Berberine Analogues as a Novel Class of the Low-Density-Lipoprotein Receptor Up-Regulators: Synthesis, Structure−Activity Relationships, and Cholesterol-Lowering Efficacy

Ying-Hong Li,[†,‡] Peng Yang,[†,‡] Wei-Jia Kong,[†,‡] Yan-Xiang Wang,[†] Chang-Qin Hu,[§] Zeng-Yan Zuo,[†] Yue-Ming Wang,[†] Hong Gao,[†] Li-Mei Gao,[†] Yan-Chun Feng,[§] Na-Na Du,[†] Ying Liu,[†] Dan-Qing Song,*[,†] and Jian-Dong Jiang*[,†]

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China, and National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100050, China

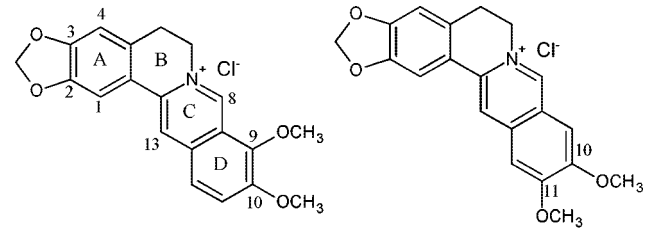
Received September 16, 2008

Twenty-nine derivatives of berberine (**1**) or pseudoberberine (**2**) were designed, semisynthesized, and evaluated for their up-regulatory activity on the low-density-lipoprotein receptor (LDLR) expression. SAR analysis revealed that (i) the methylenedioxy group at the 2- and 3-position is an essential element to keep the activity, (ii) the 7-position quaternary ammonium and planar structure of the compound are activity-required, and (iii) addition of electron-donating groups at the 7- or 13-position reduced the activity. Of the compound **1** analogues, compound **2** exhibited an increased activity on LDLR expression compared to **1**. In the hyperlipidemic rats, compound **2** (100 (mg/kg)/day) reduced blood CHO and LDL-c by 42.6% and 49.4%, respectively, more efficient than **1** did ($p < 0.01$ for both). The results were confirmed in the hyperlipidemic mice. LD_{50} of **2** in mice was over 5000 mg/kg (oral). We consider compound **2** a promising cholesterol-lowering drug candidate.

Introduction

Expression of the low-density-lipoprotein receptor (LDLR[a]) on the liver cell surface regulates homeostasis of human blood LDL cholesterol (LDL-c). Increased hepatic LDLR expression improves the clearance of blood LDL-c through a receptor-mediated endocytosis[1,2] and is strongly associated with a decreased risk of atherosclerosis and coronary heart disease.[3,4] In the previous study, we have found that berberine (**1**, Figure 1), a natural product extracted from a traditional Chinese herb Huanglian (*Coptis chinensis*), is a promising cholesterol-lowering drug.[5] Compound **1** increases LDLR expression at the post-transcriptional level through stabilization of LDLR mRNA in an extracellular signal-regulated kinase (ERK) dependent manner. Its mode of action is different from that of statins.[5] Human experiment showed that the $T_{1/2}$ (kβ) of compound **1** (orally) was 2.94 ± 0.14 h.[6] Compound **1** (oral administration) showed a significant cholesterol-lowering effect in animals as well as in hypercholesterolemic patients with almost no complaints of side effects.[5,7,8] Also, it elevated the therapeutic efficacy against hyperlipidemia in combination with statins.[8]

The structure−activity relationship (SAR) of **1** was recently initiated with the D aromatic ring analysis first.[9] We found the two vicinal methoxy at the 9- and 10-position or 10- and 11-position on the ring D to be crucial structures for LDLR up-regulation; and the compound **2** (Figure 1) bearing 10, 11-dimethoxy (called pseudoberberine as an isomer of **1**) showed a LDLR up-regulatory activity greater than **1** did. In the exploration of the chemical mechanism of this class of compounds and in the search for potent lipid-modifying agents, SAR analysis was continued by focusing our study on the aromatic ring C and side chains of the aromatic rings A and C, taking **1** or **2** as the lead compound. This report includes compound design and synthesis, SAR analysis, efficacy evaluation, and toxicity and mechanism study.



1 **2**

Figure 1. Structures of the lead compounds **1** and **2**.

Chemistry

In the present study we retained the 9,10-dimethoxy of **1** or 10,11-dimethoxy of **2** at the aromatic ring D and focused SAR analysis on the variations of the aromatic ring C as well as substituents at the 2-, 3-, 7-, and 13-position of the aromatic rings A and C, respectively. On the basis of this strategy, 15 analogues of **1** or **2** and 14 of tetrahydrogenated **1** or **2** were designed and synthesized.

The 29 derivatives were semisynthesized with **1** or **2** as starting material and were grouped as analogues of **1** or **2** (Table 1) and of tetrahydrogenated **1** or **2** (Table 2). The synthetic routes are described in the Scheme 1.

Scheme 1 includes three synthetic methods. The first one (route A) used commercially available **1** as the starting material, which reacted with phloroglucinol in H_2SO_4 (60%) at 90−95 °C to yield the key intermediate **3**.[10] The desired products **4a**−**e** were obtained with a good yield with substitution reactions of **3** with different RX in methanol in the presence of NaOH.[11−13]

* To whom correspondence should be addressed. For D.-Q.S.: phone, 86-10-63165268; fax, 86-10-63165268; e-mail, songdanqingsdq@hotmail.com. For J.-D.J.: phone, 86-10-63188423; fax, 86-10-63017302; e-mail, jiang.jdong@163.com.

† Chinese Academy of Medical Sciences and Peking Union Medical College.

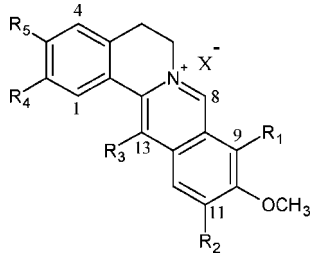
‡ These authors made equal contribution to this work.

§ National Institute for the Control of Pharmaceutical and Biological Products.

[a] Abbreviations: SAR, structure−activity relationship; LDLR, low-density-lipoprotein receptor; LDL-c, low-density-lipoprotein cholesterol; CHO, total cholesterol; HDL-c, high-density-lipoprotein cholesterol; HFHC, high fat and high cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood−urea−nitrogen; CRE, creatinine; LPDS, lipoprotein-deficient serum.

10.1021/jm801157z CCC: $40.75
Published on Web 12/17/2008

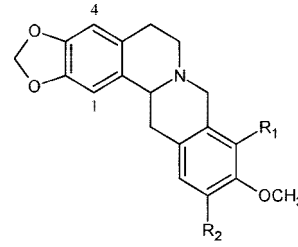
Table 1. Effect on the LDLR Expression by **1** Analogues with Variations at the 2-, 3-, and 13-Position



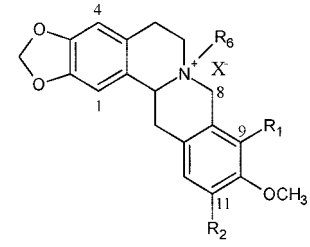
compd	R_1	R_2	R_3	R_4	R_5	X	LDLR mRNA[a]
1	OCH_3	H	H	$-OCH_2O-$		Cl	2.2 ± 0.3
2	H	OCH_3	H	$-OCH_2O-$		Cl	3.5 ± 0.9
4a	OCH_3	H	H	OH	OCH_3	I	1.0 ± 0.1
4b	OCH_3	H	H	OH	OC_2H_5	Br	0.9 ± 0.1
4c	OCH_3	H	H	OCH_3	OCH_3	I	0.8 ± 0.1
4d	OCH_3	H	H	*n*-OC_3H_7	*n*-OC_3H_7	Br	0.9 ± 0.1
4e	OCH_3	H	H	OCH_2Ph	OCH_2Ph	Br	1.0 ± 0.1
6a	OCH_3	H	CH_3	$-OCH_2O-$		Cl	1.1 ± 0.1
6b	OCH_3	H	C_2H_5	$-OCH_2O-$		Cl	1.04 ± 0.1
6c	OCH_3	H	*n*-C_3H_7	$-OCH_2O-$		Cl	0.9 ± 0.1
6d	OCH_3	H	*n*-C_4H_9	$-OCH_2O-$		Cl	0.9 ± 0.1
6e	OCH_3	H	*n*-C_6H_{13}	$-OCH_2O-$		Cl	1.0 ± 0.1
6f	OCH_3	H	$PhCH_2$	$-OCH_2O-$		Br	1.4 ± 0.1
6g	H	OCH_3	CH_3	$-OCH_2O-$		Cl	0.9 ± 0.1
6h	H	OCH_3	*n*-C_4H_9	$-OCH_2O-$		Cl	0.9 ± 0.1
6i	H	OCH_3	*n*-C_6H_{13}	$-OCH_2O-$		Cl	1.1 ± 0.1
6j	H	OCH_3	$PhCH_2$	$-OCH_2O-$		Br	1.0 ± 0.1

[a] Human liver HepG2 cells were cultured in the EMEM medium containing 0.5% of LPDS and were then incubated with **1** or **2** or their analogues (5.0 μg/mL) for 12 h at 37 °C. Up-regulation of LDLR expression was determined by the real-time RT-PCR. Abundance of LDLR mRNA in the untreated cells was defined as 1, and the levels of LDLR mRNA from the study compounds treated cells were defined as fold of the untreated control. The data shown are the mean of three separate experiments.

Table 2. Effect on LDLR Expression by **1** Analogues with Variations at the 7-Position



7a-b



8a-l

compd	R_1	R_2	R_6	X	LDLR mRNA[a]
1					2.2 ± 0.3
2					3.5 ± 0.9
7a	OCH_3	H			1.0 ± 0.1
7b	H	OCH_3			1.3 ± 0.1
8a	OCH_3	H	CH_3	I	1.1 ± 0.1
8b	OCH_3	H	CH_2CH_3	Br	1.0 ± 0.1
8c	OCH_3	H	CH_2CH_2OH	Br	1.2 ± 0.1
8d	OCH_3	H	$CH_2CH_2CH_3$	I	1.2 ± 0.1
8e	OCH_3	H	CH_2CH_2 CH_2CH_3	Br	1.1 ± 0.1
8f	OCH_3	H	$CH_2COOCH_2CH_3$	Br	0.8 ± 0.1
8g	OCH_3	H	CH_2Ph	Br	1.1 ± 0.1
8h	H	OCH_3	CH_3	I	0.9 ± 0.1
8i	H	OCH_3	CH_2CH_3	Br	0.8 ± 0.1
8j	H	OCH_3	$CH_2CH_2CH_3$	I	0.9 ± 0.1
8k	H	OCH_3	$CH_2COOCH_2CH_3$	Br	1.0 ± 0.1
8l	H	OCH_3	CH_2Ph	Br	0.9 ± 0.1

[a] Human liver HepG2 cells were cultured in the EMEM medium containing 0.5% of LPDS and were then incubated with **1** or **2** or their analogues (5.0 μg/mL) for 12 h at 37 °C. Up-regulation of LDLR expression was determined by the real-time RT-PCR. Abundance of LDLR mRNA in the untreated cells was defined as 1, and the levels of LDLR mRNA from the study compounds treated cells were defined as fold of the untreated control. The data shown are the mean of three separate experiments.

