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HYPOLIPIDEMIC BERBERINE DERIVATIVES WITH A REDUCED AROMATIC RING C

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A series of aromatic and reduced berberine derivatives were synthesized. Their hypocholesterolemic activity was studied in vitro *and* in vivo*. As a rule, berberine derivatives containing a reduced ring C were more capable of increasing the LDL receptor gene expression than the corresponding aromatic derivatives. Tests using a Triton WR1339-induced hypercholesterolemia model showed that 9-*O*-tosyltetrahydroberberine and 12-bromotetrahydroberberine possessed pronounced hypocholesterolemic activity and reduced the total cholesterol level by 33 and 27%; the triglyceride level, by 25 and 26%, respectively.*

Keywords: berberine, isoquinoline alkaloids, tetrahydroisoquinoline, LDL receptors, Triton WR1339-induced hypercholesterolemia, hypolipidemic activity.

Atherosclerosis has now reached epidemic proportions. Populations in developed countries are more susceptible to this disease because of the abundant and incorrect diet. Atherosclerosis leads to the development of various cardiovascular diseases, the most threatening of which are myocardial infarction and stroke [1]. The principal criterion of therapeutic effectiveness for treating atherosclerosis is a reduction of the total plasma cholesterol and triglycerides levels. An increase of high density lipoproteins (HDL) and decrease of low density lipoproteins (LDL) that adjust their ratio are also important for humans. A high LDL level causes cholesterol deposits to form on blood-vessel walls and atherosclerotic plaque to develop [2]. The current lead drugs are HMG-CoA-reductase inhibitors or statins, which have proved effective in the battle against this pathology. However, statins not are only highly effective but also have side effects such as elevated plasma concentrations of liver enzymes, increased myopathy and myalgia, and, in certain cases, rhabdomyolysis [3]. Furthermore, HMG-CoA-reductase catalyzes the rate-limiting step in the metabolic synthesis pathways of cholesterol, sterols, and isoprenoids. Limiting their synthesis could produce various pathologies because they are required for cell growth and differentiation [1]. Therefore, the search continues for new effective molecules that can decrease the plasma cholesterol and triglyceride levels through different mechanisms.

The isoquinoline alkaloid berberine (**1**) is broadly distributed and was capable of lowering the cholesterol and triglyceride levels in both animal models and patients with hyperlipidemia [4–6]. It was thought that the principal hypolipidemic mechanism of action of berberine was increased expression of LDL receptors (LDLRs) in liver cells [7, 8]. However, these processes were not fully studied. Another possible mechanism of action could be the ability to block enzyme PCSK9, which is an important cholesterol homeostasis regulator that binds to and subsequently degrades the LDLR [9].

The chemistry of berberine derivatives has recently developed rapidly [10–14]. However, berberine derivatives are little studied despite the large number of publications focused on the hypolipidemic activity of berberine itself. Thus, LDLR gene expression by several semisynthetic berberine derivatives was studied [15, 16]. The hypolipidemic activity of pseudoberberine [16] and berberoylpalmitate [17] was studied using Sprague–Dawley rats and C57BL/6J mice on a high-calorie and high-fat diet. However, many well-known berberine derivatives were not tested for hypolipidemic activity.

