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Novel Benzoxazine and Benzothiazine Derivatives as Multifunctional Antihyperlipidemic Agents

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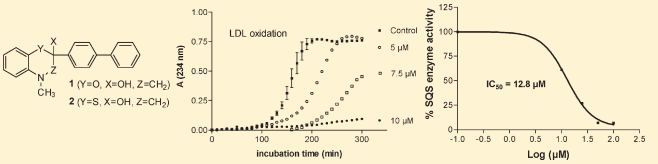
ABSTRACT: Atherosclerosis is a multifactorial disease with several mechanisms participating in its manifestation. To address this disorder, we applied a strategy involving the design of a single chemical compound able to simultaneously modulate more than one target. We hereby present the development of novel benzoxazine and benzothiazine derivatives that significantly inhibit in vitro microsomal lipid peroxidation and LDL oxidation as well as squalene synthase activity (IC_{50} of 5−16 μM). Further, these compounds show antidyslipidemic and antioxidant properties in vivo, decreasing total cholesterol, LDL, triglyceride, and MDA levels of hyperlipidemic rats by 26−74%. Finally, by determination of their in vivo concentration (up to 24 h) in target tissues (blood/liver), it is shown that compounds reach their targets in the low micromolar range. The new compounds seem to be interesting multifunctional molecules for the development of a new pharmacophore for disease-modifying agents useful in the treatment of atherosclerosis.



1. INTRODUCTION

Atherosclerosis is a severe pathology that can be directly or indirectly implicated in approximately 50% of all mortality in Western countries. It is considered the main contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function of the extremities. Over the past years, evidence has accumulated suggesting that free radicals, and the consequent lipid peroxidation they induce, could trigger most of the factors involved in atherosclerotic vascular injuries, such as cytotoxicity, inflammation, and formation of atheromatic plaques.[1] Oxidative modification of cholesterol-esterified lipids of lipoproteins (LDL) can lead to their uncontrolled uptake by macrophages. This event triggers a cascade of cellular processes that lead to the formation of fatty streaks and eventually atherosclerotic lesions in the arterial wall.[2−4]

Currently, treatment of atherosclerosis is aimed at reducing blood cholesterol and triglyceride content. The widespread clinical use of the HMGCo-A reductase inhibitors (statins) is accompanied by potential dose-limiting hepato- and myotoxicity, which may be the consequence of reduced levels of essential isoprenoid precursors, the antioxidant ubiquinone, or dolichols.[5] These levels are not affected, however, by the inhibition of the downstream enzyme of this pathway, squalene synthase (SQS), an alternative target for developing lipid-lowering agents.[6]

As multiple mechanisms are involved in the development of atherosclerosis (hyperlipidemia, oxidative stress, and inflammation), agents with at least two mechanisms of action may offer a therapeutic benefit compared to those only targeting a single mechanism.[7] We have previously developed 2-heteroaromatic[8] and polyaromatic[9] morpholine derivatives that lower cholesterol and triglyceride levels in vivo while combining in vitro antioxidant and SQS inhibitory activity. Extending our efforts toward this end, we designed compound **1** [2-(4-biphenyl)-4-methyl-3,4-dihydro-2*H*-benzo[1,4]oxazin-2-ol] that incorporates structural features of antioxidants (i.e., Trolox and **A**) as well as antihyperlipidemic (**B**) lead compounds[8,10] (Scheme 1). Moreover, compound **2** [2-(4-biphenyl)-4-methyl-3,4-dihydro-2*H*-benzo-[1,4]thiazin-2-ol] was synthesized as an isostere of compound **1**. The effect of the 2-hydroxy group on the activity of compounds **1** and **2** was also investigated; thus, compounds **3** and **4** were synthesized (Scheme 1). The pharmacological activity of the newly synthesized compounds **1**−**4** was investigated in vitro for antioxidant and SQS inhibitory activity, while intermediates **13**, **14**, and **17** were evaluated in vitro to investigate the effect of the 3-carbonyl group on lipid peroxidation. Target compounds **1** and **2** were also evaluated in vivo (antioxidant and antidyslipidemic activity, as well as determination of their time-dependent concentration in target tissues, i.e., blood/liver) and proven to be interesting molecules with combined activities useful as potential antiatherosclerotic agents.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The synthesis of compound **1** is rather straightforward (Scheme 2). The reaction of 2-methylaminophenol (**6**) with 4-bromoacetylbiphenyl (**7**) produced compound **1**. Compound **2** was synthesized following the synthetic procedure depicted in Scheme 2: ethyl (4-biphenyl)-α-bromoacetate (**10**) reacted with 2-aminobenzenethiol (**11**) to give the benzothiazine derivative **12**, N-methylation of which afforded **13**. Oxidation of compound **13** with *m*-chloroperbenzoic acid in chloroform gave a (unstable) sulfoxide (**13a**) that was rearranged (via a Pummerer reaction) during isolation with silica gel column