The second one (route B) used compound **1** or **2**[9] as starting material for a selective reduction reaction, in which $NaBH_4$ was used as a reducing agent and methanol as the solvent.[14] The key intermediates (**5a**,**b**), dihydrogenated product of **1** or **2**, were obtained at room temperature conditions, and the total amount of $NaBH_4$ was controlled strictly with an optimal level of 75% of **1** or **2** (mol/mol). The resultant intermediate (**5a**,**b**) reacted with a series of alkylaldehydes in the solvent mixture of EtOH and HOAc and then acidified with 2 N HCl to yield the desired compounds (**6a**−**e**,**g**−**i**).[14,15] Compounds **6f** and **6j** were readily obtained through a substitution reaction of benzyl bromide with the intermediate **5a** or **5b**, in which benzyl bromide was first treated with NaI in CH_3CN to improve substituent activity, followed by an oxidation reaction using *N*-bromosuccinimide (NBS).[16,17]

In the third synthetic route (route C), the key compounds **7a** and **7b**, the tetrahydrogenated product of **1** or **2**, were obtained through a complete reduction in which $NaBH_4$ was used as a reducing agent. The reaction was carried out at reflux conditions with ethanol (80%) as the solvent, and the total amount of $NaBH_4$ was at 1:1 ratio (mol/mol) to the compound **1** or **2**. Then the resultant intermediate **7** underwent a quaternization reaction with alkyl halides to achieve the corresponding quaternary ammonium salts (**8a**−**l**).[18]

Results and Discussion

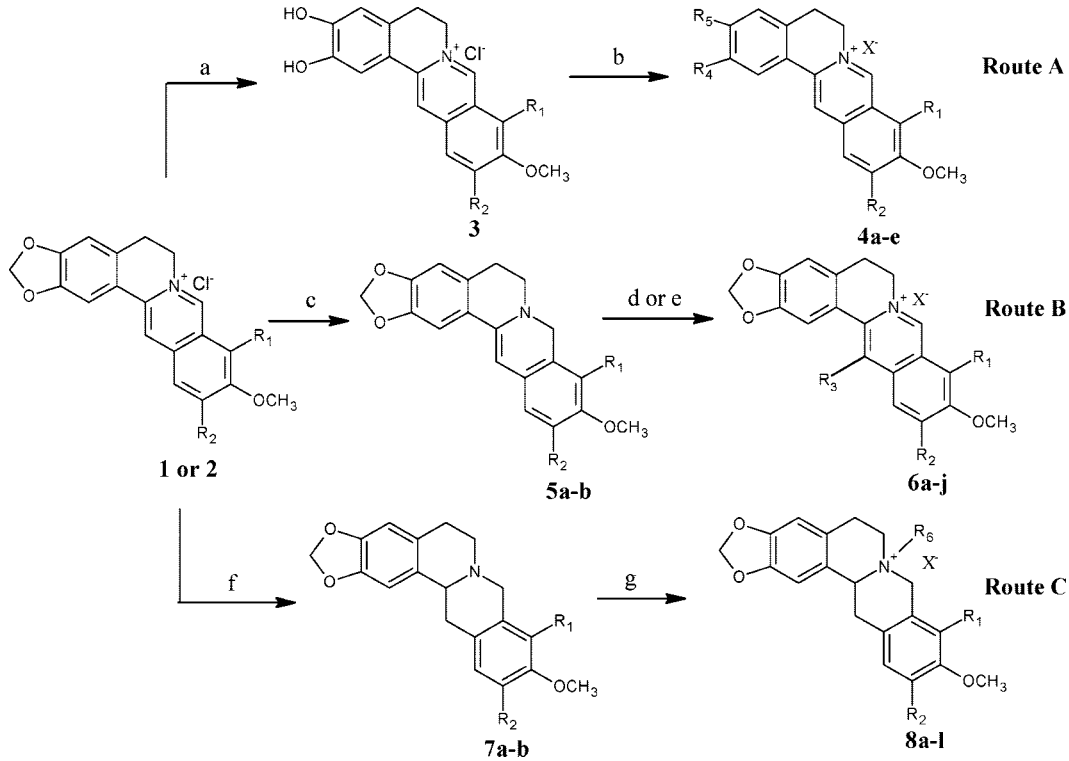
SAR Analysis. The 29 analogues were examined in human liver cells for their up-regulatory activity on LDLR mRNA expression using a specific real time RT-PCR assay. Structures of the analogues and their regulatory activity on LDLR mRNA expression are shown in Tables 1 and 2.

The SAR study was first focused on the effect of the side chains at the 2- and 3-position of the aromatic ring A (Table 1). The oxacyclopentene was opened, and a methoxyl or ethoxyl was attached at the 3-position of the compound **3**. The obtained compounds **4a** (columbamine) and **4b** showed no activity on LDLR expression. The compound **4c** (palmatine), **4d**, or **4e**, bearing dimethoxy, dipropoxy, or dibenzyloxy at the 2- and 3-position, respectively, completely lost their activity on LDLR. Therefore, all of the semisynthesized analogues with modification at the 2- and 3-position partially or completely lost their activity on LDLR, regardless of the size of the side chains. We conclude that the methylenedioxy at the 2- and 3-position plays a significant role in up-regulating LDLR expression.

Next, on the aromatic ring C of **1** or **2** the hydrogen atom at the 13-position was replaced with electron-donating groups (Table 1). Introduction of methyl, ethyl, propyl, butyl, hexyl, or benzyl at the 13-position generated compounds **6a**−**j**, all of which had their activity much less than did compound **1** or **2**. As the quaternary ammonium at the 7-position plays an important role in binding to speculated biological targets,[9,19,20] we consider that the electron-donating groups at the 13-position might reduce the electropositivity of quaternary ammonium and subsequently interfere with the bioactivity of the analogues. Meanwhile, the steric obstacle from the enlarged side chains on the 13-position could be another possible explanation.

The SAR was further explored on the variation of the aromatic ring C. The ring C was modified with hydrogenation. As shown in Table 2, the tetrahydrogenated product **7a** (canadine) or **7b** had much lower activity on the LDLR expression compared with parent compound **1** or **2**. There could be at least two chemical factors that contribute to the activity reduction after

Scheme 1. Semisynthesis of analogues of **1** and **2**[a]



[a] Reagents and conditions: (a) phloroglucinol, 60% H_2SO_4, 90−95 °C; (b) RX, NaOH, CH_3OH, reflux; (c) $NaBH_4$, K_2CO_3, CH_3OH, room temperature; (d) RCHO, HOAc, 80% ethanol, 85−95 °C; (e) $PhCH_2Br$, NaI, CH_3CN, room temperature, reflux, NBS; (f) $NaBH_4$, K_2CO_3, 80% ethanol, reflux; (g) RX, K_2CO_3, CH_3CN, reflux.

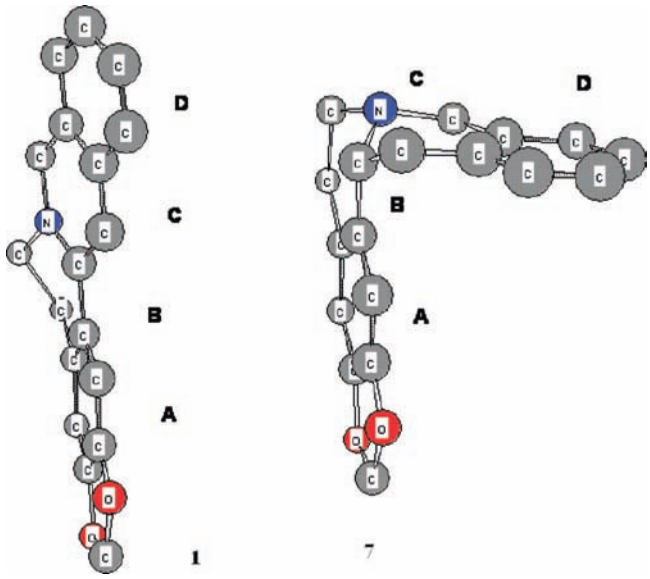


Figure 2. Modeled skeleton structures of **1** and **7** analogues. The steric structures were established on the basis of the minimum energy conformation principle, generated by ChemOffice Ultra Drawing, Modeling and Information (version 4.5), applying the calculator in molecular dynamics MOPAC component to fulfill the optimization.

hydrogenation. First, the quaternary ammonium was converted into the tertiary ammonium, resulting in a loss of the positive ion at this position, and second, the planar structure was converted into bended structure (Figure 2) at the pseudo-trans ring junction between B and C rings, causing a significant configuration change. These factors appeared to alter the interaction between the compounds (**7a** and **7b**) and speculated target(s) and reduced their activity on LDLR expression. Therefore, the quaternary ammonium and planar structure are both crucial in keeping compounds with potency.

Modification at the 7-position of the aromatic ring C was explored as well. All of the compounds (**8a**−**l**) with substituents at the 7-position exhibited an activity much less than did its parent compound **1** or **2**. It appears that the substituents at the 7-position, regardless of the size of the side chains, form a blockage that prevents the quaternary ammonium from conducting its action on the pathway molecules for LDLR expression regulation. Another possible factor is the nonplanar structure of the compounds (**8a**−**l**), which might cause the activity loss as discussed in **7a**.

Taken together, we have de novo synthesized and/or semisynthesized 48 compound **1** analogues in total, of which 19 were synthesized in our previous study[9] and 29 were in this study. Through SAR analysis we found and confirmed that compound **2** possesses an optimal structure in up-regulating LDLR expression. Its activity was higher than that of compound **1**. After overall evaluation, this compound was selected for further biological evaluation in vitro and in vivo.

