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Synthesis and highly potent hypolipidemic activity of alpha-asarone- and fibrate-based 2-acyl and 2-alkyl phenols as HMG-CoA reductase inhibitors

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ABSTRACT

In the search for new potential hypolipidemic agents, the present study focused on the synthesis of 2-acyl phenols (**6a–c** and **7a–c**) and their saturated side-chain alkyl phenols (**4a–c** and **5a–c**), and on the evaluation of their hypolipidemic activity using a murine Tyloxapol-induced hyperlipidemic protocol. The whole series of compounds **4–7** greatly and significantly reduced elevated serum levels of total cholesterol, LDL-cholesterol, and triglycerides, with series **6** and **7** showing the greatest potency ever found in our laboratory. At the minimum dose (25 mg/kg/day), the latter compounds lowered cholesterol by 68–81%, LDL by 72–86%, and triglycerides by 59–80%. This represents a comparable performance than that shown by simvastatin. Experimental evidence and docking studies suggest that the activity of these derivatives is associated with the inhibition of HMG-CoA reductase.

1. Introduction

Hyperlipidemia represents a great burden for the health care systems of many countries. A third of adults meet the criteria for metabolic syndrome,[1] the principal symptom of which is central obesity. Recent studies suggest extraordinarily high prevalence rates of hypercholesterolemia (HC) and hypertriglyceridemia (HTG),[2] also associated with obesity. In the year 2000, about 30 million adults (60.5% of the adult population) in Mexico had at least one cardiovascular risk factor,[3] and ischaemic heart disease was the second global leading cause of general mortality.[4] Obesity is an independent risk factor for cardiovascular disease (CVD) and mortality, being associated with hypertension, dyslipidemia, glucose intolerance and insulin resistance.[5] In 2007, the Frimex (Risk Factors in Mexico) survey data showed that 71.9% of the 140,017 participants were overweight or obese, 26.5% had hypertension and 40% hypercholesterolemia.[6] The prevalence of obesity in Mexico is currently about 65% in the adult population.[7,8]

Hyperlipidemia is a risk factor for cardiovascular disease, including atherosclerosis.[9,10] Genetic and environmental factors associated with obesity and diabetes may contribute to the high prevalence of dyslipidemia in Mexico and worldwide.[11] Lifestyle interventions—including diet, exercise and weight loss—represent the primary strategy during the initial stages of dyslipidemia treatment.[9,12] However, if this strategy is ineffective and/or patients begin to exhibit multiple risk factors for chronic disease, healthcare practitioners turn to lipid-lowering pharmaceuticals.[12,13]

Many synthetic drugs are available for the treatment of hyperlipidemia, but all have serious side effects. Muscle (myopathy, rhabdomyolysis) and liver toxicity are the most common adverse effects reported with hypolipidemic therapy.[14–21] Many other adverse effects have been attributed to statins such as

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rhabdomyolysis, cognitive decline, neuropathy, pancreatic and hepatic dysfunction, and increased risk of cancer.[22] Thus, there is a need to develop new synthetic hypolipidemic agents with fewer or no side effects.

α-Asarone (**1**) is a naturally occurring compound exhibiting antihyperlipidemic,[23] antifungal[24,25] and antithrombotic[26] activity (Fig. 1). The mechanism of its hypolipidemic effect has been established as the inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase,[27] which is involved in the biosynthesis of cholesterol.[28,29]