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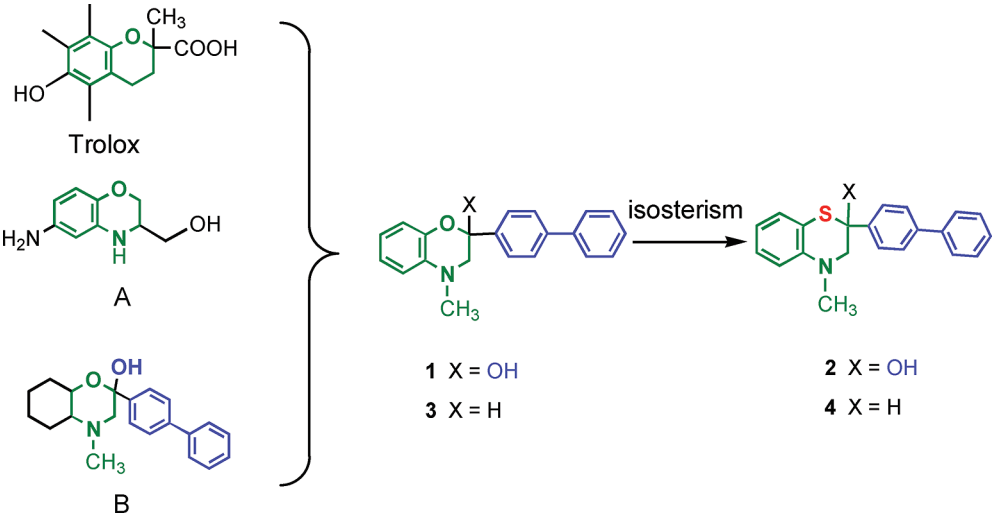
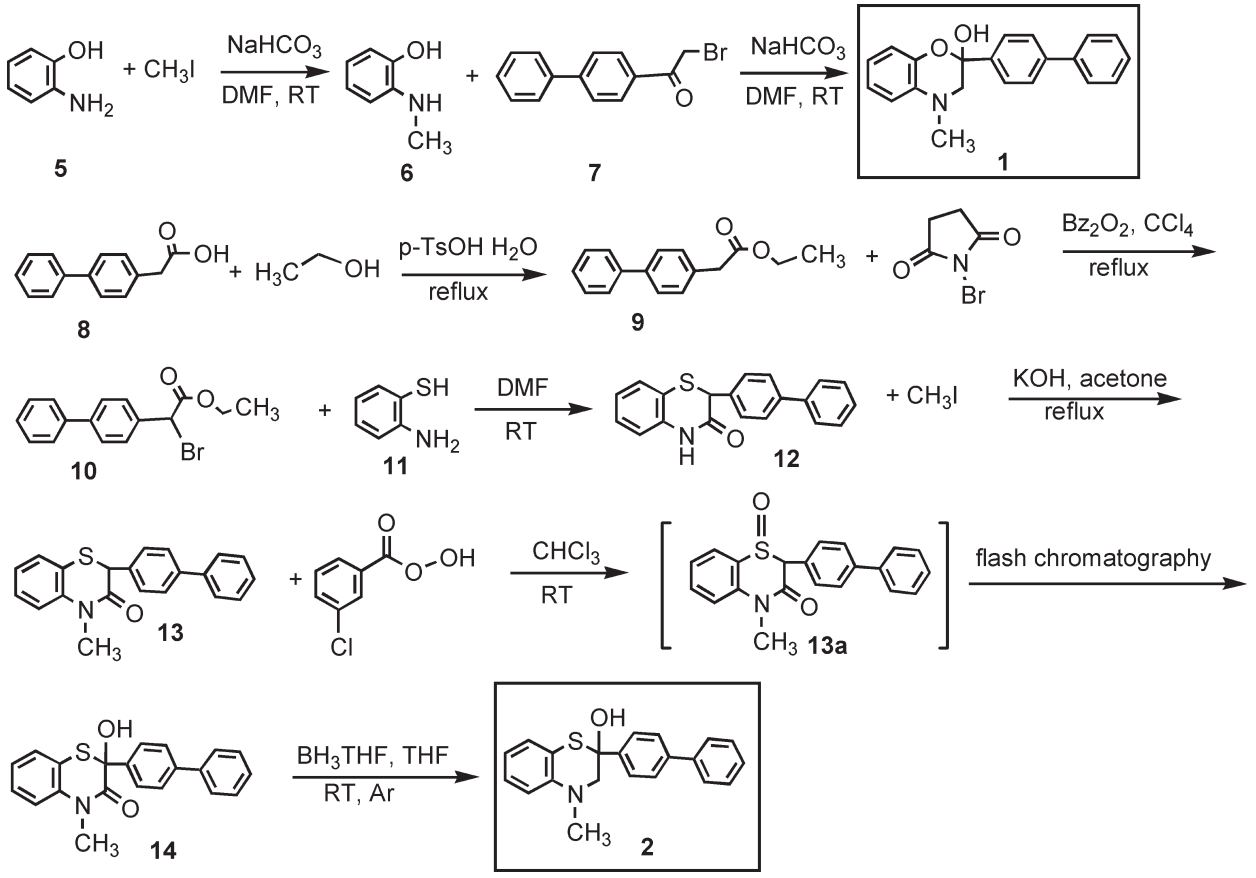
Scheme 1. Lead Structures and the Design of Target Molecules



Scheme 2. Synthetic Route for Compounds 1 and 2



chromatography to produce the 2-hydroxy compound **14**. Reduction of **14** afforded the end product **2**.

The synthesis of compounds **3** and **4** is shown in Scheme 3. Reaction of methyl (4-biphenyl)-α-bromoacetate (**15**) with 2-aminophenol (**5**) in the presence of K_2CO_3 afforded the benzoxazine derivative **16**, N-methylation of which gave **17**. Reduction of **17** and **13** with calcium borohydride ($Ca(BH_4)_2$) gave the end products **3** and **4**, respectively.[11]

^{13}C and ^{1}H NMR spectra indicated that compounds **1** and **2** are in the hemiketal and thiohemiketal forms, respectively, in solution, with no traceable amounts of the open keto alcohol or ketothiol form. Further, as verified by our theoretical studies,

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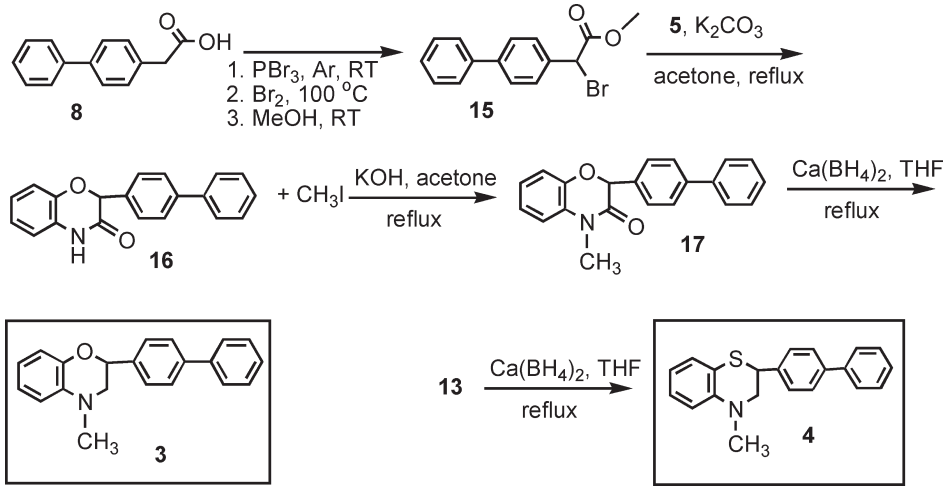
Scheme 3. Synthetic Route for Compounds 3 and 4



