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New Multifunctional Di-*tert*-butylphenoloctahydro(pyrido/benz)oxazine Derivatives with Antioxidant, Antihyperlipidemic, and Antidiabetic Action

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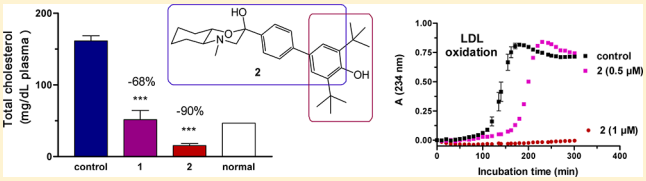
ABSTRACT: Oxidative stress, inflammation, and hyperlipidemia are common factors involved in the pathophysiology of atherosclerosis and type 2 diabetes. We have previously developed multifunctional antidyslipidemic derivatives with antioxidant and antiatherogenic properties. We now report the design, synthesis, and evaluation of two such novel derivatives that incorporate a structural moiety of the antidiabetic agent succinobucol. The new compounds exhibited a much improved in vitro antioxidant and squalene synthase inhibitory activity (at lower micromolar concentrations) as well as a significant antihyperlipidemic effect, reducing plasma total cholesterol, triglycerides, and MDA by 65−90%. Compound **2** also indicated a good anti-inflammatory activity, decreasing edema by 44%, while it was further evaluated for its antidiabetic activity using a type 2 diabetes experimental mouse model. After 7 weeks of administration, it produced a significant antihyperglycemic and antihyperlipidemic activity. In conclusion, rational drug design led to a compound combining improved antioxidant, antidyslipidemic, and antidiabetic action that may serve as a potential therapeutic strategy in metabolic syndrome disorders.



1. INTRODUCTION

Cardiovascular disease (CVD) is classified as the dominant cause of mortality in Western populations. It is mainly characterized by three pathological conditions: atherosclerosis, hypertension, and heart failure. Metabolic syndrome is the name for a group of concurring diseases or symptoms that raise the risk of cardiovascular disease, including obesity, diabetes mellitus, impaired glucose tolerance, insulin resistance, dyslipidemia (high LDL cholesterol, low HDL cholesterol, and high triglyceride levels in blood), and increased blood pressure. Ideally, drug therapy must address the biological mechanisms that are common to a number of the above diseases. Recent studies outline the role of oxidative stress in combination with hyperlipidemia in the pathogenesis and development of atherosclerosis as well as type 2 diabetes.[1] In addition, inflammatory processes are involved in the development of atherosclerosis, while continuous low grade inflammation has been linked to obesity; abdominal fat tissue appears to excrete pro-inflammatory factors that may affect other tissues in the body as well.[2]

A single-targeted therapeutic approach in multifactorial disorders such as those mentioned above is mostly considered inadequate.[3,4] On the basis of the underlying pathophysiological processes, we have previously developed multifunctional antidyslipidemic morpholine and octahydro(pyrido/benz)oxazine derivatives with antioxidant, anti-inflammatory, and antiatherogenic properties.[5−8] Here we incorporate in the pharmacophore of our biphenyloctahydrobenzoxazine lead (**a**, Chart 1) structural features of succinobucol, an antioxidant 2,6-di-*tert*-butyl-4-methylphenol derivative that was designed as an antiatherosclerotic drug and proved to have antihyperglycemic activity.[9]

Two such derivatives that were designed to combine within one structure antidyslipidemic, anti-inflammatory, and enhanced antioxidant properties were synthesized and evaluated in vitro and in vivo for their extended activity.

2. RESULTS

2.1. Chemistry. Both 3-hydroxy-3-aryloctahydropyridoxazine derivative **1** and 2-hydroxy-2-aryloctahydrobenzoxazine **2** (Scheme 1) were formed via a spontaneous cyclization of the relevant amino alcohol with the 4-bromoacetylbiphenyl derivative **b** to the corresponding hemiketal structure in good yields (~80%). As verified by spectroscopic and theoretical studies, the fused octahydro(pyrido/benz)oxazine ring systems of compounds **1** and **2** adopt a chair−chair (trans) conformation.[6,7]

2.2. In Vitro Effect on Lipid Peroxidation. The effect of the investigated derivatives on the nonenzymatic peroxidation of hepatic microsomal membrane lipids after 45 min of incubation, expressed as IC_{50} values, is shown in Figure 1. The new derivatives **1** and **2** are very active antioxidants (IC_{50} of 3.8 and 5.9 μM, respectively) compared to lead compound **a** (Chart 1) which inhibits the lipid peroxidation with an IC_{50} of 450 μM.[6,7] Under the same experimental conditions, probucol and 2,6-di-*tert*-butyl-4-methylphenol (BHT), known potent antioxidants and structurally related to succinobucol, exhibited IC_{50} values of >1 mM and 25 μM, respectively.

2.3. Effect on LDL Oxidation. Since oxidative modification of LDL can trigger a cascade of cellular processes that lead to the formation of fatty streaks and eventually atherosclerotic