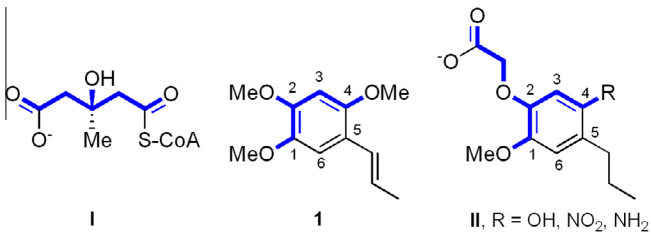
Previously, we reported the synthesis of a series of α-asarone (**1**) analogues with hypolipidemic activity.[30,31] Docking studies conducted to explore the binding mode of **1**[32] showed the potential pharmacophore groups, such as the polar methoxy groups at C-1, C-2 and C-4, and the hydrophobic side-chain, which may be responsible for the main interactions with the active site of the enzyme.[32] Moreover, it was observed that the analogues increased their affinity for the enzyme when the C-4 methoxy group was substituted with a hydroxy group.[33]

It is noteworthy that the size of the active site cavity allows the entry of a long polar group at C-2 of the benzene ring (the acetic group), which can then stabilize the interaction with the enzyme[32] (Fig. 1). In this same sense, previous studies on the synthesis of analogues of α-asarone (**1**) showed that the oxyacetic group at the C-2 position,[30] as similar to the structure found in fibrates, had a significant effect on lipid-lowering activity.[34,35] Therefore, in the current contribution we describe the synthesis of the series of compounds **4**–**7** and assess their in vivo hypolipidemic effect. An exploration of the possible action mechanism and an analysis of the docking results are provided with the aim of deepening the understanding of the molecular structure interactions between these substrates and the active site of the enzyme, which could improve the drug design and the effectiveness for this class of potential drugs.

2. Results and discussion

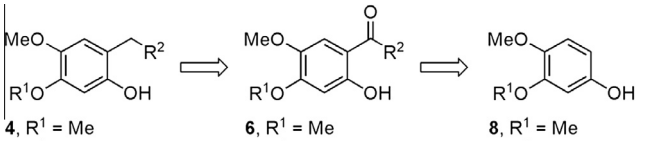
2.1. Chemistry

Upon considering the previous biological and docking studies, the design of compounds **4**–**7** was achieved on the basis of two criteria: (1) The series of compounds **4** and **6** retain the basic structure of α-asarone (**1**), but they substitute the C-4 methoxy group with a more polar hydroxy group (Scheme 1). (2) The propenyl chain is replaced by an analogous saturated hydrocarbon chain (series **4**)[36] or by a chain bearing a carbonyl group (series **6**).[37] For compounds **5** and **7**, the changes were similar to those for **4** and **6**, but a methyl acetate fragment was introduced (as a fibrate) in place of the methyl group at the C-2 position of the aromatic ring.



I 1 II, R = OH, NO_2, NH_2

Figure 1. Structures of HMG-like moiety present in the substrate (**I**) (in bold), fragment of α-asarone (**1**) (in bold), and fragment of the fibrate-like analogues (**II**) (in bold), which interact with the HMGR (the numbering of the structures was chosen for simplifying the comparison between the series of analogues, but it does not correspond to that established by the IUPAC nomenclature).



4, R^1 = Me
5, R^1 = CH_2CO_2Me
a, R^2 = Me
b, R^2 = Et
c, R^2 = *n*-Pr

6, R^1 = Me
7, R^1 = CH_2CO_2Me
a, R^2 = Me
b, R^2 = Et
c, R^2 = *n*-Pr

8, R^1 = Me
9, R^1 = CH_2CO_2Me

Scheme 1. Retrosynthesis of acyl phenols and derivatives.

The synthetic approach was envisioned starting from simple materials such as phenols (Scheme 1). Actually, compounds **4** and **6** are phenols *ortho* substituted with an alkyl or alkanoyl group, respectively, and may be found as raw materials in the perfume industry or as precursors in the synthesis of natural products. It has been observed that the aryl alkanoyl fragment is present in some biologically active molecules, though pharmacological studies on such molecules are scarce.[38,39]