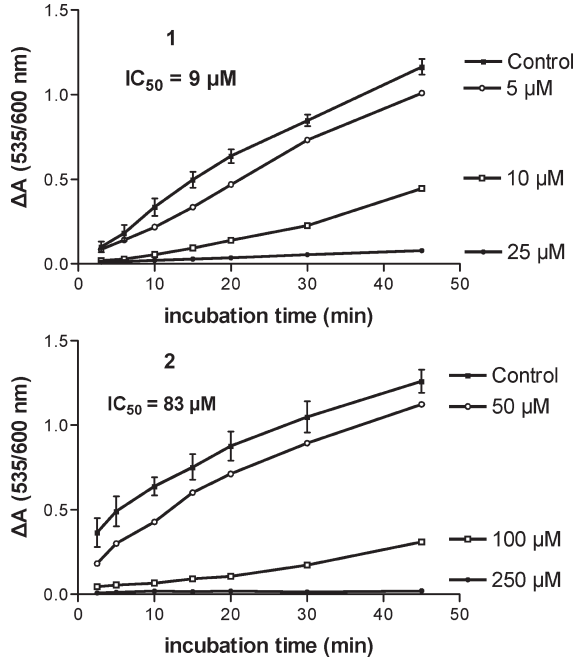


Figure 1. Time course of lipid peroxidation as affected by various concentrations of compounds **1** and **2**.

their stable conformations include equatorial position of the biphenyl substituent and axial position of the OH.

Compound **1** was stable at 4 °C for several weeks, after which it appeared to degrade as verified by TLC.

2.2. In Vitro Effect on Lipid Peroxidation. The time course of nonenzymatic lipid peroxidation, as affected by **1** and **2**, is represented in Figure 1, while IC_{50} values for **1**−**4** and intermediates **13**, **14**, and **17** are reported in Table 1. The new benzoxazine derivatives **1** and **3** are very active antioxidants (IC_{50} of 9 and 12 μM, respectively), while the benzothiazine derivatives **2** and **4** have significant antioxidant activity (IC_{50} of 83 and 39 μM, respectively) compared to lead compound B (IC_{50} = 450 μM).[8] Under the same experimental conditions, Trolox, a known potent antioxidant, and probucol exhibited IC_{50} values of 25 μM[12] and >1 mM,[8] respectively. To the best of our knowledge, only a few antioxidant compounds are reported with IC_{50} lower than 9 μM in this experimental protocol. The intermediate 3-oxobenzoxa(or thia)zine derivatives **13**, **14**, and **17** did not exhibit significant activity against lipid peroxidation. The altered conformation of the benzoxazine or benzothiazine ring in the presence of the 3-carbonyl group of compounds **13**, **14**, and **17** may contribute to reduced access, retention, and interaction with biological membranes, the site of lipid peroxidation. No apparent correlation between antioxidant activity and lipophilicity seems to apply among the tested compounds.

2.3. Effect on LDL Oxidation. Since LDL is considered the major target site for antioxidants intended as antiatherosclerotic agents, the effect of the more active derivative **1** was further investigated on Cu^{2+}-mediated oxidation of human LDL.[13] Figure 2a depicts the activity of 5, 7.5, and 10 μM **1** on LDL oxidation. Oxidation of LDL (56 μg/mL) was determined by measuring conjugate diene formation at 234 nm for 300 min, which represents the early phase peroxidation of LDL (Table 2). As reported in Table 2, the extension of the lag time of oxidation in the presence of antioxidant is interpreted as the resistance capacity of LDL particles toward oxidation. Results show a typical concentration-dependent effect of compound **1** on prolonging the control lag time (125 min) to 168 min at 5 μM (34% increase) and to 214 min at 7.5 μM (71% increase), decreasing the rate of conjugate diene formation from 8.41 (nmol/min)/mg protein (control) to 5.57 (nmol/min)/mg (34% decrease) at 5 μM and to 3.14 (nmol/min)/mg (63% decrease) at 7.5 μM, respectively (Figure 2). In the presence of 10 μM **1** the lag time was too prolonged to be recorded, while there was an almost total inhibition of LDL oxidation. Under the same experimental conditions, the lead compound **B** increased the 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%.[13] The above results render compound **1** a very strong inhibitor of LDL oxidation, a property that may be due to the incorporated structural similarity with known antioxidants (e.g., Trolox and **A**), favoring an increased interaction with LDL particles.

2.4. Effect on Squalene Synthase. All compounds were evaluated for the inhibition of rat microsomal SQS. The inhibitory

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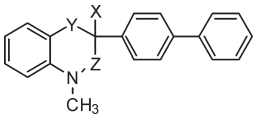
Table 1. Antioxidant [Inhibition of Lipid Peroxidation (LP)], SQS Inhibitory, And Antihyperlipidemic Effects of the Investigated Compounds and cLogP



				IC_{50} (μM)		% decrease compared to hyperlipidemic controls (56 μmol/kg ip)					
compd	X	Y	Z	inhibition of LP	microsomal SQS inhibition	TC	LDL	TG	MDA	LDL/HDL ratio	cLogP
1	OH	O	CH_2	9	12.8	62[a]	74[b]	73[a]	63[a]	0.29[b]	4.87
2	OH	S	CH_2	83	16.5	58[a]	26[c]	38[b]	31[b]	0.45[d]	5.16
3	H	O	CH_2	12	5.7						6.01
4	H	S	CH_2	39	4.9						6.35
13	H	S	CO	>1000							5.06
14	OH	S	CO	1000							4.55
17	H	O	CO	422							4.76
B				450	36	54[a]	51[b]	49[a]			4.51
simvastatin						75[a]	70[a]	0			1.98
probucol				>1000		18[b]	18[d]	11[d]			10.75
Trolox				24.8							3.09

[a] $P < 0.005$. [b] $P < 0.05$. [c] $P < 0.1$. [d] Not significant ($P > 0.1$) (Student's t test).