Compound 2 Up-Regulated LDLR Expression in Human Hepatocytes. The LDLR up-regulatory activity of **2** was first examined in a Caucasian liver cell line HepG2, in comparison with that of **1**. In the cultured HepG2 cells, compound **2** significantly increased LDLR mRNA expression, and the activity of **2** was about 1.5-fold of that of **1** in the real time RT-PCR assay (Figure 3a). With respect to the reference drug simvastatin, we found that switch of "$-OCH_3$" from the 9-position (compound **1**) to the 11-position (compound **2**) increases the effect equivalent to that of a doubled concentration of simvastatin (Figure 3b). Accordingly, LDLR protein expression was also increased on the surface of the **2**-treated hepatocytes, with a degree higher than on those treated with

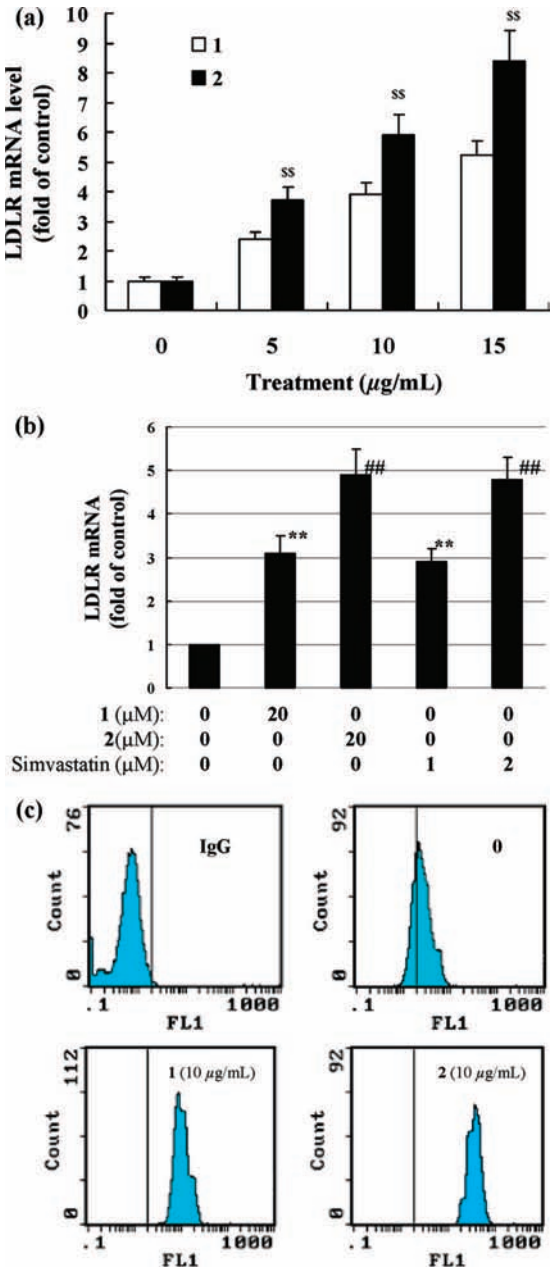


Figure 3. Up-regulation of LDLR expression by **2** in the HepG2 cells. (a) LDLR mRNA levels. HepG2 cells cultured in the LPDS containing media were left untreated or treated for 12 h with **1** or **2** at the concentrations indicated. Total cellular RNAs were extracted and reversely transcribed into cDNAs. LDLR mRNA levels were analyzed by real-time RT-PCR using GAPDH as internal control. The normalized LDLR mRNA levels were plotted as fold of the untreated control, which was designated as 1. Values are the mean ± SE of at least three independently experiments: ($$) $P < 0.01$ as compared to that of **1** at the same concentration. (b) LDLR up-regulatory activity in **1**, **2**, and simvastatin. HepG2 cells were left untreated or treated with **1**, **2**, or simvastatin as indicated for 12 h. Cellular LDLR mRNA levels were measured by real-time RT-PCR and plotted as fold of untreated control. Values are the mean ± SEM of three separate experiments: (**) $p < 0.01$ as compared to that of untreated control; (##) $p < 0.01$ as compared to that of 20 μM BBR or 1 μM simvastatin. (c) LDLR protein expression on cell surface. HepG2 cells were untreated or treated with 10 μg/mL **1** or **2** for 12 h. Cells were analyzed by flow cytometry for cell surface LDLR protein expression using a rabbit-polyclonal antibody against LDLR. Normal rabbit IgG was used as control for the background staining. Fluorescence intensities on cell surface were analyzed in a FACSCalibur system. Count on the *Y* axis is the absolute number of cells, and FL1 on the *X* axis represents the fluorescent intensity of the LDLR protein expressed on the HepG2 cell surface.

compound **1** (Figure 3c). Besides HepG2 cells, the enhanced activity of **2** was also detected in the Bel-7402 cells (not shown), a liver cell line derived from a Chinese hepatoma patient, indicating the effectiveness of the compound in cells with either Caucasian or oriental genetic background.

Lipid-Lowering Efficacy of Compound 2 in Hyperlipidemic Animals. The superior LDLR up-regulatory activity of **2** encouraged us to test its cholesterol-lowering efficacy in vivo. The rats were fed with a high-fat and high-cholesterol (HFHC) diet for 8 weeks before treatment. Compared to the normal diet group, 2.4-fold increases in blood LDL-c and total cholesterol (CHO) were detected in the HFHC-fed rats ($P < 0.01$, Figure 4a). No alteration of the high-density-lipoprotein cholesterol (HDL-c) level was found in this model.

Drug interventions lowered blood lipids. The results are shown in Figure 4a. After a 30-day treatment, compound **1** at 100 (mg/kg)/day reduced CHO and LDL-c by 27.4% and 31.6%, respectively ($P < 0.01$, compared to the untreated HFHC control). The 100 (mg/kg)/day of **2** reduced blood CHO and LDL-c by 42.6% and 49.4%, respectively, significantly higher than that of **1** ($P < 0.01$). The lipid reduction by **1** or **2** was time-dependent and became statistically significant after 10 days on therapy (Figure 4b). The LDL cholesterol-lowering efficacy of **2** was higher than that of **1** at every time-point examined. In addition, Figure 4c reveals the total cholesterol- and LDL-c-lowering effect of **2** in comparison with that of the reference drug simvastatin.

Livers were dissected from the animals at the end of experiment. Hepatic RNA was isolated and followed by LDLR mRNA expression analysis. As shown in Figure 4d, treatment with 100 (mg/kg)/day of **2** increased liver LDLR mRNA expression by 2.9-fold of the untreated HFHC control and 1.5-fold of the compound **1** treatment ($P < 0.01$). This result was consistent with that observed in cultured cells. As statins up-regulate HMG-CoA reductase mRNA expression,[8] the expression of this gene was also measured for **1** or **2**. As shown in Figure 4d, neither **1** nor **2** enhanced the expression of HMG-CoA reductase mRNA in the rat livers, confirming the mechanism difference between statins and compound **1** or **2**.

Compound **2** was safe and well tolerated in the rats. We did not observe weight loss, food intake reduction, or liver/kidney function abnormalities (Figure 4e,f) in the rats treated with compound **2**. The safety results of **2** agreed with that of compound **1**.[5,8]

Compound **2** lowered blood lipids also in a dose-dependent manner. As shown in Figure 5a, after a 17-day treatment of the hyperlipidemic rats, **2** at 100 or 300 (mg/kg)/day reduced CHO by 28.5% or 46.3% and LDL-c by 31% or 53.6%, respectively. Compound **2** at 300 (mg/kg)/day increased liver LDLR expression by 4.8-fold of the untreated control, significantly higher than did the 100 (mg/kg)/day group (Figure 5b; $P < 0.01$).

The in vivo lipid-lowering activity of **2** was confirmed in another animal model, the hyperlipidemic C57BL/6J mice, that developed hyperlipidemia after 8-weeks of HFHC diet feeding (Figure 6a,b). Treatment with compound **1** or **2** at 100 (mg/kg)/day for 21 days lowered CHO by 16.7% or 29.7% and LDL-c by 23.9% or 39%, respectively. Again, the cholesterol-lowering activity of **2** was significantly higher than that of **1** in the mice ($P < 0.01$). Furthermore, compound **2** increased the liver LDLR mRNA expression by 3.2-fold of the control (Figure 6c) and 1.5-fold of **1** ($P < 0.01$). The results in mice fully agreed with that in rats as well as in cell culture.

Safety Evaluation of 2. While the pharmacokinetics of compound **2** is still under investigation, we performed safety

