8**, a farnesyl 9-*O*-substituted berberine, showed either comparable or better cytotoxic activity against human cancer HepG2 cell line than that of berberine. Compound **8** had also shown a 104-fold antiproliferation activity in compare with berberine against human hepatoma HepG2 cell lines after 48 incubation hours. Further, in Hoechst 33258 and annexin V-FITC/PI staining analyses it induced apoptosis in HepG2 cells at lower concentration than that of berberine for 24 h. Take all; farnesyl 9-*O*-substituted berberine could be a potential candidate for new anticancer drug development.

Millions lives lost to cancer in the world each year and the number is increasing. Through, greater than 30% of cancer is considered preventable[1] and some forms of cancer are curable no matter when they are diagnosed; many others are only curable if they are caught at an early stage. Thus cancer remains a leading life threaten disease and serious peril to humanity health.[2]

Human hepatocellular carcinoma (HCC) and colorectal carcinoma are most common types of malignancy in sub-Saharan Africa and Southeast Asia like Taiwan and China. It is also the fifth most common and lethal cancer worldwide and usually treated by surgically removal followed by chemotherapy.[3,4] We have devoted our research effort in synthesizing and testing high efficient, broad-spectrum and low toxic anticarcinogen compounds for potential new drugs candidates for HCC treatment.

Berberine is a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. It is found in such plants as *Berberis*, *Berberis aristata*, *Hydrastis canadensis*, *Phellodendron amurense*, *Coptis chinensis, Tinospora cordifolia*, and to a smaller extent in *Argemone mexicana* and *Eschscholzia californica*.[5] It has a wide range of biochemical and pharmacological effects.[6–8] In China and India, berberine was widely in use as an anticancer drug to treat hepatoma,[9] breast cancer,[10] bladder cancer,[11] and colon cancer.[12] In China, especially, this natural alkaloid has been used in the traditional medicine.[13,14] Furthermore, it was reported that berberine possesses significant cytotoxicity against human cancer cell lines, HepG2 and HT-29. It caused a significant reduction of the S phase fraction of HepG2 cells[15] and an arrest of gastric cancer cell in the G2/M phase.[16] Berberine and its derivatives had also been evaluated as inhibitor of topoisomerase I and II and demonstrated with anticancer activity.[17–19] In addition, a recent study reported some effectiveness of 9-*O*-substituted berberine derivatives selectively inhibit acetylcholinesterase (AChE) activity.[20]

However, for its hydrophilic in nature, berberine is absorbed poorly in intestines and thus showed a low inhibiting effect on suppressing cancer cell growth.[21] To enhance its intestine absorption and inhibitory function, we modified berberine analogues and screened its cytotoxicity in vitro on human liver HepG2 and colon HT29 cancer cell lines. Two types of functional groups, terpenyl and alkyl derivatives were investigated for the structure–activity relationship (SAR).

In specific, we synthesized and tested compounds of 9-*O*-alkylberberine derivatives with increased alkyl chain length (butyl, octyl, and dodecyl, samples **3**, **4** & **5**) and terpenyl (isoprenyl,