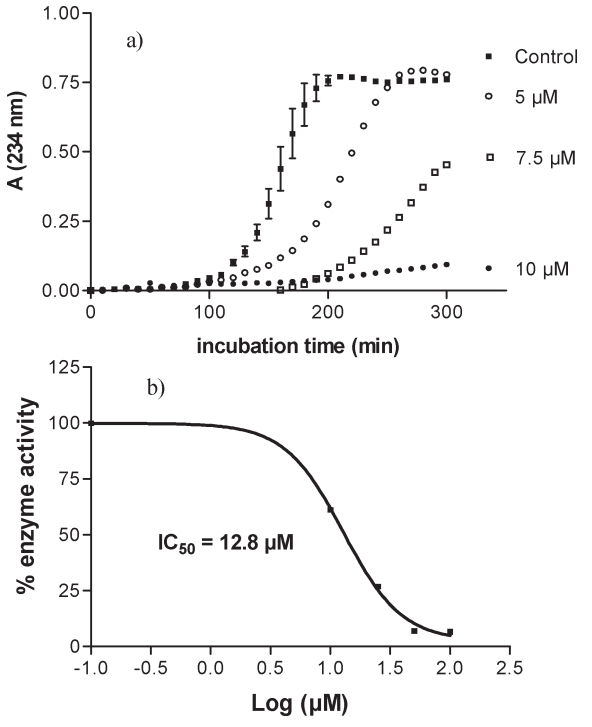


Figure 2. (a) Effect of compound **1** on the Cu^{2+}-induced oxidation of LDL. (b) Representative graph showing the activity of squalene synthase as affected by various concentrations of compound **1**.

effect on enzyme activity was measured according to the method of Amin et al.[14] Compounds **1**−**4** inhibited squalene synthase activity significantly and dose-dependently (Table 1), as shown in Figure 2b, which depicts the activity of compound **1**. It was shown that aromatic replacement of the cyclohexyl ring of reference **B** led to 3-fold higher squalene synthase inhibitory activity (IC_{50} = 12.8 μM for **1** compared to 36 μM [8] for **B**) while the isosteric replacement of oxygen with thio (compound **2**) did not alter significantly the activity of **1** (IC_{50} = 16.5 μM for **2**). To our surprise the non-hydroxyl derivatives **3** and **4** appeared to be even better squalene synthase inhibitors with IC_{50} values of 5.7 and 4.9 μM, respectively. Although the latter derivatives are more lipophilic and thus may interact more efficiently with the hydrophobic cavity of the binding site of SQS, the hydroxyl group of similar derivatives has been considered a moiety that increases interactions via hydrogen bonding.[9] In this case significant correlation between IC_{50} (or pIC_{50}) and cLogP was found (r^2 = 0.9, n = 4, P = 0.06). In order to visualize these differences, we performed rigid docking studies of compounds **1**−**4** using the available X-ray crystal structure of human SQS and Autodock 4.[9] The best fit complexes of SQS with derivatives **1** and **2** (magenda and light blue structures, Figure 3) revealed a hydrogen bond between their respective 2-hydroxyl group and GLN 212 and lipophilic interactions of their biphenyl moiety with the hydrophobic cavity[15] of what is considered the binding site of SQS (oriented "diagonally" from bottom left to top right of the protein structure in Figure 3). The more lipophilic derivatives **3** and **4** (green and yellow structures, Figure 3), lacking the 2-OH group, bind deeper in this cavity via lipophilic interactions. This difference in binding mode may possibly explain the unexpected activity of derivatives **3** and **4** compared to **1** and **2**. In all cases, binding of **1**−**4** appears to be somewhat deeper in the hydrophobic cavity of SQS compared to previously experimentally and theoretically evaluated derivatives[9] (e.g., compound **B**, blue structure in Figure 3).

2.5. Effect on Dyslipidemia. Compounds **1** and **2** were administered in a single ip dose of 56 μmol/kg (in order to allow comparison with related compounds of previous studies)[8,9] to hyperlipidemic rats (after Triton WR 1339 administration), and their effect on total cholesterol, LDL

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Table 2. Effect of 1 on the Lag Time (min) and Rate of Conjugate Diene formation in Copper-Induced Lipid Peroxidation of LDL

concentration (μM)	lag time (min)	Δt (min)	% increase lag time	rate of conjugate diene formation (nmol/min)/mg protein)	% decrease of rate of conjugate diene formation
0	125			8.4	
5	168	43	34	5.6	34
7.5	214	89	71	3.1	63
10	*a*		100		100

[a] 100% inhibition of conjugate diene formation.

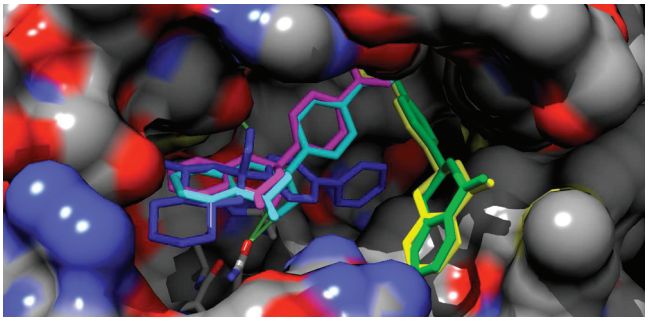

Figure 3. Binding mode of compounds **1** and **2** (magenta and light blue), **3** and **4** (green and yellow), and **B** (blue) at the active site of SQS. Hydrogen bonds between the respective 2-hydroxyl groups of **1** and **2** and the amide residual of GLN 212 are highlighted in green.

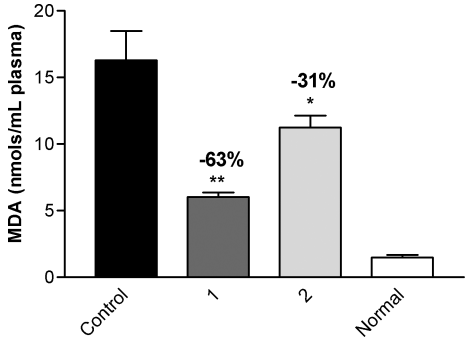

Figure 4. Effect of compounds **1** and **2** on MDA levels of hyperlipidemic rats. Statistical significance is as follows: ∗, $P < 0.05$; ∗∗, $P < 0.005$.

cholesterol, LDL/HDL ratio, and triglyceride plasma levels (mg/dL plasma) was investigated. Both compounds were found to reduce the examined parameters by 26−74% (Table 1). Under the same experimental conditions and at the same dose, probucol and simvastatin reduced plasma total cholesterol by 18 and 75%, LDL cholesterol by 18% and 70%, and triglycerides by 11% and 0%, respectively. The lead compound **B** decreased the above parameters by 54%, 51%, and 49%, respectively.[8] Compound **1** had a remarkable effect, greater than the lead compound **B**, on all lipidemic indices, comparable to that of simvastatin in total cholesterol and LDL-cholesterol parameters and even greater on triglyceride levels while the ratio LDL/HDL that is considered of interest remained significantly lower (0.29 ± 0.06) compared to control values (0.58 ± 0.02) ($P < 0.05$). Although compound **2** was somewhat less active than compound **1**, both compounds were more active than probucol, a known antioxidant molecule that has been used as an antiatherosclerotic agent.