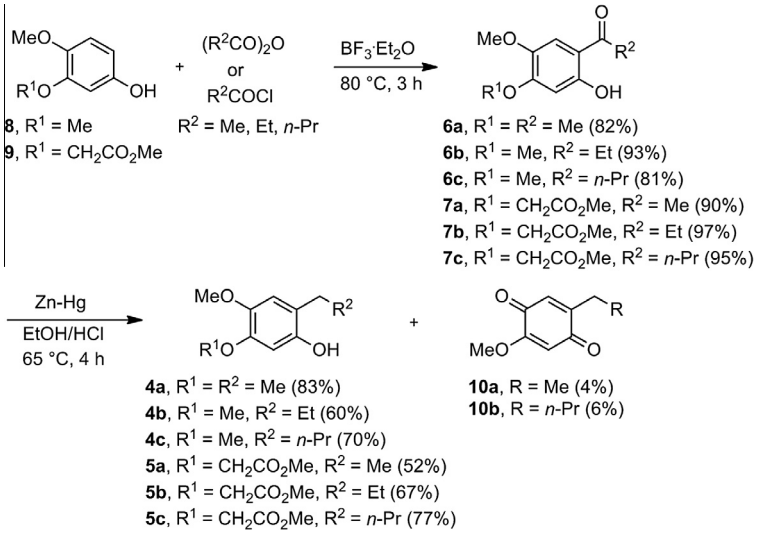
The series of compounds **6a**–**c** and **7a**–**c** were readily prepared by acylation of the corresponding phenols **8** and **9**, respectively, using the appropriate acyl chloride or acetic anhydride in the presence of boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$),[30,40] to give the corresponding 2-acyl phenols in high yields (81–97%) (Scheme 2). Reduction of compounds **6a**–-**c** and **7a**–**c** was carried out under Clemmensen conditions (Zn–Hg, HCl) to give the respective alkyl phenols **4a**–**c** and **5a**–**c** in modest to fairly good yields (52–77%) (Scheme 2). Unexpectedly, when the reaction was carried out with **6a** and **6c**, quinones **10a**–**b** were isolated as by-products in low yields. The structures of all compounds were established by ^{1}H NMR, ^{13}C NMR and mass spectrometry. The preparation of derivatives **4b** and **5b** has already been reported via a different approach.[30] Phenol **9** was not commercially available, and consequently its synthesis was carried out according to the previously developed procedure in three steps, starting from isovanilline.[30]

The ^{1}H NMR spectra of the acyl phenols **6a**–**c** show the presence of a single and fine signal of the hydroxyl proton in the 12.65–12.79 ppm range. The same protons in compounds **7a**–**c** appear at 12.57–12.68 ppm. Characteristic signals for the protons of the tetrasubstituted aromatic ring were observed as singlets. The ^{13}C NMR spectra of compounds **6** and **7** show a signal at around 204 ppm, which is characteristic of the ketonic carbonyl group. In the series of compounds **4** and **5**, the hydroxyl proton appears as a broad simple signal in the 4.53–4.97 ppm range. These signals are at low-frequency because of the lack of hydrogen bonding with the carbonyl group. Contrarily, such hydrogen bonding is present with derivatives **6** and **7**. The full assignment of proton and carbon signals was achieved by 2D NMR experiments (HMQC and HMBC).

The unambiguous structural determination of compound **7b** was carried out by X-ray diffraction analysis (Fig. 2). One can see that the acetyl and hydroxyl groups, as well as the methoxy group,[41] are coplanar with respect to the benzene ring. In this structure hydrogen bonding is formed and the methyl acetate side-chain adopts an orthogonal conformation. It is supposed that these groups are anchored to the polar amino acid residues of the enzyme active site, as suggested by the docking analysis (vide infra).

2.2. Hypolipidemic activity

Pharmacological screening for the hypolipidemic activity of these series of compounds was performed on male ICR mice with Tyloxapol-induced hyperlipidemia.[42,43] Tyloxapol (Triton WR 1339) is a non-ionic surfactant that is widely used to explore possible mechanism of lipid lowering drugs.[44–46] It causes a drastic



Scheme 2. Synthesis of acyl phenols **6a–c** and **7a–c**, reduced derivatives **4a–c** and **5a–c**, and quinones **10a–b**.