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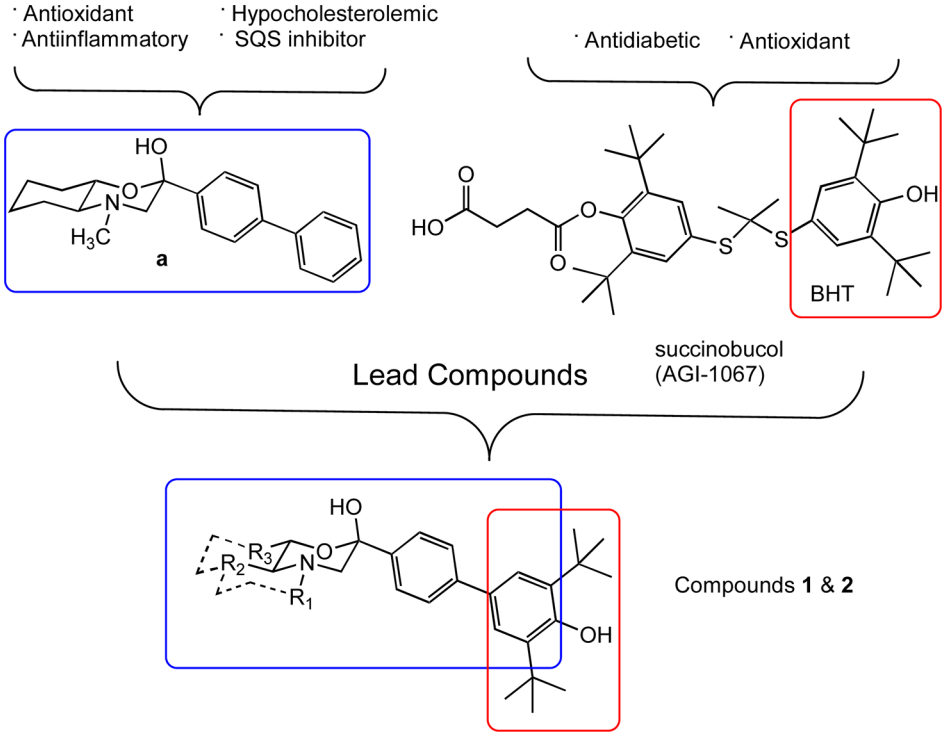
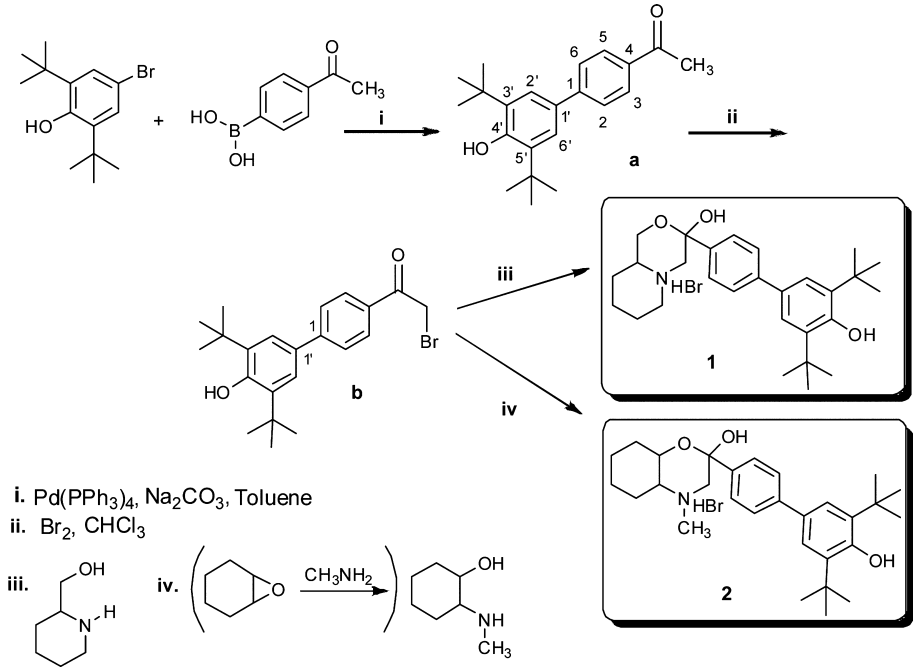
Chart 1. Design of Compounds 1 and 2 and Structures of Lead Compounds a and Succinobucol



Scheme 1. Synthetic Route Applied toward the Formation of Desired Products 1 and 2



lesions in the arterial wall, compounds **1** and **2** were also examined for their antioxidant activities on human LDL oxidation induced by cupric ions ($CuSO_4$). The metal-ion-dependent modification of LDL occurs primarily through lipid peroxidation and subsequent derivatization of apolipoprotein B lysine residues by reactive, lipid hydroperoxide-derived aldehydes.[10] The susceptibility of LDL to oxidation (in the presence or absence of our compounds) was assessed by determining (a) the time before the oxidation products (conjugate dienes) become detectable (lag time), (b) the rate of oxidation (propagation time), and (c) the maximum amount of oxidation products at 234 nm (Table 1). Figure 2 depicts the activity of 0.5 and 1 μM compounds **1** and **2** on LDL oxidation induced by 10 μM $CuSO_4$. Oxidation was significantly and dose-dependently inhibited by 0.5−1 μM compounds **1** and **2**, as shown by an increase in lag time (Table 1). Compound **1** prolonged the control lag time (110 min) to 130 min at 0.5 μM (15% increase) and to 220 min at 1 μM (100% increase). Compound **2** is shown to be a more effective antioxidant than compound **1**, prolonging the control lag time (110 min) to 170 min at 0.5 μM (55% increase),

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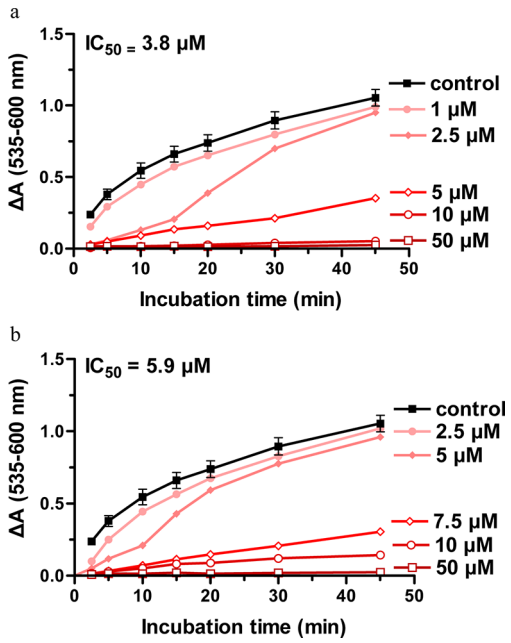

Figure 1. Time course of lipid peroxidation of rat microsomal membranes in the presence of various concentrations of compound **1** (a) and compound **2** (b).

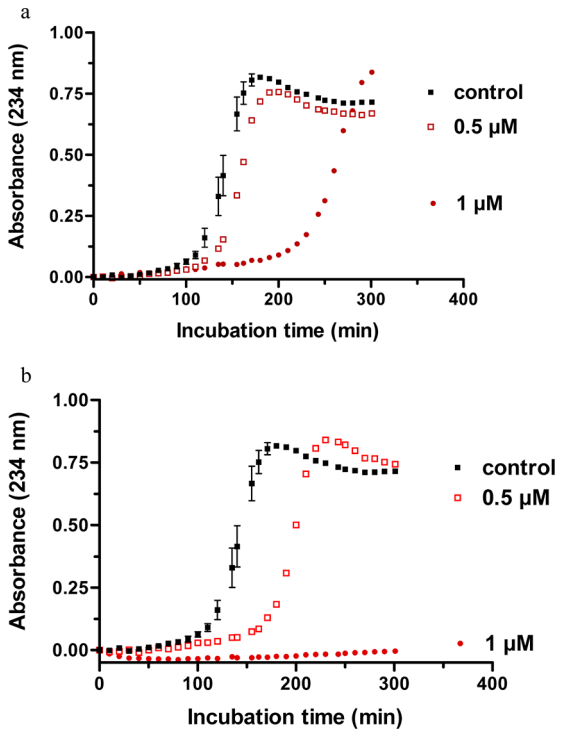

Figure 2. Inhibition of LDL oxidation in the presence of 0.5 and 1 μM compound **1** (a) and compound **2** (b).