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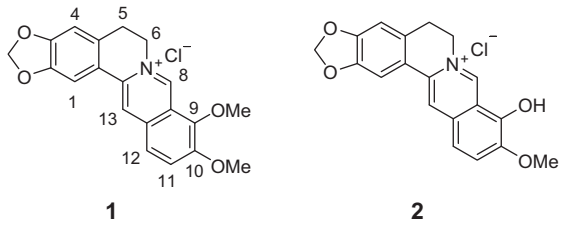
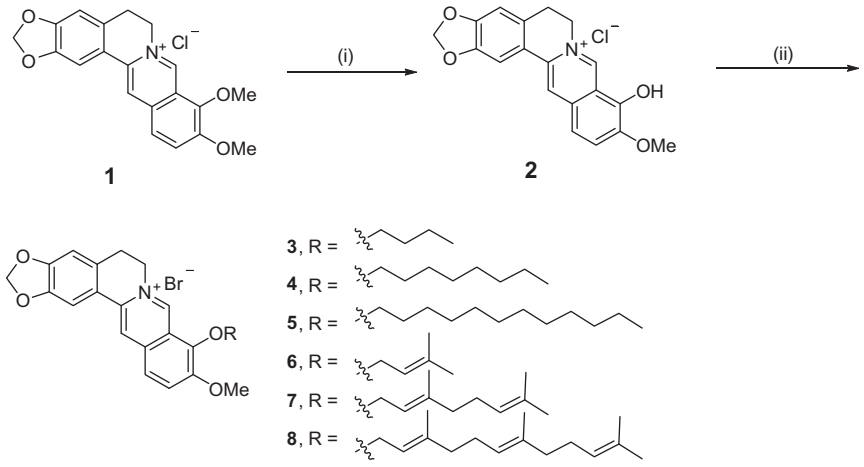


Figure 1. Chemical structures of berberine (**1**) and berberrubine (**2**).

geranyl, and farnesyl, samples **6**, **7** & **8**) substitutes. The effectiveness of anticancer activity of these compounds was carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[22] Data on berberine (sample **1**) and its more hydrophilic derivative, berberrubine (sample **2**, Fig. 1), were used as baseline for evaluating the performance of the synthetic compounds (**3**–**8**). Berberrubine is an isoquinoline alkaloid isolated from the plant *Berberis vulgaris* L.[23] and readily isolated from berberine by pyrolysis.[24] The effects of some potent compounds on human hepatoma HepG2 cells apoptosis were further evaluated.

The synthetic route of lipophilic 9-*O*-substituted berberine derivatives **3**–**8** are outlined in Scheme 1. Berberine chloride (**1**, 5.0 g) was heated at 190 °C under vacuum (20–30 mmHg) for 1–2 h underwent selective demethylation to afford berberrubine (**2**, 3.8 g) in 79% yield.[25] To a solution of berberrubine (**2**, 1 mmol) in dry CH_3CN (10 mL) was added alkylated or terpenylated bromide (1.2 mmol) and the reaction mixture was refluxed for 4–8 h, and the progress of the reaction was monitored by TLC. Then, the reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure. The crude product was chromatographed on a neutral Al_2O_3 column and eluted with $CHCl_3$/MeOH (99/1) solvent to afford the desired berberine derivatives **3**–**8** in quantitative yields (Scheme 1). The structures of berberine derivatives **3**–**8** were determined by NMR and LC–MS experiments.[26] Full signal assignment of ^1H and ^{13}C was carried out with NMR techniques including DEPT, COSY, HSQC, and HMBC analyses. All compounds were purified by flash column chromatography and were thoroughly characterized by ^1H and ^{13}C NMR spectroscopy and LC–MS spectroscopy.

The anticancer activity of the compounds studied herein was rated in half maximal inhibitory concentration (IC_{50}), which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. The data of synthesized berberine derivatives (**3**–**8**), in compare with those of berberine (**1**) and berberrubine (**2**), against two tumor cells HepG2 and HT-29 are summarized in Table 1, with cisplatin data listed as a positive control. First of all, we found that the activities of the 9-*O*-alkyl- and 9-*O*-terpenyl berberine derivatives against human cancer HepG2 and HT-29 cell lines increased with the chain length of the substitute at the C-9 position. In other words, the change in lipophilicity of the



Scheme 1. Reagents and conditions: (i) 20–30 mmHg, 190 °C, 1–2 h; (ii) K_2CO_3, alkyl or terpenyl bromide, acetonitrile, reflux, 4–8 h.