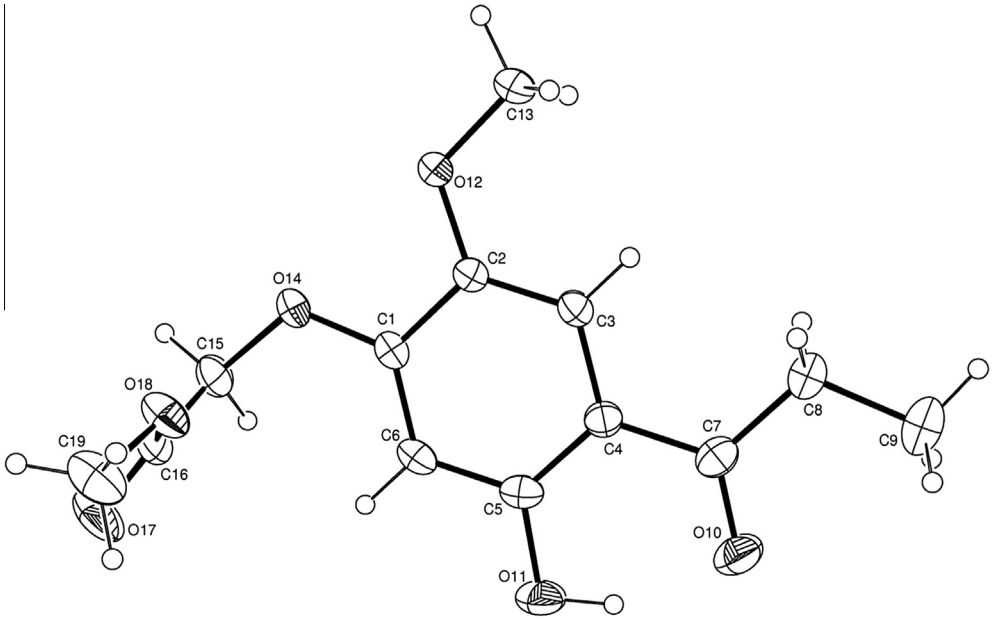


Figure 2. X-ray diffraction molecular structure of **7b** (ellipsoids at 30% probability level).

rise in serum triglyceride and cholesterol levels, mainly due to an increase in hepatic cholesterol synthesis. This in turn results from augmented HMG Co-A reductase activity[47,48] and the inhibition of the lipoprotein lipase enzyme.[49–53]

According to Korolenko et al.,[54] reducing the concentration of blood serum lipids requires the inhibition of endogenous cholesterol synthesis (elevated after treatment with Tyloxapol), a decrease in the absorption of lipoprotein lipids in the blood serum, and/or the stimulation of their excretion from the same body fluid. Several groups of compounds hold promise in this respect: fibrates (PPAR-α agonists), inhibitors of cholesterol absorption (Ezetimibe), fatty acid scavengers, and statins (HMG-CoA reductase inhibitors).

Compounds **4a–c**, **5a–c**, **6a–c** and **7a–c** (at doses of 25, 50 and 100 mg/kg) were intraperitoneally (ip) administered to hyperlipidemic mice, and then an evaluation was made of their effect on total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride serum levels (mg/dL). This procedure is similar to that used in previous studies with identical (**4b** and **5b**) and related compounds.[30]

With respect to the hyperlipidemic control group (without treatment), levels of cholesterol, LDL-cholesterol and triglycerides were sharply reduced (by 49–89%) after treatment with each of the test compounds, including simvastatin (**11**) (Table 1). This reduction was statistically significant for most of the determined values. However, there is no lineal dose–effect correlation. Although an increase in HDL-cholesterol was registered for each series of test compounds, the differences were not significant. It is noteworthy that these series display the highest activity for the α-asarone- and fibrate-based synthetic analogues previously prepared in our laboratories.[30,31,34–37]