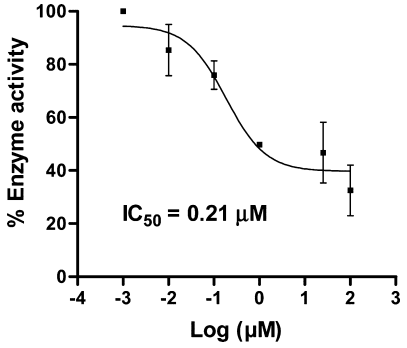

Figure 3. Representative graph showing the activity of squalene synthase as affected by various concentrations of compound **2**.

while in the presence of 1 μM the lag time was too long to be recorded (≫300 min, ≫100% increase). In addition, the presence of 1 μM compound gave a remarkable decrease in the rate of conjugate diene formation from 10.2 (nmol/min)/mg protein (control) to 6.7 (nmol/min)/mg protein (34.3% decrease) for **1** and 0 (nmol/min)/mg protein (100% decrease) for **2** (Table 1). Under the same experimental conditions, the lead compound **a** (Chart 1) increased the control lag time by 65% and decreased the rate of conjugate diene formation by 7% at 10 μM, while probucol at 5 μM increased the control lag time by 22%. The above results characterize compounds **1** and **2** as very strong inhibitors of LDL oxidation.

2.4. Effect on Squalene Synthase. Both compounds **1** and **2** inhibited significantly and dose-dependently squalene synthase activity. Inhibition of the activity of squalene synthase from rat liver microsomes by compound **2** is shown in Figure 3. IC_{50} values for **1**, **2**, and lead compound **a** are 12, 0.21, and 36 μM, respectively. It seems that the inhibitory potency markedly increased (170-fold) by substituting the second phenyl ring of the lead compound **a** with BHT's structural features. It is possible that this lipophilic moiety interacts more efficiently with the hydrophobic cavity of the binding site of SQS.

2.5. In Vivo Antidyslipidemic and Antioxidant Activity. The hypocholesterolemic/hypolipidemic as well as the antioxidant activity of the new compounds was evaluated in vivo (rat) based on the reduction of experimentally induced elevated plasma lipid and MDA levels. Experimental hyperlipidemia was successfully established 24 h after Triton WR 1339 administration (liberation of cholesterol from the liver induced by Triton WR 1339 triggers the hepatic synthesis of cholesterol),[11] with an increase in plasma total cholesterol and triglyceride levels of 260% and 580%, respectively, compared to normal values (Figure 4). Compounds **1** and **2** were

Table 1. LDL Oxidation Parameters in Presence of 0.5 and 1 μM Compounds 1 and 2

	lag phase			
LDL sample	lag time t_a, min	% increase of lag time t_a	propagation phase, rate of oxidation, (nmol dienes/min)/(mg LDL)	decomposition phase, amount of dienes, (nmol dienes)/(mg LDL)
control	110		10.2	490.9
1 (0.5 μM)	130	15	9.5	457.6
1 (1.0 μM)	220	100	6.7	506.7
2 (0.5 μM)	170	55	12.0	508.5
2 (1.0 μM)	≫300	≫100	0	0

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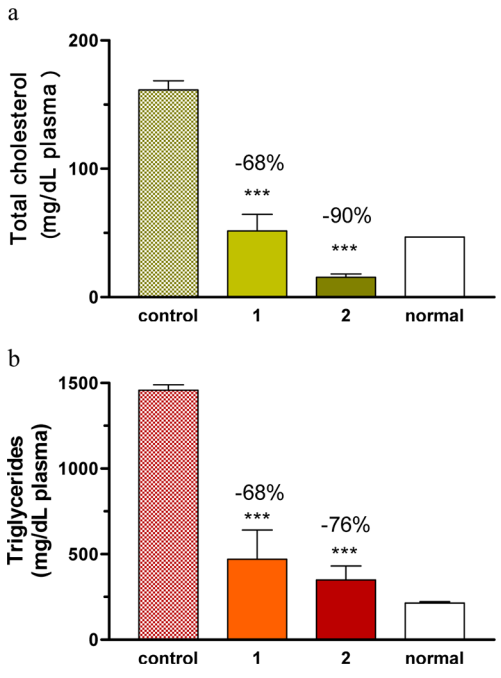

Figure 4. Percent reduction of total cholesterol and triglyceride plasma levels of experimentally induced hyperlipidemic rats after ip administration of 56 μmol/kg compounds **1** and **2**. Statistically significant difference from control: (***) $P < 0.005$.

administered in a single ip dose of 56 μmol/kg (in order to allow comparison with related compounds of previous studies) to hyperlipidemic rats. Both compounds were found able to reduce the examined parameters in the plasma of hyperlipidemic rats by 68−90%. Specifically, compound **1** decreased both total cholesterol and triglyceride levels by 68%, while compound **2** decreased the above lipid parameters by 90% and 76%, respectively (Figure 4). Under the same experimental conditions and at the same molar dose, probucol and simvastatin reduced plasma total cholesterol by 18% and 75% and triglycerides by 11% and 0%, respectively.[6,7] Lead compound **a** (2-biphenyloctahydrobenzoxazine, Chart 1) decreased the above parameters by 54% and 49%, respectively.[5] Both compounds show a greater effect on all lipidemic indices compared to that of simvastatin, probucol, and compound **a**. It appears from the results that the hypocholesterolemic/hypolipidemic activity of probucol (which contains in its structure two structural moieties of BHT) is low in this experimental protocol. This finding agrees with the lack of effect of the antioxidant BHT on plasma levels of total cholesterol and triglyceride in rabbits[12] and the modest reduction of plasma total cholesterol and triglyceride levels, at the same dose of 56 μmol/kg, by 29% and 26%, respectively, in the Triton WR 1339 induced hyperlipidemia experimental model by 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHA), the benzoic acid derivative of BHT.[13] Thus, the incorporation of the BHT structure into lead compound **a** (which showed very good hypolipidemic activity) contributed to a significant synergistic hypocholesterolemic/hypolipidemic effect. Furthermore, compounds **1** and **2** reduced malondialdehyde (MDA) content in the plasma of experimentally induced hyperlipidemic rats by 63% and 64%, respectively (Figure 5). Thus, the increased in vitro antioxidant activity of these compounds is also confirmed in vivo.