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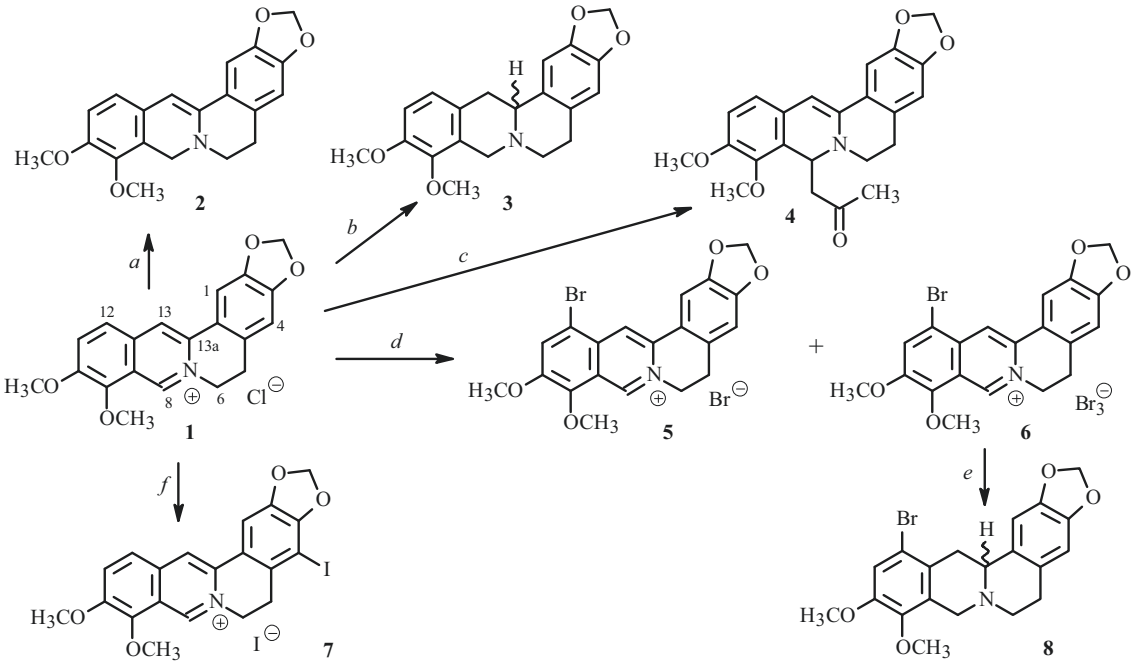
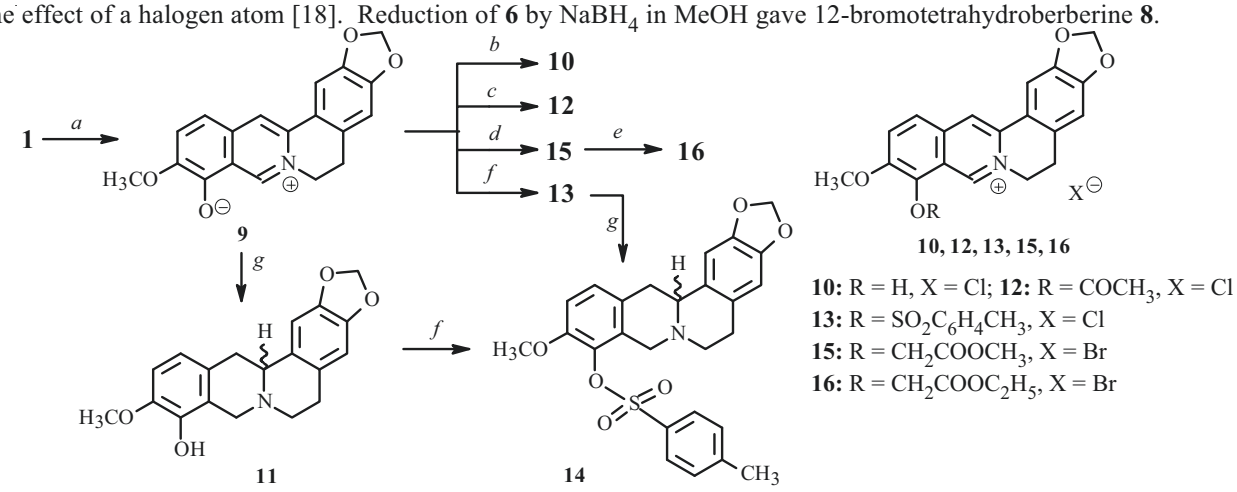


a. $LiAlH_4$, THF, RT; *b*. $NaBH_4$, MeOH, RT; *c*. H_2O, 50% NaOH, CH_3COCH_3, RT; *d*. Br_2, AcOH, 120°C; *e*. $NaBH_4$, MeOH, RT; *f*. HgO, TFA, 120°C, KI, I_2, MeOH, 80°C

Scheme 1

Preliminary studies of the influence of halo-substituted berberine derivatives on increased LDLR gene expression were published by us [18]. Herein, we present results from further research on this topic. The goal was to synthesize several aromatic and reduced berberine derivatives (both new and previously known), to study their influence on increased LDLR gene expression levels and hypocholesterolemic activity using the Triton WR1339-induced hypercholesterolemia model, and to determine the structure–activity relationship.