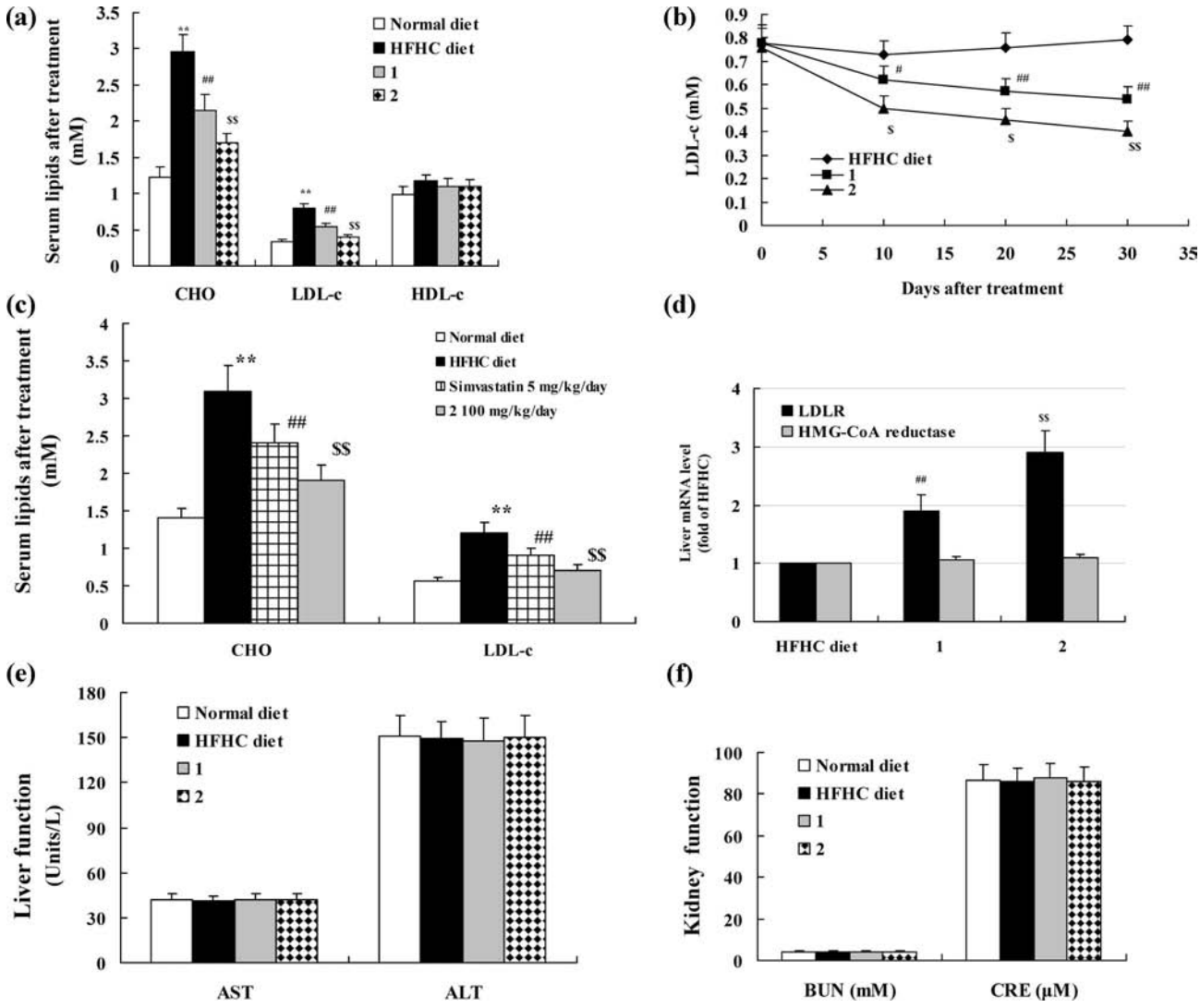


Figure 4. Lipid-lowering effect of **1** and **2** in the hyperlipidemic rats. Male Sprague−Dawley rats were fed with HFHC diet for 8 weeks. Then the animals were untreated ($n = 6$) or treated with 100 (mg/kg)/day of **1** ($n = 8$) or **2** ($n = 8$) for 30 days, respectively. (a) End-point blood lipid levels. At the end of the treatment course, blood samples were taken from the animals, and CHO, LDL-c and HDL-c levels were determined. (b) Time-dependent reduction of blood LDL-c by **1** and **2**. (c) Comparison of the cholesterol-lowering effects of compound **2** with simvastatin. Hyperlipidemic rats were untreated ($n = 6$) or treated with either 5 (mg/kg)/day of simvastatin ($n = 7$) or 100 (mg/kg)/day of compound **2** ($n = 7$). Four weeks later, serum cholesterol and LDL-c levels were assayed. Values are the mean ± SEM: (**) $p < 0.01$, as compared to that of normal diet; (##) $p < 0.01$, as compared to that of HFHC; ($$) $p < 0.01$, as compared to that of simvastatin. (d) LDLR up-regulatory activities in vivo. After the 30-day experiment, rats were sacrificed and their livers were dissected. Liver total RNAs were isolated for real-time RT-PCR assay. The expression levels of LDLR and HMG-CoA reductase mRNAs were normalized to that of GAPDH and presented as fold of the HFHC group. (e) Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the treatment with compounds **1** and **2** in rats. Hyperlipidemic rat were treated with the study compound (100 (mg/kg)/day, oral) for 30 days. Blood samples were taken before and after the treatment, and their AST and ALT levels were measured. Presented are mean and sd values. (f) Blood urea nitrogen (BUN) and creatinine (CRE) levels in the treatment with compounds **1** and **2** in rats. Hyperlipidemic rat were treated as mentioned above. Blood samples were assayed for their BUN and CRE levels. Presented are mean and sd values. Values are the mean ± SE of all of the animals in each group: (**) $P < 0.01$ vs that of the normal diet group ($n = 5$); (#) $P < 0.05$; (##) $P < 0.01$ vs that of the HFHC diet group; ($) $P < 0.05$; ($$) $P < 0.01$ vs that of **1** by one-way ANOVA and multiple comparisons.

evaluation and acute toxicity test in the Kunming mice. Compound **2** was given orally in a single-dosing experiment at 250, 1000, or 5000 mg/kg. Then the animals were closely monitored. No animal died in the 14-day treatment, indicating that the LD_{50} of **2** was larger than 5000 mg/kg. Oral administration of **2** at 250 or 1000 mg/kg did not affect the body weights (BW) of the mice. For the 5000 mg/kg group, transient BW loss (−7%) was observed in the first 2−3 days, followed by a rebound to the normal level (not shown). Furthermore, the animals were healthy in behavior and had shining fur even at the dose of 5000 mg/kg. The histological examination for liver, kidney, heart, lung, brain, and colon of the mice was done 14 days after treatment. Compound **2** showed no toxicity to the liver, kidney (Figure 7), as well as other organs (not shown), even when the dose was up to 5000 mg/kg. These results suggest compound **2** to be considerably safe in vivo.

Conclusions

In the continuation of SAR study for the compound **1** derivatives as a novel class of LDLR up-regulators, we have semisynthesized 29 analogues defined through variations of the aromatic ring C and the substituents at the 2-, 3-, 7-, and 13-position of the parent compound **1** or **2**. The SAR analysis reveals that (i) the methylenedioxy at the 2- and 3-position is

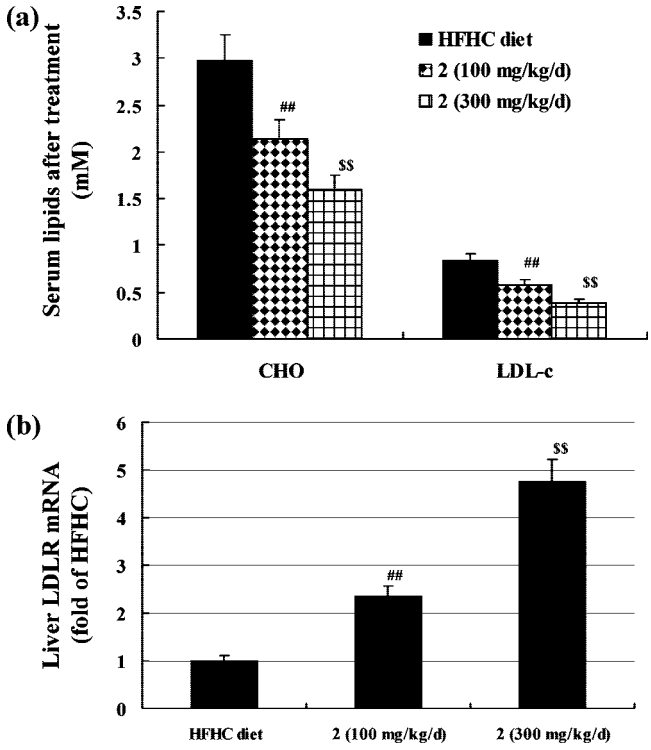


Figure 5. Dose-dependent activity of **2** in the hyperlipidemic rats. Hyperlipidemic Sprague−Dawley rats were untreated ($n = 6$) or treated with **2** at 100 (mg/kg)/day ($n = 8$) or 300 (mg/kg)/day ($n = 8$) for 17 days. (a) End-point blood levels of CHO and LDL-c. (b) Liver LDLR mRNA. At the end of treatment, animals were sacrificed and their livers were dissected. Liver total RNAs were isolated for real-time RT-PCR assay of LDLR mRNA expression levels. The normalized LDLR mRNA levels were presented as fold of the HFHC group, which was designated as 1. Values are the mean ± SE of all of the animals in each group: (##) $P < 0.01$ vs that of the HFHC diet group; ($$) $P < 0.01$ vs that of the low dose group of **2** (100 (mg/kg)/day) by one-way ANOVA and multiple comparisons.

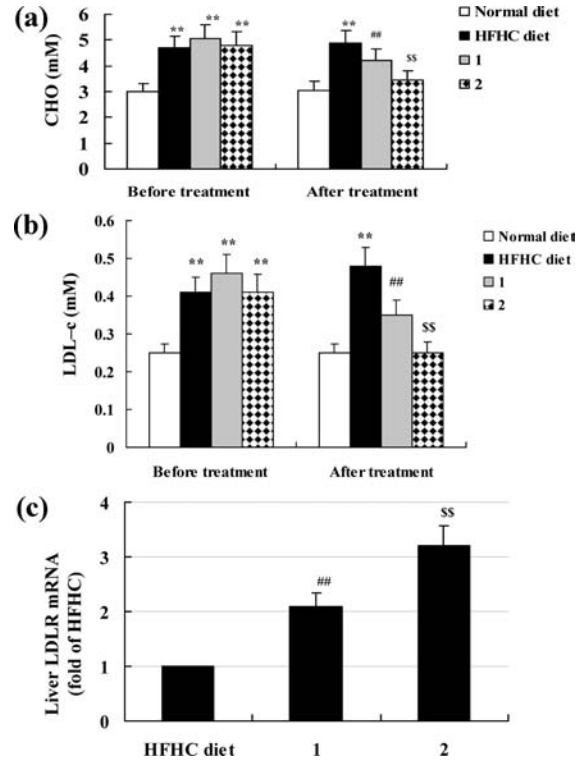


Figure 6. Lipid-lowering effect of **1** and **2** in the hyperlipidemic mice. Male C57BL/6J mice were fed with HFHC diet for 8 weeks. Then the animals were untreated ($n = 6$) or treated with 100 (mg/kg)/day of **1** ($n = 8$) or **2** ($n = 8$) for 21 days, respectively. Before and after treatment, blood samples were taken for the assay of CHO (a) and LDL-c (b) levels. At the end of treatment, livers were dissected from the animals and total liver RNAs were isolated for the real-time RT-PCR of the LDLR mRNA expression (c). The normalized LDLR mRNA levels were presented as fold of the HFHC group, which was designated as 1. Values are the mean ± SE of all of the animals in each group: (**) $P < 0.01$ vs that of the normal diet group ($n = 5$); (##) $P < 0.01$ vs that of the HFHC diet group; ($$) $P < 0.01$ vs that of **1** by one-way ANOVA and multiple comparisons.

an important element to keep the compound potent in up-regulating LDLR expression, (ii) adding electron-donating groups at the 7- or 13-position reduces the activity, and (iii) the 7-position quaternary ammonium ion and planar structure of the compound play crucial roles for its interaction with speculated target molecule(s). Among the analogues, compound **2** afforded an up-regulatory activity on LDLR expression greater than **1** did in vitro. Accordingly, **2** had a significantly enhanced lipid-lowering efficacy in vivo compared to **1**. The increased activity of **2** might result from the higher electronpositivity at the 7-position in respect to that of **1**.[9] As compound **2** shows an improved lipid-lowering activity with a mechanism different from that of statins and has a considerably good safety in vivo, it merits further investigation.