Table 1
Lipophilicity and IC_{50} values for the in vitro screening of berberine **1**, berberrubine **2**, berberine derivatives (**3**–**8**) and cisplatin against human cancer cell lines for 24 h and 48 h

Compound	Clog *P*[a]	HepG2 IC_{50}[b] (μM)		HT-29 IC_{50}[b] (μM)	
		24 h	48 h	24 h	48 h
Berberine (**1**)	−0.771	11.22 ± 1.77	8.32 ± 2.11	>20	8.45 ± 0.35
Berberrubine (**2**)	−0.707	>50	>50	>50	36.93 ± 9.60
3	0.816	1.81 ± 0.70	0.97 ± 0.21	4.41 ± 0.30	0.87 ± 0.11
4	2.932	0.19 ± 0.01	0.10 ± 0.05	0.48 ± 0.06	0.08 ± 0.02
5	5.048	0.26 ± 0.10	0.10 ± 0.03	0.93 ± 0.07	0.28 ± 0.04
6	0.931	4.67 ± 0.54	3.17 ± 0.49	10.76 ± 4.71	1.75 ± 0.63
7	2.961	0.21 ± 0.10	0.17 ± 0.02	1.21 ± 1.14	0.14 ± 0.07
8	4.993	0.21 ± 0.07	0.08 ± 0.02	1.82 ± 0.26	0.27 ± 0.07
Cisplatin[c]	−1.684	82.12 ± 2.31	36.00 ± 3.10	53.28 ± 2.64	24.10 ± 0.10

[a] Calculated log value of partition coefficient by ChemDraw Ultra 8.0.
[b] IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%. The values are means ± SD of three experiments conducted in triplicate.
[c] Positive control.