Synthesis of Berberine Derivatives. A series of berberine derivatives, several transformations of which were described by us [18, 19], were synthesized in order to study the structure–activity relationship. One or two double bonds in berberine ring C were selectively reduced in order to study the effect of aromaticity. Berberine chloride reduction by $LiAlH_4$ gave dihydroberberine **2** [20]; by $NaBH_4$ in MeOH, tetrahydroberberine **3** [21] (Scheme 1). Condensation of berberine chloride with Me_2CO produced acetonylberberine **4** [22]. Bromo-derivatives **5** and **6** and iodo-derivative **7** were prepared in order to study the effect of a halogen atom [18]. Reduction of **6** by $NaBH_4$ in MeOH gave 12-bromotetrahydroberberine **8**.



10, 12, 13, 15, 16

10: R = H, X = Cl; **12:** R = $COCH_3$, X = Cl
13: R = $SO_2C_6H_4CH_3$, X = Cl
15: R = CH_2COOCH_3, X = Br
16: R = $CH_2COOC_2H_5$, X = Br

a. 30 mmHg, 190°C, 15 min; *b*. HCl, EtOH, RT; *c*. CH_3COCl, CH_2Cl_2, RT; *d*. $BrCH_2COOCH_3$, CH_2Cl_2, 40°C; *e*. EtOH, morpholine, reflux; *f*. *p*-TosCl, NEt_3, CH_2Cl_2, RT; *g*. $NaBH_4$, MeOH, RT

Scheme 2

According to the literature, berberine is metabolized by the liver to four major products [23]. Of these, berberrubine **9** possessed the greatest effect on LDLR gene expression [17]. We synthesized berberrubine **9** [24] and its derivatives such as protonated form **10** [19], tetrahydro-derivative **11** (reduction by $NaBH_4$ in MeOH), and acetate **12** [25, 26] and *p*-toluenesulfonate

13 via reaction with acetylchloride or *p*-toluenesulfonyl chloride (Scheme 2). Tetrahydro-derivative **14** was obtained via reduction of **13** by $NaBH_4$ in MeOH or via tosylation of **11**. The methyl ester of berberoyl-*O*-acetic acid **15** was synthesized by reacting **9** with methyl bromoacetate. Refluxing **15** in EtOH in the presence of morpholine gave ethyl ester **16** [19]. The preparations of **8** from **6** and of **14** from **13** were not previously reported. The ^{13}C NMR spectrum of **14** was presented for the first time.

LDLR Gene Expression. The effects of **1–16** on LDLR gene mRNA expression levels in HepG2 hepatoma liver cells at concentrations of 20 g/mL were tested. The LDLR gene expression level was determined using the real-time reverse-transcriptase polymerase chain reaction (RT-PCR). The LDLR gene expression level after incubation with the tested compounds was calculated relative to an untreated control.

The LDLR gene expression levels for **1**–**16** at concentrations of 20 μg/mL are given below:

Compound	*Expression level, times*	*Compound*	*Expression level, times*
1	3.6 ± 0.7	**9**	1.0 ± 0.1
2	4.8 ± 1.5	**10**	1.0 ± 0.2
3	1.7 ± 0.2	**11**	2.8 ± 1.3
4	1.5 ± 0.5	**12**	1.7 ± 0.2
5	1.1 ± 0.01	**13**	3.5 ± 0.03
6	1.2 ± 0.1	**14**	3.1 ± 0.2
7	0.9 ± 0.04	**15**	0.9 ± 0.03
8	2.1 ± 0.6	**16**	1.1 ± 0.04.