Experimental Section

Chemistry. Melting points (mp) were obtained with YRT-3 melting point apparatus and were uncorrected. ^{1}H NMR spectra were performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA) in DMSO-d_6 (as internal standard on a δ scale). FAB and EI mass spectra were recorded on an Autospec UItima-TOF mass spectrometer (Micromass UK Ltd., Manchester, U.K.). Elemental analyses were from Flash 1112 series EA (Thermo Finnigan) and were within ±0.4 of the theoretical values.

Route A (for 4a−e). 2-Hydroxy-3-methoxy-9,10-dimethoxyprotoberberine Iodide (4a). To a stirred mixture of **3**[10] (360 mg, 1.0 mmol) and NaOH (100 mg, 2.5 mmol) in methanol (30 mL), methyl iodide (0.07 mL, 1.2 mmol) was added. The reaction mixture

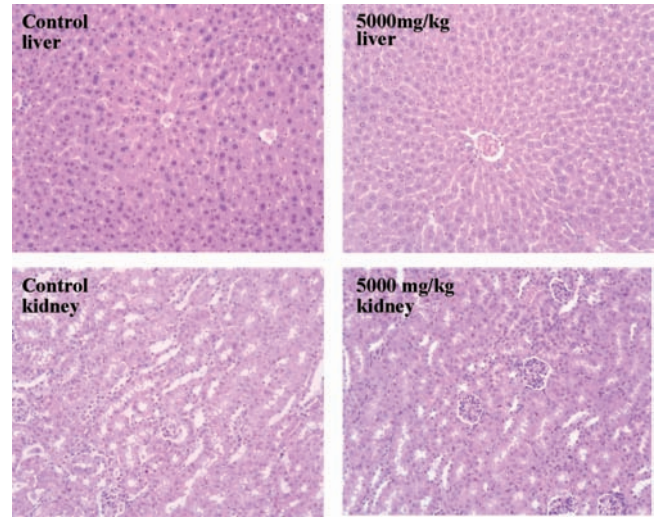


Figure 7. Histological examination of compound **2**. Male Kunming mice were divided into four groups with six mice in each. They were untreated or orally treated with **2** at 250, 1000, or 5000 mg/kg in one dose. Fourteen days later, animal organs were taken for histological examination using H&E staining.

was stirred at room temperature overnight. The solvent was removed by evaporation, and then the residue was purified by flash chromatography over silica gel, affording the title compound (237

mg, 51%) as a yellow solid, mp 214−216 °C. MS *m/z* 338 (M − I)$^+$. ^{1}H NMR: δ 3.18 (t, J = 6.0 Hz, 2H), 3.89 (s, 3H), 4.06 (s, 3H), 4.08 (s, 3H), 4.92 (t, J = 6.0 Hz, 2H), 7.05 (s, 1H), 7.54 (s, 1H), 8.05 (d, J = 9.2 Hz, 1H), 8.18 (d, J = 9.2 Hz, 1H), 8.81 (s, 1H), 9.36 (br, 1H), 9.85 (s, 1H). Anal. ($C_{20}H_{20}NO_4I$) C, H, N.

2-Hydroxy-3-ethoxy-9,10-dimethoxyprotoberberine Bromide (4b). To a stirred mixture of **3** (360 mg, 1.0 mmol) and NaOH (100 mg, 2.5 mmol) in methanol (30 mL), ethyl bromide (0.10 mL, 1.5 mmol) was added. The reaction mixture was stirred at room temperature for 0.5 h and then heated to reflux for 5 h. The solvent was removed by evaporation, and then the residue was purified by flash chromatography over silica gel, affording the title compound (155 mg, 36%) as a redlike solid, mp 172−174 °C. MS *m/z* 352 (M − Br)$^+$. ^{1}H NMR: δ 1.39 (t, J = 9.2 Hz, 3H), 3.17 (t, J = 8.4 Hz, 2H), 4.06 (s, 3H), 4.08 (s, 3H), 4.14 (q, J = 9.2 Hz, 2H), 4.91 (t, J = 8.4 Hz, 2H), 7.03 (s, 1H), 7.54 (s, 1H), 8.05 (d, J = 12.0 Hz, 1H), 8.18 (d, J = 12.0, 1H), 8.80 (s, 1H), 9.27 (br, 1H), 9.85 (s, 1H). Anal. ($C_{21}H_{22}NO_4Br$) C, H, N.

2,3-Dimethoxy-9,10-dimethoxyprotoberberine Iodide (4c). The title compound was obtained from **3** (1.0 mmol) and methyl iodide (2.5 mmol) with a procedure similar to that for compound **4b**. Yield: 78%. Yellow solid, mp 222−224 °C. MS *m/z* 352 (M − I)$^+$. ^{1}H NMR: δ 3.18 (t, J = 6.0 Hz, 2H), 3.87 (s, 3H), 3.93 (s, 3H), 4.07 (s, 3H), 4.09 (s, 3H), 4.94 (t, J = 6.0 Hz, 2H), 7.09 (s, 1H), 7.70 (s, 1H), 8.02 (d, J = 9.2 Hz, 1H), 8.20 (d, J = 9.2 Hz, 1H), 9.00 (s, 1H), 9.87 (s, 1H). Anal. ($C_{21}H_{22}NO_4I$) C, H, N.

2,3-Di-*n*-propoxy-9,10-dimethoxyprotoberberine Bromide (4d). The title compound was obtained from **3** (1.0 mmol) and propyl bromide (2.5 mmol) with a procedure similar to that for compound **4b**. Yield: 80%. Yellow solid, mp 218−220 °C. MS *m/z* 408 (M − Br)$^+$. ^{1}H NMR: δ 1.00 (t, J = 7.2 Hz, 3H), 1.03 (t, J = 7.2 Hz, 3H),1.78 (q, J = 7.2 Hz, 4H), 3.20 (t, J = 6.0 Hz, 2H), 4.02−4.11 (m, 4H), 4.06 (s, 3H), 4.09 (s, 3H), 4.93 (t, J = 6.0 Hz, 2H), 7.08 (s, 1H), 7.71 (s, 1H), 8.02 (d, J = 9.2 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 8.98 (s, 1H), 9.86 (s, 1H). Anal. ($C_{25}H_{30}NO_4Br$) C, H, N.

2,3-Dibenzyloxy-9,10-dimethoxyprotoberberine Bromide (4e). The title compound was obtained from **3** (1.0 mmol) and benzyl bromide (2.5 mmol) with a procedure similar to that for compound **4b**. Yield: 89%. Yellow solid, mp 244−246 °C. MS *m/z* 504 (M − Br)$^+$. ^{1}H NMR: δ 3.20 (t, J = 6.0 Hz, 2H), 4.07 (s, 3H), 4.09 (s, 3H), 4.93 (t, J = 6.0 Hz, 2H), 5.25 (s, 2H), 5.30 (s, 2H), 7.22 (s, 1H), 7.34−7.53 (m, 10H), 7.90 (s, 1H), 8.02 (d, J = 9.2 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 9.00 (s, 1H), 9.88 (s, 1H). Anal. ($C_{33}H_{30}NO_4Br$) C, H, N.

Route B (for 6a−j). 2,3-Methylenedioxy-9,10-dimethoxy-13-methylprotoberberine Chloride (6a). To a stirred solution of **1** (3.71 g, 10 mmol) and K_2CO_3 (3.6 g, 30 mmol) in methanol (125 mL), 5% NaOH (5 mL) solution containing $NaBH_4$ (0.30 g, 7.5 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 1 h and the precipitated product was filtered, washed with 30% ethanol (20 mL) and 80% ethanol (20 mL), and then recrystallized from absolute ethanol to provide **5a** (2.5 g, 74%) as a brownlike solid.