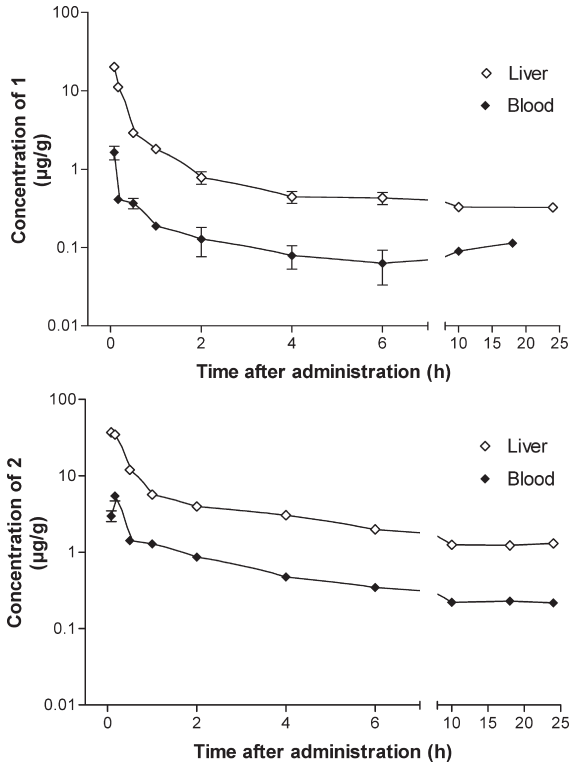

Figure 5. Concentration−time profile of compounds **1** and **2** in liver and blood after a single ip administration of 56 μmol/kg (17.8 mg/kg for **1** and 18.7 mg/kg for **2**) to mice. Values are the mean (±SEM) of four to five animals per time point.

2.6. In Vivo Antioxidant Activity. Compound **1** reduced malondialdehyde (MDA) content[16] in the plasma of experimentally induced hyperlipidemic rats by 63%, while the benzothiazine derivative **2** reduced it by 31% (Table 1 and Figure 4). The increased in vitro antioxidant and in vivo antidyslypidemic activity of **1** relative to **2** is also reflected in the greater in vivo antioxidant activity of this compound.

2.7. Pharmacokinetic Studies. The time course of the blood and liver concentrations of compounds **1** and **2** after ip injection in mice is shown in Figure 5, while corresponding pharmacokinetic/analytical parameters of both compounds are presented in Table 3. The HPLC method for **1** and **2** was linear in the range 0.1−14 μg/mL (before extraction), and recovery over the whole range was more than 95% for both liver and whole blood samples. Intraperitoneal injection of **1** and **2** at equimolar doses (56 μmol/kg in mice) showed an initial concentration of 2−5 μg/g in blood and 20−37 μg/g in liver. For both compounds these levels decreased within approximately 2 h, after which concentration levels (approximately 0.15−3 μg/g tissue)

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Table 3. Pharmacokinetic/Analytical Parameters of 1 and 2 after a Single ip Administration of 56 μmol/kg (17.8 mg/kg for 1 and 18.7 mg/kg for 2) to Mice

	1		2	
parameter	blood	liver	blood	liver
$AUC_{(5'-24h)}$ (μg·h/g)	2.0	14.3	9.7	56.1
$AUMC_{(5'-24h)}$ [a] (μg·h/g)	16.6	99.8	71.9	404.2
$AUMC/AUC_{(5'-24h)}$ (h)	8.4	7.0	7.4	7.2
concentration (μg/g) at t = 5 min	1.6	20.1	3.0	37.1
concentration (μg/g) at t = 0.5 h	0.4	2.9	1.4	12.0
$AUC_{(5'-24h)}$(liver)/$AUC_{(5'-24h)}$(blood)	7.1		5.8	
LOD[b] (μg/g)	0.06	0.05	0.02	0.01
LOQ[c] (μg/g)	0.21	0.20	0.07	0.04

[a] Area under the (first) moment curve. [b] Estimated limit of detection. [c] Estimated limit of quantification.

remained fairly stable up to 24 h, which did not allow for an accurate extrapolation in order to determine the total AUC. The pharmacokinetic parameters that were calculated based on noncompartmental analysis[17] are shown in Table 3. The liver concentrations for both compounds were consistently higher than their blood levels, a phenomenon that may favor their activity on SQS. Although in vitro activities are not always directly correlated with in vivo efficacies, it is shown here that both **1** and **2** reach their target sites (i.e., liver (for SQS activity) and blood (for protection against LDL oxidation)) at low micromolar levels, a range more or less "comparable" to their in vitro IC_{50} values.

2.8. Conclusion. We designed, synthesized, and evaluated novel compounds **1**−**4** in order to develop more active compounds aimed toward atherosclerosis. Several pharmacophore moieties found in antioxidant and antidyslipidemic lead compounds are intergrated in the structures of compounds **1** and **3**, while compounds **2** and **4** are derived from the isosteric replacement of the oxygen of compounds **1** and **3** with sulfur. Compound **1** is a powerful in vitro and in vivo inhibitor of lipid peroxidation and a potent antidyslipidemic agent, while compound **2** exhibited significant, although not higher than **1**, antioxidant and antidyslipidemic activity. The mechanism of the antidyslipidemic activity of these compounds is considered to proceed via squalene synthase inhibition: all compounds inhibited the activity of this enzyme significantly. Further, we determined the systemic exposure for compounds **1** and **2** during the time course of the in vivo experiment, which was in the lower micromolar range for both target tissues, blood and liver.

Structurally diverse benzoxazine derivatives (but very different from the compounds described here) have been shown to have antioxidant[10,18] or neuroprotective activity,[19,20] although no benzothiazine derivative has been previously evaluated for antioxidant activity. To our knowledge, this is the first time that 1,4-benzoxazine and 1,4-benzothiazine structures are shown to combine, by design in one structure, antioxidant, protective against LDL oxidation, and antidyslipidemic activity. Compounds **1**−**4** seem to be interesting multifunctional molecules for the development of a new pharmacophore for disease-modifying agents useful in the treatment of atherosclerosis.

3. EXPERIMENTAL SECTION

3.1. Materials. All commercially available chemicals are of the appropriate purity and purchased from standard sources. [^{3}H]FPP (21.5 Ci/mmol), NADPH, FPP, and BSA were purchased from Sigma-Aldrich (Germany). For the in vivo experiments, Wistar male rats (200−250 g) or SKH2 mice (26−29 g) were kept in a controlled temperature room (22 ± 2 °C), having free access to chow and tap water, under a 12 h light/dark cycle.