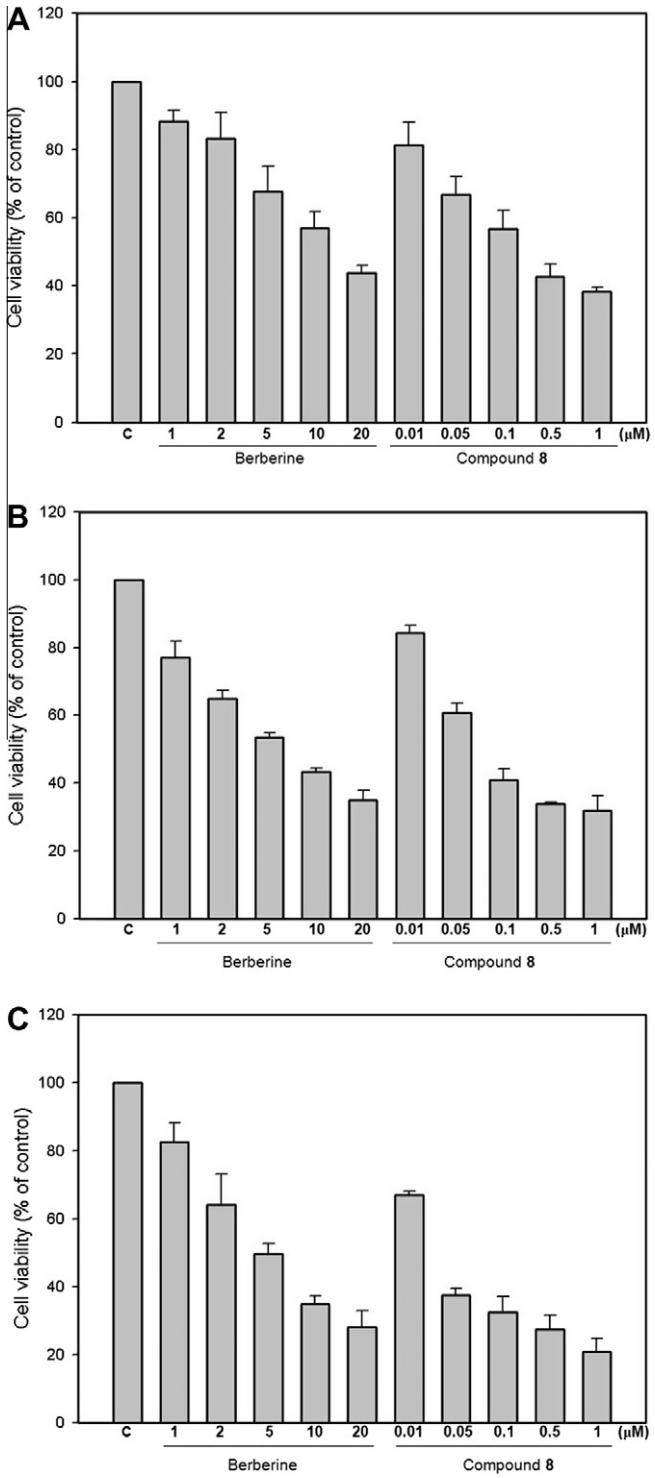


Figure 2. Cytotoxic effects of berberine (**1**) and compound **8** on human hepatoma HepG2 cell lines. HepG2 cells were treated with various concentrations of berberine (**1**) and compound **8** for (**A**) 24 h, (**B**) 48 h and (**C**) 72 h. After treatment, cell viability was estimated by MTT assay. Data are presented as means ± SD of at least three replicates from three independent experiments. c: control.

protoberberinium salts affects the anticancer activity of these derivatives. However, longer chains than octyl alkyl homologs decreased the anticancer activity as it evidenced by the moderate activities of 9-*O*-dodecylberberine bromides (**5**). Secondly, 9-*O*-terpenyl berberine derivatives were in general more active than 9-*O*-alkyl berberine derivatives and their parent molecules, berberine (**1**) and berberrubine (**2**). Most strikingly, yet, we noticed compound **8** showed a significant higher cytotoxicity against both cancer cell lines, especially HepG2 with an IC_{50} value of 0.08 μM against IC_{50} of 8.32 μM for berberine. In addition, we did not find significant cell death in normal cell lines BNL CL.2 (murine embryonic liver cells) after compounds **5** and **8** treatment, however a marked effect on cell death (under 20%) were observed only at the maximum concentration of these compounds (10 μM) after 48 h treatment (data not shown). Thus, compounds **5** and **8** appear to be capable of inducing cytotoxic effects on human liver cancer cells and human colon cancer cells without incurring significant cytotoxic effect on normal murine embryonic liver cells.

In light of its superior cytotoxicity, we extended our study in comparing the antiproliferation function between 9-*O*-farnesylberberine (**8**) and berberine on HepG2 cell for the 24, 48 and 72 h periods, at different doses (Fig. 2). The results showed, though effectiveness of both compounds was dose- and time-dependent, compound **8** exhibited 104-fold superiority to that of berberine.

To further extend our cytotoxicity study, we examined the apoptosis effect of 9-*O*-farnesylberberine (**8**). Firstly, after 24 h incubation in presence or absence of berberine or compound **8**, stained with Hoechst 33258, the topical morphological changes of HepG2 cells were examined by fluorescence microscopy (Fig. 3). Evidently, while in the control experiment, the nuclei of the human hepatoma HepG2 cells were round in shape and stained homogenously, the berberine (**1**) or 9-*O*-farnesylberberine (**8**) treated ones exhibited typical morphological features of apoptosis such as cell shrinkage, chromatin condensation and DNA fragmented.[27] These results demonstrated that both berberine (**1**) and compound **8** inhibited HCC cell proliferation in vitro.

Furthermore to gain the insight of the apoptosis action of berberine (**1**) and compound **8**, HepG2 cells were first either treated for 24 h with vehicle alone as control or with different concentrations (0.25, 0.5 or 1.0 μM, respectively) of berberine (**1**) or compound **8**. The samples were then stained with annexin V-FITC and propidium iodide (PI),[28] and the percentage of apoptotic HepG2 cells was subjected to flow cytometry analysis. As shown in Figure 4, at 1.0 μM concentration, the estimated percentage of apoptotic cells dramatically increased in both compounds at early and late apoptosis. Nonetheless, while the effect of berberine (**1**) leveled off at the concentration of 0.25 μM, apoptotic effect under compound **8** went up with treatment dosage and reached at much higher frequency than those of berberine (**1**). We attribute this outcome to the aforesaid superior cytotoxicity and antiproliferation ability of 9-*O*-farnesylberberine (**8**).