To a stirred solution of **5a** (170 mg, 5.0 mmol) in 80% ethanol (8 mL) and HOAc (2 mL), formaldehyde (2 mL) was added. The reaction mixture was heated to 85−95 °C for 5 h. The solvent was removed by evaporation, and the residue was acidified by 2 N HCl (5 mL), then stirred at room temperature for 1 h. The solid was collected by filtration and then purified by flash chromatography over silica gel, affording the title compound (74 mg, 38%) as a yellow solid, mp 198−200 °C. MS *m/z* 350 (M − Cl)$^+$. ^{1}H NMR: δ 2.92 (s, 3H), 3.10 (t, J = 5.6 Hz, 2H), 4.08 (s, 3H), 4.09 (s, 3H), 4.81 (t, J = 5.6 Hz, 2H), 6.17 (s, 2H), 7.14 (s, 1H), 7.46 (s, 1H), 8.18 (d, J = 9.6 Hz, 1H), 8.19 (d, J = 9.6 Hz, 1H), 9.89 (s, 1H). Anal. ($C_{21}H_{20}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-13-ethylprotoberberine Chloride (6b). The title compound was obtained from **5a** and acetaldehyde with a procedure similar to that for compound **6a**. Yield: 42%. Yellow solid, mp 181−183 °C. MS *m/z* 364 (M − Cl)$^+$. ^{1}H NMR: δ 1.45 (t, J = 7.2 Hz, 3H), 3.08 (t, J = 5.6 Hz, 2H), 3.35 (q, J = 7.2 Hz, 2H), 4.09 (s, 3H), 4.09 (s, 3H), 4.81 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.16 (s, 1H), 7.29 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 8.24 (d, J = 9.6 Hz, 1H), 9.92 (s, 1H). Anal. ($C_{22}H_{22}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-13-*n*-propylprotoberberine Chloride (6c). The title compound was obtained from **5a** and propaldehyde with a procedure similar to that for compound **6a**. Yield: 54%. Yellow solid, mp 201−202 °C. MS *m/z* 378 (M − Cl)$^+$. ^{1}H NMR: δ 0.99 (t, J = 7.2 Hz, 3H), 1.78 (q, J = 7.2 Hz, 2H), 3.07 (t, J = 5.6 Hz, 2H), 3.31 (m, 2H), 4.08 (s, 6H), 4.79 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.15 (s, 1H), 7.25 (s, 1H), 8.17 (d, J = 9.6 Hz, 1H), 8.22 (d, J = 9.6 Hz, 1H), 9.90 (s, 1H). Anal. ($C_{23}H_{24}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-13-*n*-butylprotoberberine Chloride (6d). The title compound was obtained from **5a** and butaldehyde with a procedure similar to that for compound **6a**. Yield: 39%. Yellow solid, mp 201−202 °C. MS *m/z* 392 (M − Cl)$^+$. ^{1}H NMR: δ 0.91 (t, J = 7.2 Hz, 3H), 1.41 (q, J = 7.2 Hz, 2H), 1.75 (m, 2H), 3.07 (t, J = 5.6 Hz, 2H), 3.35 (m, 2H), 4.08 (s, 3H), 4.09 (s, 3H), 4.79 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.15 (s, 1H), 7.28 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 8.20 (d, J = 9.2 Hz, 1H), 9.90 (s, 1H). Anal. ($C_{24}H_{26}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-13-*n*-hexylprotoberberine Chloride (6e). The title compound was obtained from **5a** and hexaldehyde with a procedure similar to that for compound **6a**. Yield: 40%. Yellow solid, mp 198−199 °C. MS *m/z* 420 (M − Cl)$^+$. ^{1}H NMR: δ 0.84 (m, 3H), 1.25 (m, 4H), 1.37 (m 2H), 1.74 (m, 2H), 3.07 (t, J = 5.6 Hz, 2H), 3.34 (m, 2H), 4.06 (s, 3H), 4.09 (s, 3H), 4.78 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.15 (s, 1H), 7.28 (s, 1H), 8.19 (s, 2H), 9.86 (s, 1H). Anal. ($C_{26}H_{30}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-13-benzylprotoberberine Bromide (6f). To a stirred suspension of NaI (310 mg, 2.06 mmol) in acetonitrile (16 mL), benzyl bromide (350 mg, 2.06 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. After **6a** (580 mg, 1.72 mmol) was added, the system was heated to reflux for 3 h, the solvent was removed by evaporation, and then the residue was redissolved in $CHCl_3$ (20 mL). The organic layer was then washed with 10% K_2CO_3 (25 mL), dried over $MgSO_4$, and filtrated. To the filtration was added NBS (367 mg, 2.06 mmol), and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed by evaporation, and the residue was purified by flash chromatography over silica gel, affording the title compound (620 mg, 72%) as a yellow solid, mp 204−206 °C. MS *m/z* 426 (M − Br)$^+$. ^{1}H NMR: δ 3.14 (t, J = 5.6 Hz, 2H), 4.02 (s, 3H), 4.11 (s, 3H), 4.74 (s, 2H), 4.87 (t, J = 5.6 Hz, 2H), 6.07 (s, 2H), 6.96 (s, 1H), 7.14−7.17 (m, 3H), 7.26−7.37 (m, 3H), 7.77 (d, J = 9.6 Hz, 1H), 8.08 (d, J = 9.6 Hz, 1H), 10.03 (s, 1H). Anal. ($C_{27}H_{24}NO_4Br$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-13-methylprotoberberine Chloride (6g). Synthetic intermediate **5b** was obtained from **2** and $NaBH_4$ with a procedure similar to that for compound **5a**. Yield: 59%. Yellow-like solid.

The title compound was obtained from **5b** and formaldehyde with a procedure similar to that for compound **6a**. Yield: 39%. Yellow solid, mp 243−244 °C. MS *m/z* 350 (M − Cl)$^+$. ^{1}H NMR: δ 2.91 (s, 3H), 3.09 (t, J = 6.0 Hz, 2H), 4.02 (s, 3H), 4.12 (s, 3H), 4.63 (t, J = 6.0 Hz, 2H), 6.06 (s, 2H), 6.97(s, 1H), 7.30 (s, 1H), 7.50 (s, 1H), 7.60 (s, 1H), 9.29 (s, 1H). Anal. ($C_{21}H_{20}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-13-*n*-butylprotoberberine Chloride (6h). The title compound was obtained from **5b** and butaldehyde with a procedure similar to that for compound **6a**. Yield: 39%. Yellow solid, 222−224 °C. MS *m/z* 392 (M − Cl)$^+$. ^{1}H NMR: δ 0.92 (t, J = 7.2 Hz, 3H), 1.41 (q, J = 7.2 Hz, 2H), 1.78 (m, 2H), 3.07 (t, J = 5.6 Hz, 2H), 3.35 (s, 2H), 4.00 (s, 3H), 4.13 (s, 3H), 4.63 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.16 (s, 1H), 7.29 (s, 1H), 7.54 (s, 1H), 7.75 (s, 1H), 9.55 (s, 1H). Anal. ($C_{24}H_{26}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-13-*n*-hexylprotoberberine Chloride (6i). The title compound was obtained from **5b** and hexaldehyde with a procedure similar to that for compound **6a**. Yield: 40%. Yellow solid, mp 195−196 °C. MS *m*/*z* 420 (M − Cl)$^+$. ^{1}H NMR: δ 0.84 (t, J = 6.8 Hz, 3H), 1.22−1.27 (m, 4H), 1.39 (m, 2H), 1.78 (m, 2H), 3.06 (t, J = 5.6 Hz, 2H), 3.34 (t, J = 6.8 Hz, 2H), 4.00 (s, 3H), 4.13 (s, 3H), 4.65 (t, J = 5.6 Hz, 2H), 6.18 (s, 2H), 7.17 (s, 1H), 7.28 (s, 1H), 7.54 (s, 1H), 7.79 (s, 1H), 9.65 (s, 1H). Anal. ($C_{26}H_{30}NO_4Cl$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-13-benzylprotoberberine Bromide (6j). The title compound was obtained from **5b** and benzyl bromide with a procedure similar to that for compound **6e**. Yield: 54%. Yellow solid, mp 218−220 °C. MS *m*/*z* 426 (M − Br)$^+$. ^{1}H NMR: δ 3.15 (t, J = 5.6 Hz, 2H), 3.74 (s, 3H), 4.00 (s, 3H), 4.71 (t, J = 5.6 Hz, 2H), 4.77 (s, 2H), 6.08 (s, 2H), 7.04 (s, 1H), 7.17 (s, 1H), 7.14−7.17 (m, 3H), 7.25−7.37 (m, 3H), 7.78 (s, 1H), 9.68 (s, 1H). Anal. ($C_{27}H_{24}NO_4Br$) C, H, N.