3.2. Synthesis. Melting points 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 Brücker Avance DRX 400 (400 MHz) and DPX 200 (200 MHz) spectrometer, respectively. The mass spectrum of **1** was obtained on an API 2000 mass 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%.

*2-(4-Biphenyl)-4-methyl-3,4-dihydro-2H-benzo[1,4]oxazin-2-ol (**1**).* To a solution of 2-hydroxyphenylmethylamine **6** (2.03 mmol) in dry DMF were added $NaHCO_3$ (2.13 mmol) and 4-bromoacetylbiphenyl **7** (2.23 mmol). After stirring at RT (4 h), EtOAc was added and the mixture was filtered, washed with water and brine, dried, and concentrated in vacuum. The product was purified by silica gel flash chromatography (PE/EtOAc, 5:1) to afford **1** as a brown oil (40%) stable for a few weeks. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.95 (s, 3H, NCH_3), 3.07 (d, J = 11.15 Hz, 1H, 3-H_{ax} benzoxazine), 3.15 (d, J = 10.96 Hz, 1H, 3-H_{eq} benzoxazine), 4.33 (brs, 1H, −OH), 6.80−6.83 (m, 2H, 5,7-H benzoxazine), 6.92−6.98 (m, 2H, 6,8-H benzoxazine), 7.24−7.33 (m, 1H, biphenyl-H), 7.38−7.42 (t, J = 7.43 Hz, 2H, biphenyl-H), 7.56 (d, J = 7.24 Hz, 2H, biphenyl-H), 7.60 (d, J = 8.22 Hz, 2H, biphenyl-H), 7.70 (d, J = 8.41 Hz, 2H, biphenyl-H). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 38.50, 59.28, 94.89, 112.95, 117.34, 120.37, 121.83, 126.49 (2C), 127.16 (2C), 127.21 (2C), 127.50, 128.81 (2C), 134.90, 138.90, 140.69, 141.79, 143.26. $C_{21}H_{19}NO_2$, M_r = 318.14, ESI-MS (m/z): 318.29 $(M + H)^+$. Elemental analysis (C, H, N): $C_{21}H_{19}NO_2 \cdot 0.2H_2O$. Calculated C 78.48, H 6.10, N 4.36. Found C 78.58, H 6.09, N 4.36.

*2-(4-Biphenyl)-4-methyl-3,4-dihydro-2H-benzo[1,4]thiazin-2-ol (**2**).* Benzothiazine derivative **14** (0.32 mmol) was dissolved in dry THF under argon, and $BH_3 \cdot THF$ (1M, 4.7 mL) was added dropwise and stirred at RT (24 h). Water was added and the mixture extracted with CH_2Cl_2 and washed with water and saturated $NaHCO_3$ solution. The residue was purified by silica gel flash chromatography (PE/DCM, 1:1) to afford **2** as a yellow semisolid (48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.05 (s, 3H, $-NCH_3$), 3.23 (d, J = 11.74 Hz, 1H, 3-H benzothiazine), 3.56 (d, J = 11.94 Hz, 1H, 3-H benzothiazine), 4.04 (brs, 1H, −OH), 6.09 (t, J = 7.34 Hz, 1H, 7-H benzothiazine), 6.88 (d, J = 8.61 Hz, 1H, 5-H benzothiazine), 7.06−7.07 (m, 2H, 6,8-H benzothiazine), 7.30−7.32 (m, 1H, biphenyl-H), 7.39 (t, J = 7.54 Hz, 2H, biphenyl-H), 7.54−7.59 (2d, 4H, biphenyl-H), 7.70 (d, J = 8.22 Hz, 2H, biphenyl-H). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 40.77, 64.16, 82.06, 113.36, 119.89, 125.38, 126.38 (2C), 126.64, 127.16 (4C), 127.49, 128.81 (2C), 130.28, 139.59, 140.55, 141.23, 143.28. Elemental analysis (C, H): $C_{21}H_{19}NOS \cdot 0.5H_2O$. Calculated C 73.65, H 5.89. Found C 73.99, H 6.02.

*2-(4-Biphenyl)-4-methyl-3,4-dihydro-2H-1,4-benzoxazine (**3**).* $Ca(BH_4)_2$ was prepared in situ by the addition of $NaBH_4$ (2.90 mmol) to a suspension of $CaCl_2$ (1.24 mmol) in dry THF (5 mL) at 0 °C.[11] Compound **17** (0.48 mmol) was added and the mixture refluxed for 30 h. The reaction mixture was then treated with brine, extracted with CH_2Cl_2, dried, and concentrated in vacuum. The residue was purified by silica gel flash chromatography (PE/DCM, 3:1) to afford **3** as a white solid (57%): mp = 112−113.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.88 (s, 3H, $-NCH_3$), 3.20−3.27 (m, 1H, 3-H benzoxazine), 3.31−3.38 (m, 1H, 3-H benzoxazine), 5.21 (d, J = 8.32 Hz, 1H, 2-H benzoxazine), 6.65−6.71 (m, 2H, 5,7-H benzoxazine), 6.84−6.89 (m, 2H, 6,8-H benzoxazine), 7.29−7.35 (m, 1H, biphenyl-H), 7.38−7.45 (m, 4H, biphenyl-H), 7.50−7.58 (m, 4H, biphenyl-H). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 38.66, 55.97, 75.30, 112.52, 116.26, 118.39, 121.68, 126.81, 126.93, 127.13, 127.36, 128.71, 128.82,

131.94, 135.87, 137.99, 138.48, 139.68, 140.06, 140.75, 144.78. Elemental analysis (C, H): $C_{21}H_{19}NO \cdot 0.25H_2O$. Calculated C 82.45, H 6.27. Found C 82.07, H 6.21.

*2-(4-Biphenyl)-4-methyl-3,4-dihydro-2H-1,4-benzothiazine (**4**).* Compound **13** (0.45 mmol) and $Ca(BH_4)_2$ were reacted under the same conditions described for **3** to give **4** as a white solid (50%): mp = 130.5−131.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.94 (s, 3H, $-NCH_3$), 3.55−3.63 (m, 2H, 2 × 3-H benzothiazine), 4.40−4.43 (2d, J = 4.60 Hz, 1H, 2-H benzothiazine), 6.65 (t, J = 7.44 Hz, 1H, 7-H benzothiazine), 6.71 (d, J = 8.22 Hz, 1H, 5-H benzothiazine), 7.01 (t, J = 7.82 Hz, 1H, 6-H benzothiazine), 7.07 (d, J = 7.63 Hz, 1H, 8-H benzothiazine), 7.27−7.31 (m, 1H, biphenyl-H), 7.37−7.40 (m, 4H, biphenyl-H), 7.52 (d, J = 7.82 Hz, 4H, biphenyl-H). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 40.17, 43.64, 58.56, 112.65, 118.05, 125.84, 126.96, 127.08(2C), 127.48 (4C), 128.34 (2C), 128.81 (2C), 138.53, 140.62, 140.91, 143.96. Elemental analysis (C, H): $C_{21}H_{19}NS \cdot 0.25H_2O$. Calculated C 78.34, H 6.12. Found C 78.48, H 6.12.