2.6. In Vivo Anti-Inflammatory Activity. The anti-inflammatory activity of the most active compound **2** was

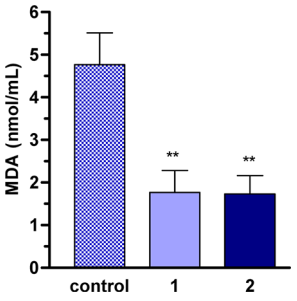

Figure 5. Plasma MDA levels (nmol/mL) of experimentally induced hyperlipidemic rats in the presence of compounds **1** and **2**. Statistically significant difference from control: (**) $P < 0.01$.

evaluated by the method of carrageenan-induced paw edema which is a nonspecific inflammation maintained by the release of histamine and serotonin and later by prostaglandins.[14,15] The examined compound was administered ip at a dose of 300 μmol/kg right after the injection of carrageenan. Compound **2** reduced the increase in edema by 44%. Naproxen, a well-known nonsteroidal anti-iflammatory drug, has been reported to give a 51% edema decrease,[16] while BHT was inactive at the same dose and experimental conditions. The results demonstrate a significant anti-inflammatory activity of compound **2**, comparable to that of naproxen at the same dose.

2.7. In Vivo Antidiabetic Activity. In the type 2 diabetes experimental animal model used, insulin resistance was induced by a high fat diet (HFD), in addition to multiple low doses of streptozotocin (STZ) that produces, via β cell death, a mild impairment of insulin secretion that is similar to that of the later stage of type 2 diabetes.[17,18] The most potent antioxidant and antidyslipidemic compound **2** was evaluated for its activity in this type 2 diabetes animal model.

2.7.1. Body Weight and Food Consumption. As shown in Figure 6a, significantly higher body weights were observed between the 14th and the 35th day for group B (HFD-STZ) compared to group C (HFD-STZ and **2**). After the 35th day

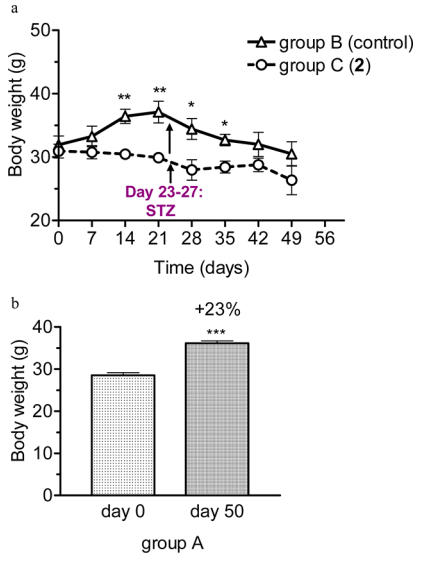

Figure 6. Body weight (a) in the obesity−diabetes type 2 mouse model after treatment with saline (group B, control) or with compound **2** (group C) and (b) in group A (fed only HFD). Statistically significant different values between the two groups: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.005$.

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and until the end of the experiment, no differences were observed in body weight gain between the two groups because of significant body weight loss of group B (reflecting a negative effect of STZ on this group). Compound **2**-treated rats showed a healthier appearance compared to control group B, while no signs of toxicity were observed. Furthermore, it seems that food intake of **2**-treated rats (group C) was similar or somewhat larger (10%) than that of the control group (group B) (average food consumption of 110 (mg/g BW)/day, results not shown). Treatment with compound **2** preserved the average body weight of mice at initial levels, as seen when compared also with mice of group A (HFD without STZ) in which a statistically significant increase of 23% in average body weight, between the first and the 50th day, was observed (Figure 6b).

2.7.2. Blood Glucose Levels. Figure 7 depicts the time course of glycemic values in nonfasting HFD-STZ mice after

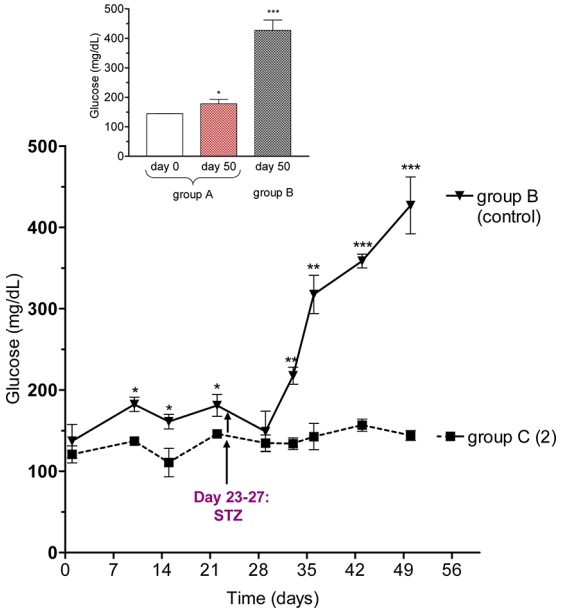

Figure 7. Blood glucose levels in the obesity–diabetes type 2 mouse model after treatment with saline (group B, control) or with compound **2** (group C) as well as in group A (HFD). Statistically significant difference between groups (or from day 0, inserted graph): (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$.

treatment with compound **2** (group C) or saline (control, group B). Glucose levels of mice in group A (HFD without STZ) increased only marginally between the first and the 50th day (Figure 7, inserted bar graph). In contrast, after STZ administration (group B) blood glucose levels progressively increased, reaching above 400 mg/dL on the 50th day. Long-term treatment of mice with compound **2** (group C) fully prevented this marked, STZ-induced rise in blood glucose levels.

2.7.3. Plasma Lipid and MDA Levels. Since there is a strong correlation among dyslipidemia, oxidative stress, and type 2 diabetes, plasma concentrations of total cholesterol (TC), LDL cholesterol (LDL), HDL cholesterol (HDL), triglycerides (TG), and MDA levels were measured on days 22, 43, and 50 (Figures 8). In the control group B, TC, HDL, and LDL levels progressively increased during the time course of the experiment while TG concentrations increased only up to day 43. In contrast, treatment with compound **2** (group C) significantly decreased TC and LDL levels while maintaining at initial levels HDL and TG levels. Specifically, on day 43, levels