In conclusion, we have synthesized several alkylated and terpenylated berberine derivatives to evaluate their efficacy as preferential cytotoxic agents against human cancer HepG2 and HT-29 cells. First of all, we found a lipophilic substitute of 9-*O*-berberine may enhance its permeability into cell membrane, and thus cytotoxicity against human cancer cell lines. Most of all, we discovered a novel berberine derivative, farnesylated berberine (**8**), which demonstrated the strongest cytotoxic activity, among 8 samples we studied herein, on human liver cancer HepG2 cell lines, but moderated for human colon cancer HT-29 cell lines. It could be a potential candidate for new anticancer drug development.

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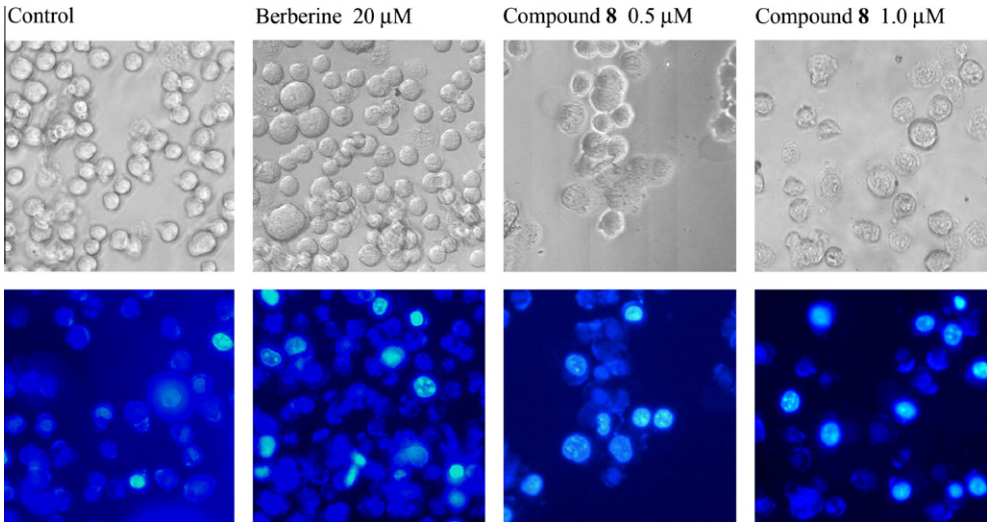

Figure 3. (Top) Cell morphological change of human liver cancer HepG2 cell lines after 24 h exposure (untreated) and with 20 μM berberine (**1**) and 0.5 or 1.0 μM compound **8**. (Bottom) Cells were stained with Hoechst 33258 and examined using a Nikon (Tokyo, Japan) fluorescence microscope. Magnification ×400.