Compounds **1**–**3**, **8**, **11**, **13**, and **14** increased LDLR gene mRNA expression by 1.7–4.8 times ($p < 0.05$) compared with the control. In contrast, **4**–**7**, **9**, **10**, **12**, **15**, and **16** did not affect LDLR gene expression. Dihydroberberine (**2**) had the highest activity, increasing LDLR gene expression by 4.8 times. This was statistically significantly greater than that of berberine (**1**) (increased expression by 3.6 times, $p < 0.05$). Aromatic tosylate **13** and its reduced derivative **14** had effects similar to that of berberine (LDLR gene mRNA expression level increased by 3.5 and 3.1 times). Tetrahydroberberrubine (**11**) (increased by 2.8 times), bromotetrahydroberberine (**8**) (2.1 times), and tetrahydroberberine (**3**) (1.7 times) were slightly less active.

Our results for several compounds differed from those published earlier. It was reported that **3** did not exhibit statistically significant activity [15] whereas **4** [15] and **10** [17] increased LDLR expression by 1.5 and 1.8 times, respectively. Compounds **11** and **14** showed relatively high activity compared with berberine (LDLR mRNA expression levels 1.8 and 2.6, respectively) [15]. We found similar LDLR mRNA expression levels for these compounds but berberine was significantly more active in our experiments. The differences in the compound activities probably varied over a certain range depending on the experimental conditions.

The influence on LDLR gene mRNA expression levels in HepG2 liver hepatoma cells of pairs of aromatic and reduced berberine derivatives **1** and **2** (increase by 3.6 and 4.8 times), **6** and **8** (1.2 and 2.1 times), and **9** and **11** (0.9 and 2.8 times) showed that reduced **2**, **8**, and **11** were more active than aromatic **1**, **6**, and **9**. The exception was reduced **3**, which was less active (1.7 times) than berberine. The pair of aromatic **13** and reduced **14** were both highly active for LDLR gene mRNA expression (3.5 and 3.1 times). Apparently, additional research should be performed to determine if this is a general trend.

Triton WR1339-induced Hyperlipidemia. The ability to reduce cholesterol and triglyceride levels *in vivo* was the next step for berberine derivatives **1**–**3**, **8**, **11**, **13**, and **14**, which possessed *in vitro* activity. For this, the well-known Triton WR1339-induced hyperlipidemia model was used [27]. It is commonly thought that Triton WR1339 causes hyperlipidemia to develop because of disruption of lipoprotein circulation that holds cholesterol and triglycerides in plasma and increases cholesterol biosynthesis in the liver. We studied only the total cholesterol and triglyceride concentrations because the lipemic plasma obtained after adding Triton WR1339 did not allow the lipoprotein contents to be measured.

All compounds were administered orally once daily for four days. On the fourth day, Triton WR1339 was injected i.p. Blood was taken for analysis after one day. Only compounds **8** and **14** showed pronounced hypolipidemic activity in the conducted experiment. Table 1 shows that the total cholesterol (TC) and triglyceride (TG) levels increased significantly from 2.79 mM (TC, control) and 2.16 mM (TG, control) to 12.28 mM (TC) and 4.86 mM (TG), respectively, in untreated animals after injecting Triton WR1339. The plasma lipid contents were statistically significantly less ($p < 0.05$) at 8.96 and 8.27 mM in animals treated beforehand with **8** and **14** (100 mg/kg), respectively. These parameters for **8** and **14** were reduced with respect to the control, which received only Triton WR1339, by 27 and 33% for TC and 26 and 25% for TG. The positive control was the statin simvastatin, the hypolipidemic effect of which at a dose of 20 mg/kg was 1.4–1.7 times greater than those of **8** and **14**.

TABLE 1. Hypolipidemic Activity of Berberine Derivatives in the Triton WR1339-Induced Hyperlipidemia Model at a Dose of 100 mg/kg

Compound	Cholesterol, mM	Change relative to control, %	Triglycerides, mM	Change relative to control, %
Untreated animals	2.79 ± 0.19		2.16 ± 0.06	
Triton WR1339 (control)	12.28 ± 0.70		4.86 ± 0.39	
Simvastatin, 20 mg/kg	6.62 ± 1.01*	–46	3.40 ± 0.42*	–30
1	11.40 ± 2.34	–7	4.47 ± 0.94	–8
2	11.02 ± 0.56	–10	6.53 ± 0.37*	34
3	14.03 ± 0.82	14	7.65 ± 0.63*	57
6	11.02 ± 1.18	–10	5.26 ± 0.07	8
7	12.65 ± 0.99	3	5.98 ± 0.62	23
8	8.96 ± 1.25*	–27	3.58 ± 0.39*	–26
10	13.81 ± 1.19	12	7.56 ± 0.53*	56
11	13.04 ± 0.88	6	3.96 ± 0.29	–19
13	13.55 ± 1.38	10	4.01 ± 0.59	–17
14	8.27 ± 0.97*	–33	3.64 ± 0.44*	–25
15	11.19 ± 0.84	–9	4.72 ± 0.55	–3

*$p < 0.05$ relative to the control, Triton WR1339.