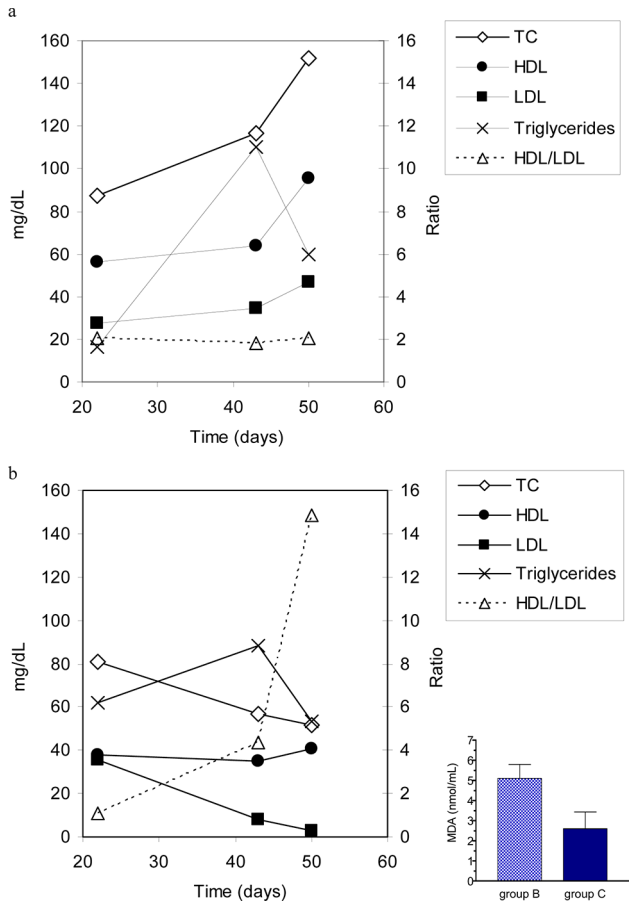

Figure 8. Plasma levels of total cholesterol (TC), LDL cholesterol (LDL), HDL cholesterol (HDL), trigelycerides (TG) and the ratio of HDL/LDL in the type 2 diabetes mouse model after treatment with (a) saline (group B) or (b) with compound **2** (group C). Inserted bar graph: MDA levels of groups B and C on day 50.

of TC and LDL of group C were reduced by 51% and 80% compared to control group B, while on day 50 the above parameters were reduced by 70% and 95%, respectively. Interestingly, the ratio HDL/LDL, which is considered of importance, differed substantially between the two groups. The HDL/LDL ratio significantly increased in group C compared to control (group B) in the time course of the experiment. Compound **2** also exhibited a strong antioxidant activity in vivo, decreasing plasma MDA levels by ~50% as measured at the end of the experiment, i.e., day 50 (Figure 8, inserted bar graph). Thus, the potent antioxidant and hypolipidemic effect shown by compound **2** in the experimentally induced hyperlipidemic rat model mentioned previously is also confirmed in this type 2 diabetes experimental animal model.

3. DISCUSSION

Vascular complications associated with type 2 diabetes confer significant morbidity and mortality. Atherosclerosis develops much earlier and progresses more rapidly in subjects with diabetes. The clustering of cardiovascular risk factors associated with type 2 diabetes is mainly responsible for accelerated atherosclerotic disease. Dyslipidemia is considered a prominent cardiovascular risk factor in treatment guidelines. Lipid-modifying therapy has an important role in attenuating diabetes-related macrovascular atherosclerotic disease, with statins being the predominant choice for therapeutic intervention.[1,19] However,

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apart from inhibiting HMG-CoA reductase, statins also suppress the production of mevalonate, a precursor of several non-sterol products that are vital for diverse cellular functions. Both known major side effects of statins (hepatotoxicity and myotoxicity) have been associated with this inhibition of synthesis of certain non-sterol products.[20−22] Furthermore, many clinical studies indicate that use of intensive-dose statin therapy compared with moderate-dose statin therapy is associated with a higher incidence of new-onset diabetes, albeit with fewer major cardiovascular events.[23,24] Although a potential mechanism to explain this phenomenon is currently lacking, it has been suggested that statin-induced myopathy may be associated with the development of muscle insulin resistance.[25] Lead structure **a** (Chart 1) is an inhibitor of squalene synthase, an enzyme that catalyzes the first commited step in the de novo cholesterol biosynthesis pathway. Squalene synthase inhibitors decrease circulating LDL cholesterol via an increased expression of hepatic LDL receptors, in a manner similar to statins. However, they leave other non-sterol products of mevalonate metabolism unaffected and therefore have at least one theoretical advantage compared with statins, as safer hypocholesterolemic and anti-atherosclerotic agents.[22,26] The design of compounds **1** and **2** was based on the incorporation of a structural moiety of the antiatherosclerotic and antidiabetic agent succinobucol (a probucol derivative) into the structure of the antidyslipidemic squalene synthase inhibitor, compound **a** (Chart 1). The BHT moiety was selected from succinobucol's structure because of its potent antioxidant effect against lipid peroxidation. Compounds **1** and **2** exhibited a very strong hypocholesterolemic/hypolipidemic effect, decreasing plasma concentrations of total cholesterol and triglycerides by 68−90% in hyperlipidemic rats (Figure 4).

Reactive oxygen species (ROS) are implicated in the damage of various cell types, including endothelial (in atherosclerosis) and pancreatic β cells (in diabetes), acting by various mechanisms, e.g., via lipid peroxidation.[27,28] LDL oxidation is a crucial event in the initiation and development of atherosclerosis leading to abnormalities in endothelium function and the formation of atherosclerotic lesions. Moreover, oxidative stress, among others, is proposed to be responsible for the progressive loss of β cells due to the particularly high sensitivity of these cells to excessive ROS.[29,30] Compounds **1** and **2** were shown to be potent inhibitors of in vitro lipid peroxidation at very low concentrations (IC_{50} values of **1** and **2** are 3.8 and 5.9 μM, respectively, compared to reference compounds **a** and BHT with IC_{50} values of 450 and 25 μM, respectively). The lipophilic character of compounds **1** and **2** (ClogP values of 6.67 and 7.30, respectively) may facilitate their presence in biological membranes, adding to the strong antioxidant properties of the di-*tert*-butylphenol moiety and contributing to the enhanced protection against lipid peroxidation. Furthermore, both **1** and **2** provide an effective protection against LDL oxidation in vitro, prolonging the initiation of LDL lipid peroxidation and significantly decreasing the rate of conjugate diene formation at 0.5 and 1 μM. The incorporation of the antioxidant and lipophilic structure of BHT in the lead compound **a** (Chart 1) may also favor a better interaction of the new compounds (i.e., compared to compound **a** and probucol) with LDL particles and a more efficient protection from oxidation.

The enhanced antioxidant activity of the new compounds is also exhibited in vivo, as they decrease plasma MDA levels in hyperlipidemic rats by ∼65%. This activity of compound **2** is furthermore accompanied by a significant anti-inflammatory activity in vivo, reducing carrageenan-induced edema by 44%.