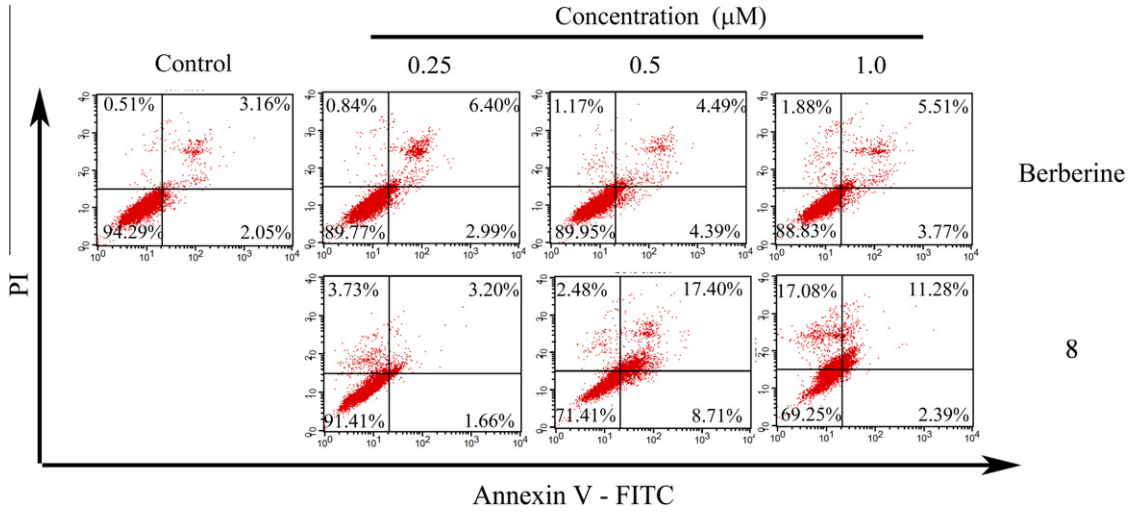

Figure 4. Induction of apoptosis by berberine (**1**) or compound **8**. FACS analysis of annexin V-FITC and PI double-stained cells. HepG2 cells were untreated or treated with 0.25–1.0 μM berberine (**1**) or compound **8** after 24 h. After treatment, cells were double-stained with annexin V-FITC/PI and analyzed by flow cytometry. Cells appearing in the lower right quadrant show positive annexin V-FITC staining, which indicates phosphatidylserine translocation to the cell surface, and no DNA staining by PI, which demonstrates intact cell membranes, both features of early apoptosis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.098.