The remaining compounds did not reduce the TC and TG levels. Conversely, the groups receiving **2** and **3** showed statistically significant ($p < 0.05$) increases of TG by 34 and 57%, respectively. It is noteworthy that the compounds that increased LDLR gene expression the most and berberine itself (**1**) did not exhibit hypolipidemic activity in the *in vivo* experiment.

Thus, we studied the hypolipidemic activity of several aromatic and reduced berberine derivatives. Reduced berberine derivatives **2**, **14**, **11**, and **8**; aromatic derivative **13**; and berberine (**1**) itself showed the best results for increased LDLR gene expression *in vitro*. The ability to increase LDLR gene expression was an important but insufficient property. Highly promising **1**, **2**, **11**, and **13** were inactive for decreasing the cholesterol level in the WR1339-induced hypercholesterolemia model. On the other hand, **14** and, rather unexpectedly, **8** showed a pronounced ability to decrease the cholesterol and triglyceride levels. These were berberine derivatives with a reduced ring C and contained a 9-*O*-*p*-toluenesulfonate (**14**) or a Br atom on C-12 (**8**). Further research on hypolipidemic agents among berberine derivatives containing one or both of these groups or their close analogs is highly promising.

EXPERIMENTAL

Melting points were measured on a Mettler Toledo FP900 apparatus. UV spectra were recorded in EtOH (c 10^{-4} M) on an HP 8453 UV-Vis instrument. IR spectra were taken from KBr pellets on a Vector 22 instrument. Mass spectra were measured in a Bruker micrOTOF-Q hybrid quadrupole–time-of-flight mass spectrometer. The mass detection parameters were electrospray ionization at atmospheric pressure (API-ES), positive ions scanned in the range m/z 100–3000, drying gas (N_2) flow rate 4 L/min and temperature 220°C, and sprayer pressure 1.0 bar. PMR and ^{13}C NMR spectra were recorded on a Bruker AM-400 instrument (400.13 and 100.61 MHz) for 5-10% solutions in DMSO-d_6. Residual solvent resonances were used as standards. Column chromatography was performed over basic Al_2O_3 LL40/250. HPLC used a Milikhrom A-02 microcolumn chromatograph (ZAO EkoNova, Novosibirsk) and a standard chromatography column packed with Pronto SIL-120-5-C18 AQ reversed-phase sorbent (2 × 75 mm, 5 μm) at 35°C, 30–36 atm, and flow rate 150 μL/min. The injected volume was 2 μL. Gradient elution used solvents A (0.1% trifluoroacetic acid in H_2O) and B (MeOH) from 100% A to 100% B over 25 min with simultaneous multi-wavelength detection at 220, 240, 260, 280, 320 and 360 nm.

Berberine chloride [mp 180°C (dec.)] was isolated from *Berberis sibirica* roots (species defined by Cand. Chem. Sci. I. V. Nechepurenko) collected in Ongudaysky District, Altai Republic (Russia) in July 2014. The spectral characteristics of berberine chloride agreed with those in the literature [28]. Elemental analyses corresponded to those calculated for all synthesized compounds and berberine chloride monohydrate.