With respect to the dose in mg/kg/day, acyl phenols **6** and **7** were the most active compounds from the series. Since the reduction in serum cholesterol levels was similar for the **6a–c** and **7a–c** series, the hypocholesterolemic activity cannot be associated with

Table 1
Effect of the test compounds on the change in the percentage of serum lipids in male ICR mice[a]

Compound	Dose mg (mmol)/kg/day	Cholesterol	LDL-Chol	HDL-Chol	Triglycerides
Normal diet		−85.18 ± 3.90*	−93.17 ± 11.8*	52.54 ± 4.90	−82.75 ± 7.48*
Simvastatin	17 (0.04)	−63.46 ± 3.15*	−84.31 ± 4.13*	292.20 ± 3.10*	−56.96 ± 1.72*
Tyloxapol	400	100 ± 24.49[b]	100 ± 27.05[c]	100 ± 20.34[d]	100 ± 24.40[e]
4a + Tyloxapol	25 (0.14)	−67.13 ± 2.70*	−72.47 ± 3.85*	33.90 ± 6.44	−76.79 ± 4.95*
	50 (0.28)	−73.41 ± 4.62*	−78.34 ± 4.62*	25.42 ± 1.68	−86.97 ± 1.19*
	100 (0.56)	−75.85 ± 1.74*	−83.93 ± 1.54*	71.19 ± 6.77	−81.65 ± 1.47*
4b + Tyloxapol	25 (0.13)	−71.84 ± 2.79*	−78.15 ± 3.85*	49.15 ± 8.94	−82.75 ± 1.38*
	50 (0.26)	−66.70 ± 3.75*	−71.61 ± 4.14*	25.42 ± 3.26	−73.85 ± 2.75*
	100 (0.52)	−69.22 ± 5.23*	−73.63 ± 5.97*	15.25 ± 3.81	−77.98 ± 3.58*
4c + Tyloxapol	25 (0.12)	−70.10 ± 2.96*	−74.78 ± 3.56*	23.73 ± 7.48	−82.57 ± 3.21*
	50 (0.24)	−67.04 ± 4.36*	−70.93 ± 5.10*	11.86 ± 2.88	−79.17 ± 2.94*
	100 (0.48)	−70.10 ± 2.18*	−74.69 ± 2.50*	23.73 ± 5.85	−83.94 ± 1.47*
5a + Tyloxapol	25 (0.10)	−79.08 ± 3.57*	−86.14 ± 4.43*	40.68 ± 6.37	−72.94 ± 11.0*
	50 (0.20)	−69.22 ± 7.06*	−75.26 ± 8.37*	28.81 ± 10.62	−59.63 ± 17.90
	100 (0.40)	−78.20 ± 2.62*	−82.58 ± 3.27*	−3.39 ± 0.59	−75.87 ± 3.39*
5b + Tyloxapol	25 (0.10)	−79.77 ± 2.79*	−89.32 ± 2.60*	91.53 ± 10.53	−84.13 ± 2.84*