Route C (for 7a,b, 8a−l). 2,3-Methylenedioxy-9,10-dimethoxytetrahydroprotoberberine (7a). To a stirred refluxing solution of **2** (3.71 g, 10 mmol) and K_2CO_3 (3.6 g, 30 mmol) in 80% ethanol (60 mL), $NaBH_4$ (0.4 g, 10 mmol) was added portionwise. The reaction mixture was allowed to react at this reflux temperature for 20 min, then at room temperature for 4 h, and generated a lot of white solid. The solid was collected by filtration and then recrystallized from 95% ethanol twice to afford the title compound (2 g, 59%) as a white needle, mp 174−176 °C. MS *m*/*z* 340 (M + H)$^+$. ^{1}H NMR: δ 2.43 (q, J = 3.2 Hz, 1H), 2.55 (q, J = 4.0 Hz, 1H), 2.61 (m, 1H), 2.85−2.93 (m, 1H), 3.08 (q, J = 3.6 Hz, 1H), 3.27−3.36 (m, 2H), 3.35 (d, J = 16.0 Hz, 1H), 3.71 (s, 3H), 3.76 (s, 3H), 4.04 (d, J = 16.0 Hz, 1H), 5.93 (d, J = 2.4 Hz, 2H), 6.65 (s, 1H), 6.84 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.4 Hz, 1H), 6.90 (s, 1H). Anal. ($C_{20}H_{21}NO_4$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxytetrahydroprotoberberine (7b). The title compound was obtained from **2** and $NaBH_4$ with a procedure similar to that for compound **7a**. Yield: 63%. White solid, mp 176−177 °C. MS *m*/*z* 340 (M + H)$^+$. ^{1}H NMR: δ 2.39−2.44 (m, 1H), 2.52−2.61 (m, 2H), 2.85−2.93 (m, 1H), 3.03 (q, J = 3.6 Hz, 1H), 3.26 (d, J = 10.8 Hz, 1H), 3.39 (d, J = 10.8 Hz, 1H), 3.47 (d, J = 14.8 Hz, 1H), 3.70 (s, 6H), 3.85 (d, J = 14.8 Hz, 1H), 5.94 (s, 2H), 6.65 (s, 1H), 6.65 (s, 1H), 6.69 (s, 1H), 6.89 (s, 1H). Anal. ($C_{20}H_{21}NO_4$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-methyltetrahydroprotoberberine Iodide (8a). To a stirred solution of **3** (340 mg, 1.0 mmol) and K_2CO_3 (410 mg, 3.0 mmol) in acetonitrile (40 mL), methyl iodide (3.0 mL, 5.0 mmol) was added. The reaction mixture was stirred at 40 °C for 36 h and filtered to remove undissolved substitutes. The filtrate was evaporated under vacuum, and the residue was washed with ether, then recrystallized from methanol/acetone, affording the title compound (328 mg, 68%) as a white-like solid, mp 233−234 °C. MS *m*/*z* 354 (M − I)$^+$. ^{1}H NMR: δ 2.93 (q, J = 12.0 Hz, 1H), 3.08 (q, J = 9.2 Hz, 1H), 3.14−3.18 (m, 2H), 3.63−3.68 (m, 1H), 3.77 (s, 3H), 3.80 (s, 3H), 3.83 (s, 3H), 3.87−3.94 (m, 1H), 4.72 (d, J = 16.4 Hz, 1H), 4.84 (d, J = 16.4 Hz, 1H), 5.00 (q, J = 4.8 Hz, 1H), 6.05 (d, J = 5.6 Hz, 2H), 6.89 (s, 1H), 6.97 (d, J = 8.8 Hz, 1H), 7.09 (s, 1H), 7.15 (d, J = 8.8 Hz, 1H). Anal. ($C_{21}H_{24}NO_4I$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-ethyltetrahydroprotoberberine Bromide (8b). The title compound was obtained from **7a** and ethyl bromide with a procedure similar to that for compound **8a**. Yield: 26%. Yellow-like powder, mp 218−219 °C. MS *m*/*z* 368 (M − Br)$^+$. ^{1}H NMR: δ 1.20 (t, J = 7.2 Hz, 3H), 3.00−3.08 (m, 4H), 3.68−3.76 (m, 1H), 3.79 (s, 3H), 3.73 (s, 3H), 4.06−4.12 (m, 3H), 4.52 (d, J = 16.4 Hz, 1H), 4.86 (d, J = 16.4 Hz, 1H), 5.16 (q, J = 5.6 Hz, 1H), 6.05 (d, J = 6.4 Hz, 2H), 6.88 (s, 1H), 7.06 (s, 1H), 7.09 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H). Anal. ($C_{22}H_{26}NO_4Br$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-*β*-ethanoltetrahydroprotoberberine Bromide (8c). The title compound was obtained from **7a** and ethanol bromide with a procedure similar to that for compound **8a**. Yield: 29%. Yellow-green solid, mp 238−239 °C. MS *m*/*z* 384 (M − Br)$^+$. ^{1}H NMR: δ 3.03−3.18 (m, 5H), 3.70 (m, 1H), 3.77 (s, 3H), 3.83 (s, 3H), 3.94−3.98 (m, 1H), 4.01 (q, J = 5.2 Hz, 1H), 4.24 (q, J = 5.2 Hz, 1H), 4.55 (d, J = 15.6 Hz, 1H), 5.07 (q, J = 5.2 Hz, 1H), 5.35 (d, J = 15.6 Hz, 1H), 5.37 (t, J = 4.8 Hz, 2H), 6.05 (d, J = 7.2 Hz, 2H), 6.88 (s, 1H), 7.07 (s, 1H), 7.09 (d, J = 8.4 Hz, 1H), 7.14 (d, J = 8.4 Hz, 1H). Anal. ($C_{22}H_{26}NO_5Br$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-*n*-propyltetrahydroprotoberberine Iodide (8d). The title compound was obtained from **7a** and propyl iodide with a procedure similar to that for compound **8a**. Yield: 60%. White-like solid, mp 165−166 °C. MS *m*/*z* 382 (M − I)$^+$. ^{1}H NMR: δ 0.76 (t, J = 7.2 Hz, 3H), 1.55−1.81 (m, 1H), 1.79 (m, 1H), 2.89 (t, J = 8.4 Hz, 2H), 3.05−3.10 (m, 3H), 3.68−3.79 (m, 1H), 3.78 (s, 3H), 3.83 (s, 3H), 4.01 (q, J = 4.8 Hz, 1H), 4.13 (q, J = 4.0 Hz, 1H), 4.55 (d, J = 16.4 Hz, 1H), 4.88 (d, J = 16.4 Hz, 1H), 5.14 (q, J = 5.2 Hz, 1H), 6.05 (d, J = 8.0 Hz, 2H), 6.88 (s, 1H), 7.07 (s, 1H), 7.09 (d, J = 8.8 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H). Anal. ($C_{23}H_{28}NO_4I$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-*n*-butyltetrahydroprotoberberine Bromide (8e). The title compound was obtained from **7a** and butyl bromide with a procedure similar to that for compound **8a**. Yield: 22%. White-like solid, mp 130−131 °C. MS *m*/*z* 396 (M − Br)$^+$. ^{1}H NMR: δ 0.87 (t, J = 7.2 Hz, 3H), 1.18−1.28 (m, 2H), 1.71 (m, 1H), 1.87 (m, 1H), 3.05−3.14 (m, 3H), 3.38−3.40 (m, 2H), 3.65−3.75 (m, 2H), 3.78−3.83 (m, 1H), 3.79 (s, 3H), 3.81 (s, 3H), 4.65 (d, J = 12.4 Hz, 1H), 4.74 (q, J = 5.6 Hz, 1H), 4.80 (d, J = 12.4 Hz, 1H), 6.03 (d, J = 8.0 Hz, 2H), 6.88 (s, 1H), 7.07 (s, 1H), 7.09 (d, J = 9.2 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H). Anal. ($C_{24}H_{30}NO_4Br$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-ethylacetatetetrahydroprotoberberine Bromide (8f). The title compound was obtained from **7a** and ethyl acetate bromide with a procedure similar to that for compound **8a**. Yield: 71%. White-like solid, mp 142−144 °C. MS *m*/*z* 426 (M − Br)$^+$. ^{1}H NMR: δ 1.21 (t, J = 7.2 Hz, 3H), 3.10−3.25 (m, 2H), 3.42 (q, J = 5.6 Hz, 1H), 3.75−3.94 (m, 2H), 3.76 (s, 3H), 3.86 (s, 3H), 4.14−4.26 (m, 2H), 4.22 (q, J = 5.6 Hz, 1H), 4.40−4.49 (m, 2H), 4.90 (d, J = 16.4 Hz, 1H), 4.99 (d, J = 16.4 Hz, 1H), 5.07 (q, J = 5.6 Hz, 1H), 6.02 (d, J = 6.8 Hz, 2H), 6.88 (s, 1H), 6.92 (s, 1H), 7.01 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 8.4 Hz, 1H). Anal. ($C_{24}H_{28}NO_6Br$) C, H, N.

2,3-Methylenedioxy-9,10-dimethoxy-7-benzyltetrahydroprotoberberine Bromide (8g). The title compound was obtained from **7a** and benzyl bromide with a procedure similar to that for compound **8a**. Yield: 70%. White powder, mp 180−181 °C. MS *m*/*z* 430 (M − Br)$^+$. ^{1}H NMR: δ 3.23 (q, J = 14.4 Hz, 1H), 3.38 (q, J = 12.4 Hz, 1H), 3.64−3.71 (m, 3H), 3.74 (s, 3H), 3.87 (s, 3H), 4.10−4.24 (m, 1H), 4.19 (m, 2H), 4.36 (d, J = 16.4 Hz, 1H), 4.48 (d, J = 16.4 Hz, 1H), 5.34 (q, J = 5.6 Hz, 1H), 6.08 (d, J = 5.6 Hz, 2H), 6.95 (s, 1H), 7.14 (s, 1H), 7.14−7.25 (m, 4H), 7.45−7.52 (m, 3H). Anal. ($C_{27}H_{28}NO_4Br$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-7-methyltetrahydroprotoberberine Iodide (8h). The title compound was obtained from **7b** and methyl iodide with a procedure similar to that for compound **8a**. Yield: 40%. White-like solid, mp 197−198 °C. MS *m*/*z* 354 (M − I)$^+$. ^{1}H NMR: δ 2.90 (q, J = 12.8 Hz, 1H), 3.00−3.08 (m, 2H), 3.38 (q, J = 5.6 Hz, 1H), 3.63−3.72 (m, 1H), 3.74 (s, 3H), 3.77 (s, 3H), 3.79 (s, 3H), 3.90 (q, J = 5.6 Hz, 1H), 4.55 (d, J = 12.4 Hz, 1H), 4.59 (d, J = 12.4 Hz, 1H), 5.02 (q, J = 5.6 Hz, 1H), 6.02 (s, 2H), 6.78 (s, 1H), 6.83 (s, 1H), 6.92 (s, 1H), 6.93 (s, 1H). Anal. ($C_{21}H_{24}NO_4I$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-7-ethyltetrahyroprotoberberine Bromide (8i). The title compound was obtained from **7b** and ethyl bromide with a procedure similar to that for compound **8a**. Yield: 30%. White-like solid, mp 226−227 °C. MS *m*/*z* 368 (M − Br)$^+$. ^{1}H NMR: δ 1.34 (t, J = 7.2 Hz, 3H), 2.97−3.15 (m, 2H), 3.33−3.36 (m, 1H), 3.44−3.48 (m, 1H), 3.60−3.64 (m, 2H), 3.72 (s, 3H), 3.74 (s, 3H), 3.91−4.00 (m, 2H), 4.59 (d, J = 13.2 Hz, 1H), 4.71 (d, J = 13.2 Hz, 1H), 5.20 (q, J = 5.6 Hz, 1H), 6.05 (d, J = 6.8 Hz, 2H), 6.82 (s, 1H), 6.91 (s, 1H), 6.95 (s, 1H), 7.02 (s, 1H). Anal. ($C_{22}H_{26}NO_4Br$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-7-*n*-propyltetrahydroprotoberberine Iodide (8j). The title compound was obtained from **7b** and propyl iodide with a procedure similar to that for compound **8a**. Yield: 28%. White-like solid, mp 235−236 °C. MS *m*/*z* 382 $(M - Br)^+$. ^{1}H NMR: δ 0.76 (t, J = 7.2 Hz, 3H), 1.66−1.72 (m, 1H), 1.74−1.80 (m, 1H), 2.84−2.94 (m, 2H), 3.05−3.10 (m, 3H), 3.70−3.74 (m, 1H), 3.76 (s, 3H), 3.77 (s, 3H), 3.94−4.00 (m, 2H), 4.58 (d, J = 15.6 Hz, 1H), 4.71 (d, J = 15.6 Hz, 1H), 5.17 (q, J = 5.6 Hz, 1H), 6.05 (d, J = 6.0 Hz, 2H), 6.88 (s, 1H), 6.89 (s, 1H), 6.92 (s, 1H), 7.02 (s, 1H). Anal. ($C_{23}H_{28}NO_4I$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-7-ethylacetatetetetrahydroprotoberberine Bromide (8k). The title compound was obtained from **7b** and ethyl acetate bromide with a procedure similar to that for compound **8a**. Yield: 60%. White solid, mp 140−141 °C. MS *m*/*z* 426 $(M - Br)^+$. ^{1}H NMR: δ 1.23 (t, J = 7.2 Hz, 3H), 3.07−3.26 (m, 2H), 3.44 (q, J = 5.6 Hz, 1H), 3.74 (s, 6H), 3.82 (s, 2H), 3.96 (q, J = 7.2 Hz, 1H), 4.17 (m, 2H), 4.42 (s, 2H), 4.80 (d, J = 15.6 Hz, 1H), 4.96 (d, J = 15.6 Hz, 1H), 5.04 (q, J = 5.6 Hz, 1H), 6.05 (d, J = 8.0 Hz, 2H), 6.88 (s, 1H), 6.92 (s, 1H), 6.94 (s, 1H), 7.03 (s, 1H). Anal. ($C_{24}H_{28}NO_6Br$) C, H, N.

2,3-Methylenedioxy-10,11-dimethoxy-7-benzyltetrahydroprotoberberine Bromide (8l). The title compound was obtained from **7b** and benzyl bromide with a procedure similar to that for compound **8a**. Yield: 50%. White solid, mp 178−179 °C. MS *m*/*z* 430 $(M - Br)^+$. ^{1}H NMR: δ 3.19 (m, 1H), 3.30−3.36 (m, 2H), 3.53 (q, J = 5.6 Hz, 1H), 3.56−3.66 (m, 2H), 3.74 (s, 3H), 3.78 (s, 3H), 4.06−4.22 (m, 3H), 4.53 (d, J = 15.6 Hz, 1H), 5.38 (q, J = 5.6 Hz, 1H), 6.08 (d, J = 3.6 Hz, 2H), 6.83 (s, 1H), 6.96 (s, 1H), 7.01 (s, 1H), 7.09 (s, 1H), 7.21 (s, 1H), 7.23 (s, 1H), 7.46−7.55 (m, 3H). Anal. ($C_{27}H_{28}NO_4Br$) C, H, N.