Succinobucol (or AGI-1067, Chart 1), the monosuccinic acid ester of probucol, was designed to retain probucol's antioxidant properties with reduced adverse effects that were attributed to its spiroquinone metabolite. Unlike probucol, AGI-1067 inhibits human aortic smooth muscle cell proliferation and the progression of atherosclerosis, lowering LDL levels (in animals) while maintaining (or even elevating) HDL levels. The potential mechanism of action of succinobucol on glycemic improvement is uncertain. It has been hypothesized that its antioxidant and anti-inflammatory actions may reduce tissue damage and inflammation from oxidative stress and free-radical accumulation that are implicated in pancreatic β-cell destruction, insulin resistance, and subsequent development of diabetes.[9,31−33]

Taking into consideration the promising antioxidant, hypolipidemic, and anti-inflammatory activities of compound **2**, it was evaluated for its antidiabetic effect using a mouse model of type 2 diabetes with reduced β-cell mass, obtained by the combined administration of multiple low doses of streptozotocin (STZ) and high fat diet (HFD).[17,18] HFD increased significantly the body weight in mice of group A (23% increase of average body weight at 50 days) , as well as in control mice (group B) until the 23th day (19% increase). The subsequent administration of streptozotocin in control mice (group B) at days 23−27 led to the progressive loss of body weight to initial levels on day 50 (Figure 6a). However, administration of compound **2** (group C) maintained body weight (during the time course of the experiment) without affecting the feeding pattern of the animals. Furthermore, at the end of the experiment, accumulation of visceral fat mass in control mice (group B) was macroscopically observed despite the restoration (from day 21 to day 50) of the average body weight at initial levels. In contrast, no such fat accumulation was observed in mice treated with compound **2** (group C). The above-mentioned differences may be attributed to the potential effect of compound **2** on lipid metabolism or a different lipolytic pathway. Noteworthy, obesity has recently been found to be characterized by a low grade inflammatory process involving stimulated adipocytes, macrophages, and other regulatory immune cells, resulting in the excretion of proinflammatory compounds from the fat tissue.[2] An additional effect observed in such activated fat tissues is a reduced insulin sensitivity of the fat cells, contributing to diabetes type 2. Several compounds with anti-inflammatory action have been shown to reduce obesity in animal models.[34] The apparent "antiobesity" activity of compound **2** is accompanied by a very strong antihyperglycemic effect, conserving glucose levels of group C at physiological levels in relation to the increased glucose levels of control group B (Figure 7). Thus, administration of compound **2** inhibited the pathological increase of glucose levels and forecoming diabetes compared to control group (group B). Further, the potent antioxidant and hypolipidemic activity of compound **2** was also confirmed in this experimental animal model of type 2 diabetes. The marked antidiabetic activity of compound **2** is possibly explained by the attenuation of prolonged oxidative-stress-induced tissue damage and inflammation as well as the protection of pancreatic β cells against oxidative stress. The molecular mechanism of its antihypercholesterolemic/antihyperlipidemic activity is considered to be via squalene synthase inhibition.

In conclusion, in the present study a multitarget-directed approach led us to rationally design compounds **1** and **2**, which are characterized by a unique multimodal profile. In particular,

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compound **2** was able (a) to potently inhibit in vitro lipid peroxidation and LDL oxidation, (b) to inhibit in vitro squalene synthase activity in the submicromolar scale, (c) to decrease in vivo lipidemic parameters and oxidative stress, (d) to reduce in vivo the increase in edema induced by carrageenan, and (e) to suppress the onset and progress of diabetes mellitus with a potential antiobesity effect as well. To the best of our knowledge, this is the first time that a squalene synthase inhibitor combines simultaneously hypolipidemic, antioxidant, and antidiabetic activity. The very potent and appropriately balanced activities of the new compounds render them interesting multifunctional molecules useful in the treatment of metabolic syndrome disorders.

4. EXPERIMENTAL SECTION

Materials. All commercially available chemicals are of the appropriate purity and purchased from standard sources. For the in vivo experiments, Wistar male rats (200−250 g) and SKH-2 male mice were used. Animals were kept in a temperature controlled room (22 ± 2 °C), having free access to laboratory chow and tap water, under a 12 h light/dark cycle.

Synthesis. Melting points (mp) were determined with a digital Electrothermal IA 9000 series apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker Avance DRX 400 (400 MHz) and DPX 200 (200 MHz) spectrometer, respectively. Mass spectra were obtained on a 4000 QTRAP MS/MS spectrometer. Purity of tested compounds was established by elemental analyses performed by the Service Central de Microanalyse, France (analysis of C, H) and is ≥95%.

4-Acetyl-3′,5′-di-*tert*-butyl-4′-hydroxybiphenyl (a). To a stirring solution of 4-acetylphenylboronic acid (6.2 mmol, 1.0 g) in 25 mL of toluene were added a saturated aqueous solution of Na_2CO_3 (12.5 mL), a solution of 4-bromo-2,6-bis-*tert*-butylphenol (6.78 mmol, 1.9 g) in 12 mL of absolute ethanol, and 0.12 mmol (0.14 g) of $Pd(PPh_3)_4$, followed by 4 h of reflux in the presence of argon. After the reaction mixture reached room temperature, ice was added and was extracted with ethyl acetate. The organic layer was washed with 1 N HCl, water, and saturated NaCl aqueous solution and dried over Na_2SO_4. The mixture was separated via column chromatography (flash) (petroleum ether/dichloromethane, 3/1). Yield 66%, mp = 143.5−146.0 °C.[35] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.52 (s, 18H, 2 × -C$(CH_3)_3$), 2.65 (s, 3H, -CH_3), 5.39 (s, 1H, -OH), 7.46 (s, 2H, 2′-H and 6′-H biphenyl), 7.65 (d, J = 8.54 Hz, 2H, 2-H and 6-H biphenyl), 8.03 (d, J = 8.54 Hz, 2H, 3-H and 5-H biphenyl).

4-Bromoacetyl-3′,5′-di-*tert*-butyl-4′-hydroxybiphenyl (b). To a stirring solution of 4-acetyl-3′,5′-di-*tert*-butyl-4′-hydroxybiphenyl (1.85 mmol, 0.6 g) in 10 mL of anhydrous chloroform was added dropwise a solution of Br_2 (2.03 mmol) in 10 mL anhydrous chloroform. After 2 h of being stirred, the reaction mixture was washed with water, 5% $NaHCO_3$, water and dried over Na_2SO_4. The mixture was separated via column chromatography (flash) (petroleum ether/dichloromethane, 3/1). Yield 68%, mp = 144.5−145.5 °C.[36] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.50 (18H, s, 2 × -C$(CH_3)_3$), 4.49 (2H, s, -CH_2), 5.41 (1H, s, -OH), 7.31 (2H, s, 2′-H and 6′-H biphenyl), 7.71 (2H, d, J = 8.56 Hz, 2-H and 6-H biphenyl), 8.10 (2H, d, J = 8.56 Hz, 3-H and 5-H biphenyl).