*2-Hydroxyphenylmethylamine (**6**)*[21]. A solution of 2-aminophenol **5** (18 mmol) in dry DMF was treated with $NaHCO_3$ (19 mmol) and CH_3I (22 mmol) and stirred at room temperature for 5 h. After the usual workup the residue was purified by silica gel flash chromatography (petroleum ether/ethyl acetate, 5:1) to afford **6** as a white solid (50%): mp = 102.5−105 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.61 (s, 3H, CH_3), 4.65 (brs, 1H, NH), 6.29−6.34 (m, 2H, 3,5-H Ar), 6.54−6.58 (m, 2H, 4,6-H Ar), 9.07 (s, 1H, −OH).

*Ethyl (4-Biphenyl)acetate (**9**)*[22]. Compound **9** was synthesized according to the procedure described previously.[22] Colorless oil (89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.21 (t, J = 7.05 Hz, 3H, $-CH_3$), 3.60 (s, 2H, $-CH_2CO$), 4.12 (q, J = 7.05 Hz, 2H, OCH_2), 7.26−7.31 (m, 3H, biphenyl-H), 7.36−7.39 (m, 2H, biphenyl-H), 7.49−7.54 (m, 4H, biphenyl-H).

*Ethyl (4-Biphenyl)-α-bromoacetate (**10**)*[23]. Compound **10** was synthesized according to the procedure described previously.[23] Dark yellow oil (91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.25 (t, J = 7.14 Hz, 3H, $-CH_3$), 4.20 (q, J = 7.24 Hz, 2H, $-OCH_2$), 5.33 (s, 1H, −CHBr), 7.29−7.34 (m, 1H, biphenyl-H), 7.37−7.42 (m, 2H, biphenyl-H), 7.51−7.58 (m, 6H, biphenyl-H).

*2-(4-Biphenyl)-2H-1,4-benzothiazin-3(4H)-one (**12**).* A solution of ethyl (4-biphenyl)-α-bromoacetate **10** (2.7 mmol) and 2-aminobenzenethiol **11** (4.1 mmol) in dry DMF was stirred at room temperature for 25 h.[24] The reaction mixture was then poured into ice−water. The precipitated solid was filtered off, triturated with acetonitrile, filtered off, and dried to afford **12** as a white solid (70%): mp = 235−236.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 4.68 (s, 1H, 2-H benzothiazine), 6.81 (d, J = 8.02 Hz, 1H, 8-H benzothiazine), 6.96 (dt, J_1 = 1.17 Hz, J_2 = 7.62 Hz, 1H, 7-H benzothiazine), 7.11 (dt, J_1 = 1.18 Hz, J_2 = 7.83 Hz, 1H, 6-H benzothiazine), 7.25−7.29 (m, 2H, 5-H benzothiazine and biphenyl-H), 7.34 (d, J = 7.83 Hz, 2H, biphenyl-H), 7.38 (d, J = 8.22 Hz, 2H, biphenyl-H), 7.45−7.48 (m, 4H, biphenyl-H), 8.55 (s, 1H, NH). Elemental analysis (C, H): $C_{20}H_{15}NOS$. Calculated C 75.68, H 4.76, Found C 75.35, H 4.71.

*2-(4-Biphenyl)-4-methyl-2H-1,4-benzothiazin-3(4H)-one (**13**).* An excess of KOH (4.1 mmol) was added to a solution of 2-(4-biphenyl)-2*H*-1,4-benzoxazin-3(4*H*)-one **12** (1.1 mmol) in dry acetone.[25] The mixture was refluxed, while methyl iodide (1.7 mmol) in dry acetone was added dropwise. The mixture was then stirred at 60 °C for 15 min. Acetone was filtered, evaporated in vacuum, and diethyl ether was added. The organic layer was washed with water and brine, dried, and concentrated in vacuum. The residue was purified by silica gel flash chromatography (petroleum ether/dichloromethane, 2:3) to afford **13** as a yellow solid (84%): mp = 170−171.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.37 (s, 3H, $-NCH_3$), 5.69 (s, 1H, 2-H benzoxazine), 6.69−6.76 (m, 2H, 5,6-H benzoxazine), 6.89−6.92 (m, 1H, 8-H benzoxazine), 6.96−6.99 (m, 2H, biphenyl-H), 7.01−7.04 (m, 1H, 7-H benzoxazine), 7.33 (d, J = 8.32 Hz, 2H, biphenyl-H), 7.40−7.44 (m, 3H, biphenyl-H), 7.47 (d, J = 8.32 Hz, 2H, biphenyl-H). Elemental analysis (C, H): $C_{21}H_{17}NOS \cdot 0.1CH_2Cl_2$. Calculated C 73.94, H 5.09, Found C 73.74, H 5.16.

*2-(4-Biphenyl)-2-hydroxy-4-methyl-4H-benzo[1,4]thiazin-3-one (**14**).* A stirred solution of 2-(4-biphenyl)-4-methyl-2*H*-1,4-benzothiazin-3-(4*H*)-one **13** (0.9 mmol) in dry $CHCl_3$ was treated with *m*-chloroperbenzoic acid, 77% (0.9 mmol), and the mixture was stirred for 3 h at room temperature.[26] The solution was washed with water and saturated $NaHCO_3$ solution. The organic layer was dried and concentrated in vacuum. The residue was purified by silica gel flash chromatography (*n*-hexane/ethyl acetate, 8:1) to afford **14** as a yellow solid (64%): mp = 138.0−140.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.57 (s, 3H, $-NCH_3$), 5.13 (brs, 1H, −OH), 6.90−6.96 (m, 2H, 5,6-H benzothiazine), 7.11 (t, J = 7.83 Hz, 1H, 7-H benzothiazine), 7.23−7.26 (m, 2H, 8-H benzothiazine and biphenyl-H), 7.30−7.37 (m, 4H, biphenyl-H), 7.41−7.44 (m, 4H, biphenyl-H). Elemental Analysis (C, H): $C_{21}H_{17}NO_2S \cdot 0.5$ hexane. Calculated C 73.81, H 6.21, Found C 73.62, H 6.30.