12-Bromotetrahydroberberine (8). A suspension of **6** (2.50 g) in MeOH (50 mL) at 20°C was stirred on a magnetic stirrer, treated in portions with $NaBH_4$ (0.73 g, 5 eq), and stirred at room temperature for 3 h. The solvent was vacuum

distilled. The residue was dissolved in CH_2Cl_2 and passed over a layer of Al_2O_3. Eluent fractions containing product were combined and evaporated *in vacuo*. The residue was recrystallized from MeCN to afford **8** (1.28 g, 80%). $C_{20}H_{20}NO_4Br$, mp 162.5°C. Mass spectrum (API-ES, *m/z*): 420.062 ($M^+ + 1$), calcd 420.063. ^1H NMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.54–2.68 (2H, m, H-5, 6), 3.03–3.20 (3H, m, H-5, 6, 13), 3.25 (1H, dd, J = 16.7, 4.0, H-13), 3.45–3.51 (2H, m, H-8, 14), 3.81 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 4.19 (1H, d, J = 15.9, H-8), 5.91 (2H, s, OCH_2O), 6.57 (1H, s, H-4), 6.77 (1H, s, H-1), 7.01 (1H, H-11). ^{13}C NMR spectrum (100 MHz, $CDCl_3$, δ, ppm): 29.36 (C-5), 37.43 (C-13), 50.93 (C-6), 53.83 (C-8), 55.89 (10-OCH_3), 59.41 (C-14), 60.12 (9-OCH_3), 100.72 (OCH_2O), 105.51 (C-1), 108.29 (C-4), 114.61 (C-11), 118.40 (C-12), 120.00 (C-8a), 126.84 (C-12a), 127.54 (C-4a), 130.29 (C-1a), 144.26 (C-9), 145.92 , 146.08 (C-2, 3), 150.65 (C-10).

Tetrahydroberberrubine (11). A suspension of **9**–EtOH solvate (1:1, 2.00 g) in MeOH (20 mL) at 0°C was stirred on a magnetic stirrer, treated in portions with $NaBH_4$ (0.83 g, 4 eq), and stirred at room temperature for 1.5 h. The solvent was vacuum distilled. The residue was dissolved in CH_2Cl_2 and passed over a layer of Al_2O_3. Eluent fractions containing product were combined and evaporated *in vacuo*. The residue was recrystallized from MeCN to afford **11** (0.682 g, 78%). The spectral data agreed with those published [29].

9-Demethyl-9-*O*-tosyltetrahydroberberine (14). A) A suspension of **13** (230 mg) in MeOH (5 mL) at 20°C was stirred on a magnetic stirrer, treated in portions with $NaBH_4$ (68 mg, 4 eq), and stirred at room temperature for 2 h. The solvent was vacuum distilled. The residue was dissolved in CH_2Cl_2 and passed over a layer of Al_2O_3. Eluent fractions containing product were combined and evaporated *in vacuo*. The residue was recrystallized from MeOH to afford **14** (67 mg, 31%).

B) A solution of **11** (800 mg) in CH_2Cl_2 (10 mL) was stirred on a magnetic stirrer at room temperature, treated dropwise with a solution of *p*-toluenesulfonyl chloride (705 mg) in CH_2Cl_2 (10 mL) and NEt_3 (0.51 mL), stirred for 1 h, left overnight, and evaporated. The residue was chromatographed over a column of silica gel [CH_2Cl_2 and CH_2Cl_2–MeOH (100:1) eluents]. Eluent fractions containing product were combined and evaporated *in vacuo*. The residue was recrystallized from MeCN to afford **14** (843 mg, 71%). $C_{26}H_{25}NO_6S$, mp 184.3°C. Mass spectrum (API-ES, *m/z*): 480.149 ($M^+ + 1$), calcd 480.148. ^1H NMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.45 (3H, s, CH_3), 2.52–2.68 (2H, m, H-5, 6), 2.78 (1H, dd, J = 16.3, 11.4, H-13ax), 3.00–3.12 (2H, m, H-5, 6), 3.22 (1H, dd, J = 16.3, 3.7, H-13eq), 3.45 (3H, s, OCH_3), 3.54 (1H, dd, J = 11.4, 3.7, H-14), 3.57 (1H, d, J = 15.8, H-8), 4.18 (1H, d, J = 15.9, H-8), 5.90 (2H, s, OCH_2O), 6.56 (1H, s, H-4), 6.68 (1H, s, H-1), 6.69 (1H, d, J = 8.2, H-12), 6.99 (1H, d, J = 8.2, H-11), 7.33 (2H, d, J = 8.2, H-3′, 5′), 7.86 (2H, d, J = 8.2, H-2′, 6′). ^{13}C NMR spectrum (100 MHz, $CDCl_3$, δ, ppm): 23.92 (CH_3), 31.76 (C-5), 38.37 (C-13), 53.32 (C-6), 56.34 (C-8), 57.68 (OCH_3), 61.63 (C-14), 103.03 (OCH_2O), 107.69 (C-1), 110.69 (C-4), 112.94 (C-12), 129.88 (C-11), 130.03, 130.30 (C-12a, 4a), 130.65 (C-2′, 6′), 131.54 (C-3′, 5′), 132.88, 132.89 (C-8a, 1a), 136.92 (C-1′), 137.73 (C-4′), 146.99 (C-9), 148.22, 148.40 (C-2, 3), 152.07 (C-10).