Biological Methods. Cell Culture. HepG2 and Bel-7402 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C with 5% CO_2. At 24 h before drug treatment, cells were trypsinized and grown in RPMI-1640 containing 10% lipoprotein-deficient serum (LPDS, Sigma). When cells reached about 80% confluence, media were switched to RPMI-1640 containing 0.5% LPDS and supplemented with **1** (Sigma) or **2** or simvastatin (Merck, Rahway, NJ) at the concentrations indicated. Cells were treated for 12 h before harvest for RNA extraction.

RNA Extraction and Real-Time RT-PCR. Total RNAs were isolated from cells or animal livers using the Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX) according to the supplier's protocol. For reverse transcription, 1 μg of total cellular or liver RNA from each sample was used as template in a 20 μL reaction system (Promega, Madison, WI). The reverse transcription reactions were conducted at 42 °C for 30 min and then inactivated at 95 °C for 5 min. Real-time RT-PCR was performed with these cDNAs using the Applied Biosystems 7500 real-time RT-PCR system and the 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). In each of the 20 μL real-time RT-PCR reaction, 2 μL of cDNA, 10 μL of the Universal PCR Master Mix, and 1 μL of the 20 × TaqMan gene expression assay reagent were used. Each experiment was repeated at least 3 times. The comparative threshold cycle (C_T) method was used in relative gene expression quantification with the GAPDH as the endogenous control.

Flow Cytometry. Cells were detached and fixed in 2% paraformaldehyde at 4 °C overnight. After blocking, cells were incubated on ice for 1 h with a rabbit-polyclonal antibody against LDLR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with a dilution of 1:25. Normal rabbit IgG was used as a control for background staining. Then cells were washed and stained on ice for 30 min with a FITC-conjugated goat-antirabbit IgG (Santa Cruz) with a dilution of 1:50. After washing, the fluorescent intensities on the cell surface were analyzed in a FACSCalibur system (BD Biosciences, San Jose, CA).

Animal Experiments. Male Sprague−Dawley rats (180 ± 10 g) and C57BL/6J mice (21 ± 2 g) were obtained from the Institute of Laboratory Animal Science (Beijing, China). Animals were cared according to the institutional guidelines of the Chinese Academy of Medical Sciences. Animals were housed in an air-conditioned room with 3−5 rats/mice per cage, and with a 12 h light and 12 h dark cycle. After 1 week of accommodation period, all of the animals were fed with HFHC diet containing 2% cholesterol, 10% yolk powder, 15% lard, and 0.2% of sodium cholate for 8 weeks. Five of the rats and mice were fed with regular rodent chow serving as normal diet controls. The HFHC as well as the regular rodent chow was from Institute of Laboratory Animal Science (Beijing, China).

Hyperlipidemic animals (rats or mice) were untreated (n = 6) or treated with 100 (mg/kg)/day of **1** (n = 8) or **2** (n = 8) orally for 30 days for rat experiment or 21 days for mouse experiment. Study compounds were suspended in saline (1 mL/rat, 0.2 mL/mice) prior to use. Compound **1** or **2** was orally administered to the animals using gavage twice a day at 8 a.m. and 5 p.m., respectively, and simvastatin was given once a day at 5 p.m. Before, after, and during the treatment, blood samples were taken by tail snip after fast. CHO, LDL-c, HDL-c, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CRE) levels were assayed using commercially available kits. In another set of experiments, in order to observe the dose-dependent effect of **2**, the hyperlipidemic rats were untreated (n = 6) or treated with **2** at 100 (mg/kg)/day (n = 8) or 300 (mg/kg)/day (n = 8), respectively, for 17 days. Before and after treatment, blood lipid levels were assayed.

At the end of the treatment course, animals were sacrificed; their livers were dissected and quickly frozen at −80 °C. Liver tissues were homogenized with the Ultraspec RNA lysis solution, and total RNAs were isolated. After reversely transcribed into cDNAs, the liver mRNA levels of LDLR and HMG-CoA reductase were analyzed with real-time RT- PCR.

Acute Toxicity. Male Kunming mice (19 ± 1 g) were purchased from the Institute of Laboratory Animal Science. They were fed with regular rodent chow and housed in an air-conditioned room. Then they were randomly divided into four groups with six mice each, and received saline (control) or **2** (at 250, 1000, or 5000 mg/kg). **2** was given orally in a single-dosing. After treatment, animals were weighed every day during the experiment. Fourteen days later, animals were sacrificed; their livers, kidneys, hearts, lungs, brains, and colons were fixed in 10% formaldehyde at room temperature for histological examination using hematoxylin and eosin (H&E) staining.

Statistical Analysis. The in vitro gene expression data were analyzed by the Student's t-test. For animal experiments, after validation of the test for homogeneity of variance, results were examined by one-way ANOVA followed by the Newman−Keuls test for multiple comparisons. $P < 0.05$ was considered as statistically significant.

Acknowledgment. This work was supported by the IMB Research Foundation.

Supporting Information Available: Element analysis data of the studied analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Stamler, J.; Daviglus, M. L.; Garside, D. B.; Dyer, A. R.; Greenland, P.; Neaton, J. D. Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. *J. Am. Med. Assoc.* **2000**, *284*, 311–318.

(2) Anderson, K. M.; Castelli, W. P.; Levy, D. Cholesterol and mortality: 30 years of follow-up from the Framingham study. *J. Am. Med. Assoc.* **1987**, *257*, 2176–2180.

(3) Grundy, S. M.; Cleeman, J. I.; Merz, C. N., Jr.; Clark, L. T.; Hunninghake, D. B.; Pasternak, R. C., Jr.; Stone, N. J. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. *Circulation* **2004**, *110*, 227–239.

(4) Kong, W. J.; Liu, J.; Jiang, J. D. Human low-density lipoprotein receptor gene and its regulation. *J. Mol. Med.* **2006**, *84*, 29–36.

(5) Kong, W. J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H.; Wang, S.; Wu, J.; Wang, Y.; Li, Z.; Liu, J.; Jiang, J. D. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat. Med.* **2004**, *10*, 1344–1351.
(6) Li, B. X.; Zhang, M. S.; Bao, L. H. Study on the pharmacokinetics of berberine after oral administration in human being. *J. Harbin Med. Univ.* **1995**, *29*, 382–384.
(7) Zhao, W.; Xue, R.; Zhou, Z. X.; Kong, W. J.; Jiang, J. D. Reduction of blood lipid by berberine in hyperlipidemic patients with chronic hepatitis or liver cirrhosis. *Biomed. Pharmacother.* **2008**, *62*, 730–731.
(8) Kong, W. J.; Wei, J.; Zuo, Z. Y.; Wang, Y. M.; Song, D. Q.; You, X. F.; Zhao, L. X.; Pan, H. N.; Jiang, J. D. Combination of simvastatin with berberine improves the lipid-lowering efficacy. *Metabolism* **2008**, *57*, 1029–1037.
(9) Yang, P.; Song, D. Q.; Li, Y. H.; Kong, W. J.; Wang, Y. X.; Gao, L. M.; Liu, S. Y.; Cao, R. Q.; Jiang, J. D. Synthesis and structure−activity relationships of berberine analogues as a novel class of low-density lipoprotein receptor up-regulators. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4675–4677.
(10) Feng, Z.; Norio, N.; Teruaki, A.; Masao, H. Pharmacokinetics of berberine and its main metabolites in conventional and pseudo germ-free rats determined by liquid chromatography ion trap mass spectrometry. *Drug Metab. Dispos.* **2006**, *34*, 2064–2072.
(11) Pan, J. F.; Yu, C.; Zhu, D. Y.; Zhang, H.; Ren, J. Y. Preparation of Six Isoquinoline Alkaloid Salt. CN 1314347, 2001.
(12) Wang, L. J.; Ye, X. L.; Li, X. G.; Sun, Q. L.; Yu, G.; Cao, X. G.; Liang, Y. T.; Zhang, H. S.; Zhou, J. Z. Synthesis and antimicrobial activity of 3-alkoxyjatrorrhizine derivatives. *Planta Med.* **2008**, *74*, 290–292.
(13) Zhang, W. J.; Ou, T. M.; Lu, Y. J.; Huang, Y. Y.; Wu, W. B.; Huang, Z. S.; Zhou, J. L.; Wong, K. Y.; Gu, L. Q. 9-Substituted berberine derivatives as G-quadruplex stabilizing ligands in telomeric DNA. *Bioorg. Med. Chem.* **2007**, *15*, 5493–5501.
(14) Iwasa, K.; Kamigauchi, M.; Sugiura, M.; Nanba, H. Antimicrobial activity of some 13-alkyl substituted protoberberinium salts. *Planta Med.* **1997**, *63*, 196–198.
(15) Ikekawa, T.; Ikeda, Y. Antitumor activity of 13-methylberberrubine derivatives. *J. Pharmacobio-Dyn.* **1982**, *5*, 469–474.
(16) Park, K. D.; Lee, J. H.; Kim, S. H.; Kang, T. H.; Moon, J. S.; Kim, S. U. Synthesis of 13-(substituted benzyl)berberine and berberrubine derivatives as antifungal agents. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3913–3916.
(17) Ikekawa, T.; Shimada, F.; Jong-Chol, C.; Uebaba, K. 13-Propylberberine Salts. EP 184118 A1, 1986.
(18) Wang, R. F.; Xu, G. Y.; Hua, W. Y.; Peng, S. X. Synthesis and anti-arrhythmic activity of some tetrahydroberberine quaternary ammonium compounds. *Chin. J. Med. Chem.* **1996**, *4*, 235–248.
(19) Qin, Y.; Pang, J. Y.; Chen, W. H.; Cai, Z.; Jiang, Z. H. Synthesis, DNA-binding affinities, and binding mode of berberine dimers. *Bioorg. Med. Chem. Lett.* **2006**, *14*, 25–32.
(20) Abidi, P.; Chen, W.; Kraemer, F. B.; Li, H.; Liu, J. W. Identification of medicinal plant goldenseal as a natural cholesterol-lowering agent: mechanisms of actions and new modulators of LDL receptor expression. *J. Lipid Res.* **2006**, *47*, 2134–2147.

JM801157Z