	50 (0.20)	−80.21 ± 2.44*	−87.97 ± 2.41*	57.63 ± 10.53	−81.93 ± 2.01*
	100 (0.40)	−74.02 ± 9.33*	−81.23 ± 10.39*	50.85 ± 6.86	−71.38 ± 11.56*
5c + Tyloxapol	25 (0.09)	−70.62 ± 7.50*	−77.77 ± 9.34*	11.86 ± 2.88	−20.46 ± 11.7
	50 (0.18)	−59.46 ± 8.81*	−62.75 ± 9.82*	−3.39 ± 0.65	−49.82 ± 16.3
	100 (0.36)	−79.95 ± 1.83*	−89.51 ± 1.92*	89.83 ± 12.03	−82.75 ± 2.2*
6a + Tyloxapol	25 (0.13)	−71.84 ± 6.19*	−76.23 ± 7.8*	−4.75 ± 1.70	−59.63 ± 11.38*
	50 (0.26)	−80.09 ± 1.61*	−87.01 ± 2.12*	44.07 ± 16.10	−80.92 ± 2.29
	100 (0.52)	−80.12 ± 2.7*	−87.68 ± 1.92*	55.93 ± 28.81	−82.11 ± 2.29*
6b + Tyloxapol	25 (0.12)	−81.69 ± 2.79*	−86.72 ± 3.37*	6.78 ± 1.29	−80.09 ± 2.11*
	50 (0.24)	−81.95 ± 1.22*	−87.68 ± 1.64*	16.95 ± 3.93	−81.01 ± 2.11*
	100 (0.48)	−81.43 ± 1.74*	−88.35 ± 1.44*	40.68 ± 5.88	−80.92 ± 3.03*
6c + Tyloxapol	25 (0.12)	−74.28 ± 5.75*	−80.65 ± 7.41*	33.90 ± 9.87	−69.54 ± 8.26*
	50 (0.24)	−81.40 ± 1.05*	−87.97 ± 1.73*	30.51 ± 6.34	−77.25 ± 4.59*
	100 (0.48)	−83.26 ± 0.96*	−88.74 ± 1.35*	8.47 ± 0.98	−79.36 ± 3.30*
7a + Tyloxapol	25 (0.10)	−74.72 ± 2.62*	−81.62 ± 4.52*	8.47 ± 1.46	−28.62 ± 5.12
	50 (0.20)	−72.71 ± 4.18*	−76.90 ± 4.14*	1.69 ± 0.34	−74.77 ± 9.72*
	100 (0.40)	−75.33 ± 5.67*	−85.76 ± 6.26*	13.56 ± 3.85	−51.38 ± 27.1
7b + Tyloxapol	25 (0.10)	−68.18 ± 5.84*	−72.18 ± 6.83*	−3.39 ± 0.77	−59.82 ± 21.28*
	50 (0.20)	−66.87 ± 5.32*	−76.03 ± 8.28*	66.10 ± 11.47	−31.93 ± 39.81
	100 (0.40)	−74.46 ± 4.80*	−81.33 ± 4.43*	45.76 ± 10.64	−75.41 ± 3.48*
7c + Tyloxapol	25 (0.09)	−78.38 ± 4.10*	−84.70 ± 4.91*	30.51 ± 7.92	−73.12 ± 1.18*
	50 (0.18)	−49.26 ± 13.16*	−54.28 ± 15.59*	20.34 ± 9.74	−27.80 ± 2.47
	100 (0.36)	−73.23 ± 5.23*	−79.40 ± 6.83*	35.59 ± 8.90	−73.58 ± 7.79*