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26. To a solution of berberrubine **2** (1 mmol) in dry acetonitrile (10 mL) was added various alkyl or terpenyl bromide (1.2 mmol) and the reaction mixture was refluxed for 4–8 h. The progress of the reaction was monitored by TLC. Then, the reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure. The crude product was chromatographed on a silica gel column and eluted with ethyl acetate/methanol (2/1) solvent to afford the desired berberine derivatives **3**–**8**. 9-*O*-Butylberberine bromide (**3**): yellowish brown solid, yield: 86%; UV (MeOH) λ_{max} (log ε): 431 (3.77), 350 (4.41), 267 (4.43), 231 (4.45), 205 (4.41) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.75 (s, 1H, H-8), 8.94 (s, 1H, H-13), 8.20 (d, J = 9.1 Hz, H-12), 7.99 (d, J = 9.1 Hz, H-11), 7.80 (s, 1H, H-1), 7.09 (s, 1H, H-4), 6.17 (s, 2H, –OCH_2O–), 4.95 (t, J = 6.3 Hz, 2H, H-6), 4.29 (t, J = 6.8 Hz, 2H, H-1′), 4.05 (s, 3H, –OCH_3), 3.21 (t, J = 6.3 Hz, 2H, H-5), 1.86 (p, J = 7.2 Hz, 2H, H-2′), 1.52 (sex, J = 7.4 Hz, 2H, H-3′), 0.99 (t, J = 7.4 Hz, 3H, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.4, 149.8, 147.7, 145.3, 142.9, 137.5, 133.0, 130.7, 126.7, 123.3, 121.7, 120.5, 120.2, 108.4, 105.4, 102.1, 73.9, 57.0, 55.3, 31.5, 26.3, 18.6, 13.8; LC–MS (ESI$^+$, m/z) $C_{23}H_{24}NO_4^+$: 378.33 [M–Br]$^+$. 9-*O*-Octylberberine bromide (**4**): bright yellow solid, yield: 74%; UV (MeOH) λ_{max} (log ε): 432 (3.79), 350 (4.42), 268 (4.44), 230 (4.47), 204 (4.48) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.75 (s, 1H, H-8), 8.95 (s, 1H, H-13), 8.19 (d, J = 9.1 Hz, H-12), 7.99 (d, J = 9.1 Hz, H-11), 7.80 (s, 1H, H-1), 7.09 (s, 1H, H-4), 6.17 (s, 2H, –OCH_2O–), 4.95 (t, J = 6.2 Hz, 2H, H-6), 4.28 (t, J = 6.8 Hz, 2H, H-1′), 4.06 (s, 3H, –OCH_3), 3.21 (t, J = 6.2 Hz, 2H, H-5), 1.87 (p, J =7.2 Hz, 2H, H-2′), 1.48 (p, J = 7.3 Hz, 2H, H-3′), 1.33 (m, 8H, H-4′–H-7′), 0.88 (t, J = 6.8 Hz, 3H, H-8′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.4, 149.8, 147.7, 145.3, 142.9, 137.5, 133.0, 126.7, 123.3, 121.7, 120.5, 120.2, 108.4, 105.4, 102.1, 74.2, 57.0, 55.3, 31.2, 29.5, 28.8, 28.7, 26.3, 25.3, 22.1, 14.0; LC–MS (ESI$^+$, m/z) $C_{27}H_{32}NO_4^+$: 434.41 [M–Br]$^+$. 9-*O*-Dodecylberberine bromide (**5**): dark yellowish brown solid, yield: 61%; UV (MeOH) λ_{max} (log ε): 431 (3.42), 351 (4.03), 267 (4.07), 230 (4.10), 202 (4.19) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.77 (s, 1H, H-8), 8.98 (s, 1H, H-13), 8.21 (d, J = 9.1 Hz, H-12), 8.00 (d, J = 9.1 Hz, H-11), 7.82 (s, 1H, H-1), 7.10 (s, 1H, H-4), 6.18 (s, 2H, –OCH_2O–), 4.96 (t, J = 6.1 Hz, 2H, H-6), 4.28 (t, J = 6.8 Hz, 2H, H-1′), 4.05 (s, 3H, –OCH_3), 3.21 (t, J = 6.1 Hz, 2H, H-5), 1.88 (d, J = 7.4 Hz, H-2′), 1.47 (q, J = 7.5 Hz, 2H, H-3′), 1.29 (m, 16 H, H-4′–H-11′), 0.86 (t, J = 6.8 Hz, 3H, H-12′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.4, 149.8, 147.7, 145.3, 142.9, 137.5, 133.0, 130.7, 126.7, 123.3, 121.7, 120.5, 120.3, 108.4, 105.5, 102.1, 74.2, 57.0, 55.3, 48.6, 31.3, 29.5, 29.1, 29.1, 28.9, 28.7, 26.3, 25.3, 22.1, 14.0; LC–MS (ESI$^+$, m/z) $C_{31}H_{40}NO_4^+$: 490.50 [M–Br]$^+$. 