Cell Cultivation. HepG2 cells were cultivated at 37°C and 5% CO_2 in 25-mL vials with DMEM/F12 medium containing FBS (10%), glutamine (0.03%), and kanamycin at a concentration of 100 μg/mL. Cells in the exponential growth phase were seeded 24 h before the experiment in wells of a 24-well plate and cultivated in DMEM/F12 medium containing lipoprotein-deficient serum (LPDS, 10%, Sigma) and glutamine (0.03%) up to an 80% density monolayer. Then, the growth medium was replaced by DMEM/F12 without serum with added **1**–**16** at concentrations of 20 μg/mL. Medium in the control sample was replaced by DMEM/F12 without serum. Cells were incubated with drugs for 12 h, after which the growth medium was removed. Cells were lysed by TRIzol reagent (Invitrogen) for subsequent mRNA isolation.

Assay of LDLR Gene mRNA by Real-time RT-PCR. Total cellular RNA was isolated according to the manufacturer's recommendations. cDNA was synthesized by reverse transcriptase (RT) at 42°C for 45 min in a reaction mixture (20 μL) containing Tris-HCl (10 mM, pH 8.5), $MgCl_2$ (5 mM), DTT (10 mM), KCl (100 mM), dNTP (0.2 mM), Stat-9 primer (10 ng/μL), RNA polymerase (MoMLV, 200 U), and total RNA (200 ng to 1 μg). The real-time PCR method determined in each of the cDNA samples the relative amount of cDNA of the LDLR gene. Primer F: 5′-ACTGCAAGGACAAATCTGACGA-5′ and R: 5′-CCTCGCAGAGTGTCACATTAAC-5′ was used for amplification. cDNA samples were amplified in a reaction mixture (20 μL) containing Tris-HCl (10 mM, pH 8.9), $MgCl_2$ (2.5 mM), KCl (55 mM), dNTP (0.2 mM), Taq polymerase (0.5 U), and SYBR Green I (Invitrogen) at a 1:25,000 dilution in addition to the corresponding pair of F and R primers (each 300 nM) and cDNA sample. The results were normalized relative to a coefficient that was calculated as the average geometric amount of mRNA for POLR2A, GAPDH, RPL32, and ACTB genes.

Laboratory Animals. The experiments used white male laboratory mice (20–35 g) from the Experimental Animal Nursery at the Institute of Cytology and Genetics, SB, RAS, and were kept under standard conditions. Experiments with animals were conducted according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasburg, 1986). Each group consisted of 10 animals.

Triton WR1339-induced Hyperlipidemia. The tested compounds were mixed with several drops of Tween-80 and distilled H_2O. The resulting suspension was injected intragastrically once per day for four days calculated as 0.2 mL/10 g of body mass at a dose of 100 mg/kg. All test and control animals were injected i.p. one hour after the last drug injection with Triton WR1339 solution (7%, Tyloxapol, CAS 25301-02-4, Sigma) in normal saline calculated as 0.1 mL/10 g of body mass. The animals were fasted after this and sacrificed after 24 h. Blood from jugular veins was collected to produce serum. Biochemical analysis used standard diagnostic kits (Olvex-diagnosticum) and a StatFax 3300 spectrophotometer (USA). Results were processed statistically using the Statistica 7.0 program using *t*-criteria to assess the significance of differences. The data were presented as averages ± standard errors of the mean (SE).

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