General Procedure for the Preparation of the Final Products. The final products **1** and **2** were obtained by the reaction of 3 mmol of 2-piperidinemethanol or *trans*-2-methylaminocyclohexanol with 1.2 mmol of 4-bromoacetyl-3′,5′-di-*tert*-butyl-4′-hydroxybiphenyl (**b**) in anhydrous acetone (40 mL) at room temperature with stirring for 24 h. Acetone was then distilled off. Ether was added to the residue, and the mixture was washed with saturated NaCl aqueous solution and dried over K_2CO_3. The products were isolated as hydrobromide salts and purified by recrystallization (acetone/ethyl ether).

3-(3′,5′-Di-*tert*-butyl-4′-hydroxy-4-biphenyl)octahydropyrido-[2,1-*c*][1,4]oxazin-3-ol Hydrobromide (1). Yield 77%, mp = 197.2−199.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.35 (s, 18H, 2 × -C$(CH_3)_3$), 1.49−1.52 (m, 2H, 8-H_{ax}, 9-H_{ax}), 1.75−1.85 (m, 2H, 2 × 7-H), 1.92−2.01 (m, 2H, 8-H_{eq}, 9-H_{eq}), 2.56−2.62 (dt, J_1 = 13.45 Hz, J_2 = 2.11 Hz, 1H, 6-H_{ax}), 2.79−2.83 (1H, d, J = 12.72 Hz, 4-H_{ax}), 3.17 (m, 1H, 9a-H_{ax}), 3.31−3.33 (m, 1H, 6-H_{eq}), 3.44 (d, J = 12.71 Hz, 1H, 4-H_{eq}), 3.78 (dd, J_2 = 13.40 Hz, J_1 = 3.42 Hz, 1H, 1-H_{eq}), 4.39−4.46 (dd, J_1 = 13.30 Hz, J_2 = 11.01 Hz, 1H, 1-H_{ax}), 5.17 (s, 1H, 2-OH), 6.71 (s, 1H, 4′-OH), 7.24 (s, 2H, 2′-H and 6′-H biphenyl), 7.55 (d, J = 1.96 Hz, 2H, 3-H and 5-H biphenyl), 7.57 (d, J = 1.86 Hz, 2H, 2-H and 6-H biphenyl), 11.38 (brs, 1H, -NH). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 22.07 (6-C), 22.36 (7-C), 24.14 (8-C), 30.30 (6 C, 6 × -C(**C**$H_3)_3$), 34.45 (2 C, 2 × -**C**$(CH_3)_3$), 55.23 (5-C), 61.25 (1-C), 61.43 (4-C), 62.70 (8α-C), 94.53 (3-C), 124.00 (2′,6′-C biphenyl), 126.23 (2,6-C biphenyl), 126.96 (3,5-C biphenyl), 131.65 (1′-C biphenyl), 136.31 (3′,5′-C biphenyl), 137.95 (4-C biphenyl), 143.18 (1-C biphenyl), 153.81 (4′-C biphenyl). $C_{28}H_{39}NO_3$, ESI-MS (m/z) $(M + H)^+$, calculated: 438.3. Found: 438.2. Anal. Calcd for $C_{28}H_{40}BrNO_3$ (%): C, 64.86; H, 7.78. Found (%): C, 65.09; H, 7.84.

2-(3′,5′-Di-*tert*-butyl-4′hydroxy-4-biphenyl)-4-methyloctahydro-1,4-benzoxazin-2-ol Hydrobromide (2). Yield 80%, mp = 163.5−164.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.32−1.54 (m, 20H, 2 × 7-H and 2 × -C$(CH_3)_3$), 1.71−2.15 (5H, m, 5-H_{ax}, 8-H_{ax}, 2 × 6-H, 8-H_{eq}), 2.20−2.30 (m, 1H, 5-H_{eq}), 2.82 (s, 3H, N-CH_3), 3.01 (m, 2H, 3-H_{ax} and 4α-H_{ax}), 3.67 (d, J = 12.63 Hz, 1H, 3-H_{eq}), 4.52 (m, 1H, 8α-H_{ax}), 5.30 (s, 1H, 2-OH), 6.29 (s, 1H, 4′-OH), 7.37 (s, 2H, 2′-H and 6′-H biphenyl), 7.55 (d, J = 8.19 Hz, 2H, 3-H and 5-H biphenyl), 7.69 (d, J = 7.85 Hz, 2H, 2-H and 6-H biphenyl), 11.62 (brs, 1H, -NH). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.12−1.47 (m, 4H, 8-H_{ax}, 2 × 7-H, 5-H_{ax}), 1.42 (s, 18H, 2 × -C$(CH_3)_3$), 1.75−1.93 (m, 3H, 2 × 6-H, 8-H_{eq}), 2.22−2.24 (bd, J = 10.93 Hz, 1H, 5-H_{eq}), 2.75 (s, 3H, -NCH_3), 3.05−3.07 (m, 1H, 4a-H_{ax}), 3.21 (t, J = 10.92 Hz, 1H, 3-H_{ax}), 3.57 (d, J = 12.29 Hz, 1H, 3-H_{eq}), 4.08 (t, J = 10.24 Hz, 1H, 8a-H_{ax}), 7.13 (s, 1H, 2-OH), 7.31 (s, 2H, 2′-H and 6′-H biphenyl), 7.42 (s, 1H, 4′-OH), 7.60 (s, 4H, 2-H and 6-H, 3-H and 5-H biphenyl), 9.93 (brs, 1H, -NH). ^{13}C NMR (200 MHz, DMSO-d_6) δ (ppm): 23.82 (6,7-C), 24.50 (5-C), 24.71 (8-C), 30.77 (6 C, 6 × -C(**C**$H_3)_3$), 31.07 (2 C, 2 × -**C**$(CH_3)_3$), 35.07 (N-CH_3), 61.43 (3-C), 66.26 (4α-C), 70.28 (8α-C), 94.14 (2-C), 123.64 (2,6-C biphenyl), 126.67 (3, 5, 2′, 6′-C biphenyl), 131.69 (1′-C biphenyl), 139.42 (1-C biphenyl), 140.07 (3′,5′-C biphenyl), 142.62 (4-C biphenyl), 154.40 (4′-C biphenyl). $C_{29}H_{41}NO_3$, ESI-MS (m/z) $(M + H)^+$, calculated: 452.3. Found: 452.2. Anal. Calcd for $C_{29}H_{42}BrNO_3 \cdot 1.5H_2O$ (%): C, 62.23; H, 8.12. Found (%): C, 62.20; H, 7.87.