9-*O*-Isoprenylberberine bromide (**6**), yellowish brown solid, yield: 68%; UV (MeOH) λ_{max} (log ε): 432 (3.65), 351 (4.25), 268 (4.29), 230 (4.34), 204 (4.43) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.78 (s, 1H, H-8), 8.92 (s, 1H, H-13), 8.18 (d, J = 9.1 Hz, 1H, H-12), 7.99 (d, J = 9.1 Hz, 1H, H-11), 7.79 (s, 1H, H-1), 7.08 (s, 1H, H-4), 6.16 (s, 2H, –OCH_2O–), 5.62 (t, J = 6.56 Hz, 1H, H-2′), 4.94 (t, J = 6.24 Hz, 2H, H-6), 4.84 (d, J = 7.2 Hz, H-1′), 4.06 (s, 3H, –OCH_3), 3.20 (t, J = 6.2 Hz, 2H, H-5), 1.71 (s, 3H, H-4′), 1.66 (s, 3H, H-3a′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.7, 149.8, 147.7, 145.4, 142.4, 138.8, 137.4, 132.9, 130.7, 126.5, 123.4, 122.1, 120.5, 120.2, 119.9, 108.4, 105.4, 102.1, 72.5, 70.2, 63.1, 57.0, 55.3, 26.4, 25.4, 17.9; LC–MS (ESI$^+$, m/z) $C_{24}H_{24}NO_4^+$: 390.22 [M–Br]$^+$. 9-*O*-Geranylberberine bromide (**7**), bright yellow solid, yield: 76%; UV (MeOH) λ_{max} (log ε): 433 (3.82), 352 (4.45), 268 (4.47), 230 (4.50), 205 (4.53) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.81 (s, 1H, H-8), 8.92 (s, 1H, H-13), 8.19 (d, J = 9.1 Hz, 1H, H-12), 8.00 (d, J = 9.1 Hz, 1H, H-11), 7.80 (s, 1H, H-1), 7.09 (s, 1H, H-4), 6.18 (s, 2H, –OCH_2O–), 5.63 (t, J = 6.9 Hz, 1H, H-2′), 4.96 (m, 3H, H-6, 6′), 4.89 (t, J = 6.9 Hz, 2H, H-1′), 4.07 (s, 3H, –OCH_3), 3.20 (t, J = 6.3 Hz, 2H, H-5), 1.97 (m, 4H, H-4′, 5′), 1.64 (d, J = 0.6 Hz, 1H, H-3a′), 1.58 (s, 3H, H-8′), 1.50 (d, J = 0.6 Hz, 1H, H-7a′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.8, 149.8, 147.7, 145.5, 142.3, 137.4, 132.9, 131.0, 130.6, 126.4, 123.6, 123.4, 122.2, 120.4, 120.2, 119.5, 108.4, 105.4, 102.1, 70.0, 57.0, 55.2, 26.3, 25.9, 25.4, 17.5 16.2; LC–MS (ESI$^+$, m/z) $C_{29}H_{32}NO_4^+$: 458.28 [M–Br]$^+$. 9-*O*-Farnesylberberine bromide (**8**), bright yellow solid, yield: 65%; UV (MeOH) λ_{max} (log ε): 432 (3.68), 351 (4.27), 268 (4.30), 230 (4.34), 203 (4.48) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.78 (s, 1H, H-8), 8.92 (s, 1H, H-13), 8.17 (d, J = 9.2 Hz, 1H, H-12), 8.00 (d, J = 9.2 Hz, 1H, H-11), 7.78 (s, 1H, H-1), 7.07 (s, 1H, H-4), 6.16 (s, 2H, –OCH_2O–), 5.62 (t, J = 7.2 Hz, 1H, H-2′), 4.99 (t, J = 7.0 Hz, 1H, H-6′), 4.94 (m, 3H, H-6, 10′), 4.88 (d, J = 7.3 Hz, 1H, H-1′), 4.05 (s, 3H, –OCH_3), 3.19 (t, J = 6.3 Hz, 2H, H-5), 1.88 (m, 8H, H-4′, 5′, 8′, 9′), 1.62 (s, 3H, H-3a′), 1.60 (s, 3H, H-12′), 1.51 (s, 3H, H-7a′), 1.47 (s, 3H, H-11a′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 150.9, 149.9, 147.8, 145.6, 142.6, 142.4, 137.4, 134.8, 133.0, 130.7, 126.5, 124.1, 123.6, 123.5, 122.4, 120.5, 120.3, 119.6, 108.5, 105.5, 102.2, 70.0, 57.0, 55.4, 26.5, 26.2, 25.9, 25.5, 17.6, 16.3, 15.8; LC–MS (ESI$^+$, m/z) $C_{34}H_{40}NO_4^+$: 526.30 [M–Br]$^+$.

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