*Methyl (4-Biphenyl)-α-bromoacetate (**15**)*[27]. Compound **15** was synthesized according to the literature.[27] Yellow oil (96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.83 (s, 3H, $-OCH_3$), 5.42 (d, J = 2.56 Hz, 1H, −CHBr), 7.38−7.51 (m, 3H, biphenyl-H), 7.54−7.66 (m, 6H, biphenyl-H).

*2-(4-Biphenyl)-2H-1,4-benzoxazine-3(4H)-one (**16**).* A mixture of 2-aminophenol **5** (5.30 mmol), methyl (4-biphenyl)-α-bromoacetate **15** (4.82 mmol), K_2CO_3 (5.78 mmol), and acetone (25 mL) was refluxed for 2 h.[27] The reaction mixture was diluted with water and extracted with EtOAc. The organic phase was washed with water, dried, and concentrated in vacuum, and cold $CHCl_3$ was added to the residue. The precipitated solid was triturated with acetone, filtered off, and dried to afford **16** as a white solid (31%): mp = 250 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 5.74 (s, 1H, 2-H benzoxazine), 6.28−6.55 (m, 1H, 6-H benzoxazine), 6.85−6.87 (m, 3H, 5,7,8-H benzoxazine), 6.93−6.95 (m, 1H, biphenyl-H), 7.38 (d, J = 7.34 Hz, 2H, biphenyl-H), 7.52−7.60 (m, 6H, biphenyl-H), 10.90 (s, 1H, NH).

*2-(4-Biphenyl)-4-methyl-2H-1,4-benzoxazin-3(4H)-one (**17**).* 2-(4-Biphenyl)-2*H*-1,4-benzothiazin-3(4*H*)-one **16** (1.9 mmol) and methyl iodide (2.8 mmol) were reacted under the same conditions[25] described for **13** to give **17** as a yellow semisolid (80%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.48 (s, 3H, -NCH_3), 4.64 (s, 1H, 2-H benzothiazine), 6.96 (t, J = 7.58 Hz, 1H, 6-H benzothiazine), 7.02 (d, J = 8.07 Hz, 1H, 5-H benzothiazine), 7.17 (m, 1H, 7-H benzothiazine), 7.24−7.36 (m, 6H, 8-H benzothiazine and biphenyl-H), 7.42 (d, J = 8.07 Hz, 2H, biphenyl-H), 7.46 (d, J = 7.58 Hz, 2H, biphenyl-H).

3.3. In Vitro Microsomal Lipid Peroxidation. Heat-inactivated hepatic microsomes from untreated rats were prepared as described previously. The inhibitory effect of compounds on lipid peroxidation was assessed spectrophotometrically (535 against 600 nm) as TBAR material.[8]

3.4. Isolation and in Vitro LDL Oxidation. Blood was collected from a normolipidemic human volunteer, and LDL was isolated by discontinuous density gradient (using KBr) ultracentrifugation.[28]

LDL (56 μg of protein/mL) in PBS was incubated at 37 °C in the absence or presence of various concentrations of **1** (in 10% DMSO−H_2O). Oxidation was initiated by 10 μM $CuSO_4$. Conjugated dienes were determined every 10 min for 5 h as the increase in absorbance at 234 nm and calculated using the extinction coefficient of 29 500.[13]

3.5. In Vitro Squalene Synthase Activity Assay. SQS activity was evaluated by determining the amount of [^{3}H]FPP converted to squalene as previously described.[9,14]

3.6. Docking Studies. Preparation of ligands, protein crystal structure (1EZF), and docking runs were performed with Spartan04 (Wavefunction Inc., Irvine, CA, 2004), Chimera, and Autodock 4/Autodock Tools[29,30] and according to previous methodology.[9]

3.7. In Vivo Evaluation of Antidyslipidemic and Antioxidant Activity. Triton WR 1339[8] was given ip to rats (200 mg/kg), and 1 h later the test compounds (56 μmol/kg) were given. After 24 h, blood was collected from the aorta and used for the determination of lipidemic parameters, using commercially available kits, and MDA content, by measuring the complex of MDA with *N*-methyl-2-phenylindole (586 nm).[16] Values are the means from six to eight rats, while all standard errors are within 10% of the respective values.

3.8. Pharmacokinetic Studies. Compounds **1** and **2** were administered ip to mice in a single dose of 56 μmol/kg. At predetermined time points (5, 10, 30 min and 1, 2, 4, 6, 10, 18, and 24 h) after administration of compounds and under sevoflurane anesthesia, blood (from the heart) was collected in heparinized tubes. Liver samples were weighed and homogenized after diluting three times with ice-cold Tris-HCl (50 mM, pH 7.4) buffer. All samples were kept on ice until processed. Whole blood (for animals administered compound **1**) or plasma isolated by centrifugation at 4 °C (for compound **2**) and liver homogenates were extracted with twice the volume of acetonitrile, and the mixtures were centrifuged at 4 °C. The supernatants were maintained at 4 °C until the injection of 20 μL onto a Waters Symmetry Shield ResElut C8 reverse-phase column (5 μm, 3 mm × 150 mm) with a guard column, thermostated at 35 °C using an Aligent 1100 HPLC equipped with a UV−vis variable wavelength detector. The mobile phase (60% acetonitrile and 40% of 10 mM acetic acid) was eluted at a 0.7 mL/min with compounds **1** and **2** being detected at 295 and 255 nm, respectively, and analyzed using Chemstation software 1990−2003. Retention times were 6.9 min for **1** and 9.8 min for **2**. The recovery of compounds **1** and **2** from whole blood and liver homogenate was determined by comparing the peak areas obtained for mixtures of either the compound-free biological matrix or water (for calibration standards) with the appropriate stock solutions of each compound to reach final concentrations in the range 0.10−14 μg/mL and after subjecting them to the extraction procedure described above, including the isolation of plasma by centrifugation for samples with compound **2** only. Final results are expressed as microgram of corresponding compound per gram of tissue (blood or liver).

3.9. Protein Determination. The protein content of microsomal and LDL fractions was determined according to Lowry's method.[31]

3.10. 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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ABBREVIATIONS USED

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-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; MDA, malondialdehyde

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