In Vitro Lipid Peroxidation. Heat-inactivated hepatic microsomes from untreated rats were prepared as described.[5] The incubation mixture contained microsomal fraction (corresponding to 2.5 mg of hepatic protein per mL or 4 mM fatty acid residues), ascorbic acid (0.2 mM) in Tris-HCl/KCl buffer (50 mM/150 mM, pH 7.4), and the studied compounds (50−1 μM) dissolved in DMSO. The reaction was initiated by addition of a freshly prepared $FeSO_4$ solution (10 μM), and the mixture was incubated at 37 °C for 45 min.[7] Lipid peroxidation of aliquots was assessed spectrophotometrically (535 nm against 600 nm) as TBAR. Both compounds and solvents were found not to interfere with the assay. Each assay was performed in duplicate, and IC_{50} values represent the mean concentration of compounds that inhibits the peroxidation of control microsomes by 50% after 45 min of incubation. All standard errors are within 10% of the respective reported values.

Isolation and in Vitro LDL Oxidation. Blood was collected from a normolipidemic volunteer. EDTA was used as anticoagulant (1 mg/mL blood). After low-speed centrifugation (3100 rpm, 20 min, 20 °C) of whole blood to obtain plasma, LDL was isolated from the plasma by discontinuous density gradient ultracentrifugation.[37] Briefly, the density of plasma was increased to 1.019 with KBr and then was centrifuged at 40 102 rpm at 13 °C for 10 h. After the top layers containing chylomicrometer and very-low-density lipoprotein (VLDL) were removed, the density of remaining plasma fractions was increased to 1.063 with KBr and they were recentrifuged at 40 102 rpm at 13 °C for an additional 10 h. The LDL fraction in the top of the tube was collected and dialyzed against three changes of phosphate buffer (pH 7.4) in the dark at 4 °C to remove KBr and EDTA. The solution of LDL in PBS was stored at 4 °C and used within 3 weeks.

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Human LDL (56 μg protein/mL) in PBS was incubated at 37 °C in the absence or presence of various concentrations of compounds **1** and **2** (in 10% DMSO−water) for 5 min before the start of oxidation. Oxidation was initiated by the addition of 10 μM $CuSO_4$. The formation of conjugate dienes was determined spectrophotometrically every 10 min for 5 h as the increase in absorbance at 234 nm and calculated using the extinction coefficient of 29 500.[38]

In Vitro Squalene Synthase Activity Assay. SQS activity was evaluated by determining the amount of [^{3}H]FPP converted to squalene as previously described.[7,39]

In Vivo Evaluation of Antidyslipidemic and Antioxidant Activity. An aqueous solution of Triton WR 1339 was given ip to rats (200 mg/kg),[11] and 1 h later, the test compounds (56 μmol/kg), finely suspended/dissolved with the help of Tween-80 (of a final concentration of ∼1%) in saline, or saline only, were administered ip. After 24 h, blood was taken from the aorta and used for the determination of plasma total cholesterol (TC) and triglyceride (TG) levels, using commercially available kits, as well as MDA content, by measuring the complex of MDA with *N*-methyl-2-phenyl indole (586 nm).[40] Values are the mean from 8 to 10 rats (per compound), while all standard errors are within 12% of the respective reported values.

Determination of in Vivo Anti-Inflammatory Effects. As a measure of the in vivo anti-inflammatory effect of compound **2**, the inhibition of phlogistic-induced paw edema was determined. Healthy 2- to 4-month-old male SKH-2 mice were used in groups of five animals weighing 20−30 g. A single dose of 0.3 mmol/kg test compound was administered ip right after the injection of 0.05 mL of the phlogistic agent carrageenan (2% w/v solution in saline) id into the right foot paw, the left paw serving as control. The percentage of edema inhibition is calculated for each animal by the swelling caused by the phlogistic agent after 3.5 h (given as percentage of weight increase of right hind paw in comparison to noninjected left hind paw), as previously described.[7,14,15]

Evaluation of Compound 2 in a Type 2 Diabetes Experimental Mouse Protocol. The 7- to 8-week-old male SKH-2 mice were acclimated for 1 week and allowed water and normal rodent chow ad libitum. Subsequently they were divided in three groups: group A, HFD (high fat diet); group B, HFD + STZ (high fat diet + streptozotocin); group C, HFD + STZ + compound **2**. From day 1 to day 50 mice of all groups were allowed ad libitum a high-fat diet (fat = 35.5% w/w). Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and injected ip after 4 h of fasting to groups B and C in (multiple) low doses (35 mg/kg/day × 5 days) from day 23 to day 27.[17,18,41] Compound **2** was administered ip at a dose of 56 μmol/kg twice daily during the whole experimental period (i.e., for 50 days) to group C, while similarly, saline was injected to group B.

Glucose Levels. Blood glucose concentration was determined every 5−10 days with a hand-held glucometer (Precision Xtra Plus, Abbott). Blood samples were applied directly to the glucose strip from nonfasted mice to measure nonfasting levels of blood glucose.

Body Weight and Food Consumption. Body weights were measured, and food consumption was estimated once weekly.

Lipidemic and Oxidative Parameters. For lipid analysis, blood was drawn on days 23, 43, and 50 of the experiment. Total cholesterol, HDL cholesterol, LDL cholesterol, TG, and MDA plasma levels were determined using commercially available kits or as described earlier above.

Protein Determination. The protein content of microsomal and LDL fractions was determined according to Lowry's method.[42]

Statistical Analysis. Data are expressed as the mean ± SD. Where indicated, statistical comparisons were made using Student's *t* test, and a statistically significant difference was inferred if $P < 0.05$.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CVD, cardiovascular disease; SQS, squalene synthase; HMGCo-A, 3-hydroxy-3-methylglutaryl-CoA; FPP, farnesyl pyrophosphate; PSPP, presqualene pyrophosphate; rt, room temperature; EtOAc, ethyl acetate; PE, petroleum ether; DCM, dichloromethane; BSA, bovine serum albumin; NADPH, nicotinamide adenine dinucleotide phosphate; IC_{50}, inhibitory concentration (for 50% of the reaction); TC, total cholesterol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; TG, triglyceride; MDA, malondialdehyde; STZ, streptozotocin; HFD, high fat diet

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