[a] Expressed as percentage of the group treated with Tyloxapol (mean ± standard error); $n = 6$.
[b] 114.7 mmol/L.
[c] 103.9 mmol/L.
[d] 5.9 mmol/L.
[e] 10.9 mmol/L.
* Significantly different from the Tyloxapol group, $p < 0.05$.

the length of the side-chain.[55] Although the hypolipidemic activity of all compounds in these series was very high, the best activity pattern was found for compound **6b**. The latter (at 25 mg/kg) significantly decreased total cholesterol (81%), LDL-cholesterol (86%) and triglycerides (80%).

The effect of the carbonyl group in **6b** can be assessed by comparing the activity of this compound with that of analogue **4b**. With both these compounds, triglyceride levels were reduced in almost equal magnitude. However, compound **4b** (with a saturated chain) had a lesser effect than **6b** at the same dosage (25 mg/kg), lowering total cholesterol by 71% and LDL-cholesterol by 78%. The opposite hypolipidemic effect was previously observed for structurally close homologues,[55] compounds with an alkenyl side-chain that were more active than the acyl analogues.

A dose–effect relationship cannot be established for the series as a whole. However, in some cases the highest dose (100 mg/kg) produced the highest effect (Table 1). Moreover, the significant decrease in LDL levels with almost all compounds is relevant, considering that this type of lipid is regarded as an important risk factor for the development of atheroma formation and coronary disease.[56]

In Table 1, doses have also been indicated in molar scale. It is interesting to note that the lowest dose of the series **5a**–**c** and **7a**–**c** proved to be the most potent in lowering the cholesterol and LDL levels. Therefore, the molar dose scale shows a different potency sequence of the four series with respect to the weight dose scale. Although the members of each series have relative analogous molar doses, their potency follows a similar pattern to that observed from the point of view of the weight dose scale.

2.3. Stability of compounds 4–7 under pH-variable enzymatic conditions

For the in vivo administration route, the enzymes of the gastro intestinal tract represent the first physiological barrier to the potential activity of the test compounds. Therefore, in order to determine if such compounds undergo structural changes in the presence of these enzymes, an in vitro digestion simulation was carried out using pepsin and trypsin/chymotrypsin.[57] Compounds **4a**, **4c**, **5a**, **5c**, **6a**, **6c**, **7a** and **7c** were selected for these tests.

The retention times and recovery percentages of compounds **4**, **5**, **6** and **7** are summarized in Table 2. The results suggest that the compounds are highly stable under acidic conditions (pH 2.0) or pepsin treatment, evidenced by the fact that these conditions did not cause the formation of by-products. The exceptions were **4a** and **4c**, in which quinones **10a** and **10b**, respectively, were

observed in trace amounts, but mostly the test compounds were recovered (93–98%). When treatment was carried out under basic conditions (pH 8.3) and with a mixture of trypsin and α-chymotrypsin, additional products were observed only for compounds **4a** and **4c**. Derivative **4a** displayed four peaks, at 6.94 (54.5%, assigned to **4a**), 6.35 (22.7%, assigned to **10a**), 2.15 (17.4%, not isolated and characterized) and 1.89 (5.1%, not isolated and characterized) min. In the case of **4c**, a large quantity (82.8%) was recovered and an additional low peak (11.4%, assigned to **10b**) was detected at 13.14 min.

Since both α-chymotrypsin and trypsin are alkaline endoproteases that hydrolyze peptide bonds at the C-terminus of aromatic amino acids (tryptophan, tyrosine and phenylalanine),[58] they should be expected to produce changes in the structure of compounds **5** and **7**, such as hydrolysis of the methyl phenoxyacetate group. However, the most affected compounds were those of series **4**. In the case of **4a**, we were able to establish only the structure of the major by-product resulting from digestion, which had a retention time of 6.35 min that corresponds to quinone **10a** (Scheme 2). The structure of the other two by-products was not established because of the impossibility of isolating them in a sufficiently pure quantity. Similarly, the retention time of the by-product of digestion of **4c** corresponds to quinone **10b**. This suggests that the oxidation process is promoted under both acidic (see reaction in Scheme 2) and basic (α-chymotrypsin and trypsin) conditions.

In spite of the partial decomposition of series **4** by the enzymes, the hypolipidemic results did not show any anomalous behavior of this series of compounds compared to the other series. This suggests that there is no significant hydrolysis or decomposition of series **4**–**7** during the in vivo protocol. However, we cannot rule out the formation of quinones **10a**–**b** and the contribution that they might have on the hypolipidemic effect, since analogous compounds have proved to exhibit potent antitumor[59] and botulinum neurotoxin serotype A inhibition[60] activity. Although a detailed study of these compounds is beyond the scope of the present work, an assessment of their biological activity is contemplated for the near future.

2.4. Evaluation of series 4–7 as human HMG-CoA reductase inhibitors

Similar to the action mechanism of statins and α-asarone (**1**), according to a previous study many series of α-asarone- and fibrate-based analogues act through the inhibition of the HMG-CoA reductase enzyme.[30] The protocol used for determining this inhibition was based on the enzymatic activity of HMGR shown by *Schizosaccharomyces pombe*, which is an analogue of human HMGR (HMGRh). In the case of the homologue series of **4**–**7**, the specific enzymatic activity was evaluated for HMGRh, which is commercially available. Once having established the protocol for determining the inhibition of HMGRh, the activity of derivatives **4b**, **5b**, **6b**, and **7c** was evaluated (Table 3). Simvastatin (**11**) was used as the positive control. There was no statistically significant difference between the test compounds and the positive controls, **1** and **11**.

Compared to **11**, similar activity was found for **5b** and **7c**, while a lower IC_{50} was observed for **c**ompounds **4b** and **6b**. The higher activity of compounds **4b** and **6b** with respect to **5b** and **7c** may be associated with the fact that the former two compounds have a C-2 methoxy group in the benzene ring, while the latter two have a methyl acetyloxy group. On the other hand, there was no notable effect caused by the polar acyl moiety or the saturated side-chain present in some derivatives. There is not a complete correlation between the inhibitory activity produced by the test compounds on HMGRh and the in vivo hypolipidemic activity. However, all test compounds evaluated proved to be inhibitors of HMGRh, which is probably also true for the other derivatives that were not evaluated.

Table 3
The 50% inhibitory concentration (IC_{50}) values of the activity of alpha-asarone- and fibrate-based 2-acyl and 2-alkyl phenols as human-HMG-CoA reductase inhibitors

Entry	Compound	Enzyme IC_{50} (μM)
1	Simvastatin (**11**)	6.11 ± 1.53
2	α-Asarone (**1**)	5.86 ± 1.97
3	**4b**	4.79 ± 0.68
4	**5b**	6.11 ± 1.43
5	**6b**	4.94 ± 0.47
6	**7c**	6.43 ± 1.21

Table 4
Glide docking results of α-asarone and fibrate analogues tested with human HMGR

Compound	pIC_{50}	Glide XP (kcal/mol)
4b	7.64	−1.49
5b	7.40	−4.52
6b	7.61	−2.24
7c	7.35	−5.68
Simvastatin (**11**)	7.40	−11.44
α-Asarone (**1**)	7.44	−2.84

Table 2
HPLC analysis of products from enzymatic digestion of compounds **4**–**7**[a]

Treated compound	Pepsin t_r (min)	Recovery treated compound and side products (%)	Trypsin/α-chymotrypsin t_r (min)	Recovery treated compound and side products (%)
4a	6.95	**4a** (94.6)	6.94	**4a** (54.5)
			6.35	**10a** (22.7)
			2.15	(17.4)[b]
			1.89	(5.1)[b]
4c	12.45	**4c** (93.0)	12.44	**4c** (82.8)
			13.14	**10b** (11.4)
5a	7.78	**5a** (96.4)	7.78	**5a** (99.0)
5c	13.81	**5c** (97.5)	13.80	**5c** (95.4)
6a	8.57	**6a** (95.3)	8.58	**6a** (97.0)
6c	13.84	**6c** (95.6)	13.83	**6c** (97.5)
7a	8.67	**7a** (94.0)	8.68	**7a** (97.6)
7c	13.16	**7c** (96.5)	13.18	**7c** (99.0)

[a] Column chromatography: Zorbax Eclipse Plus HT C18 column (4.6 mm × 100 mm, 3.5 μm); Elution: (a) Flow rate: 1.0 mL/min (40 °C), (b) solvent gradient: solvent B (acetonitrile) in solvent A (aqueous solution of formic acid, 0.2%) from 20% to 50% for 18 min, and an isocratic stage of 50% of solvent B for 2 min; Detection: UV (254 nm).
[b] The structure of the compound was not established.

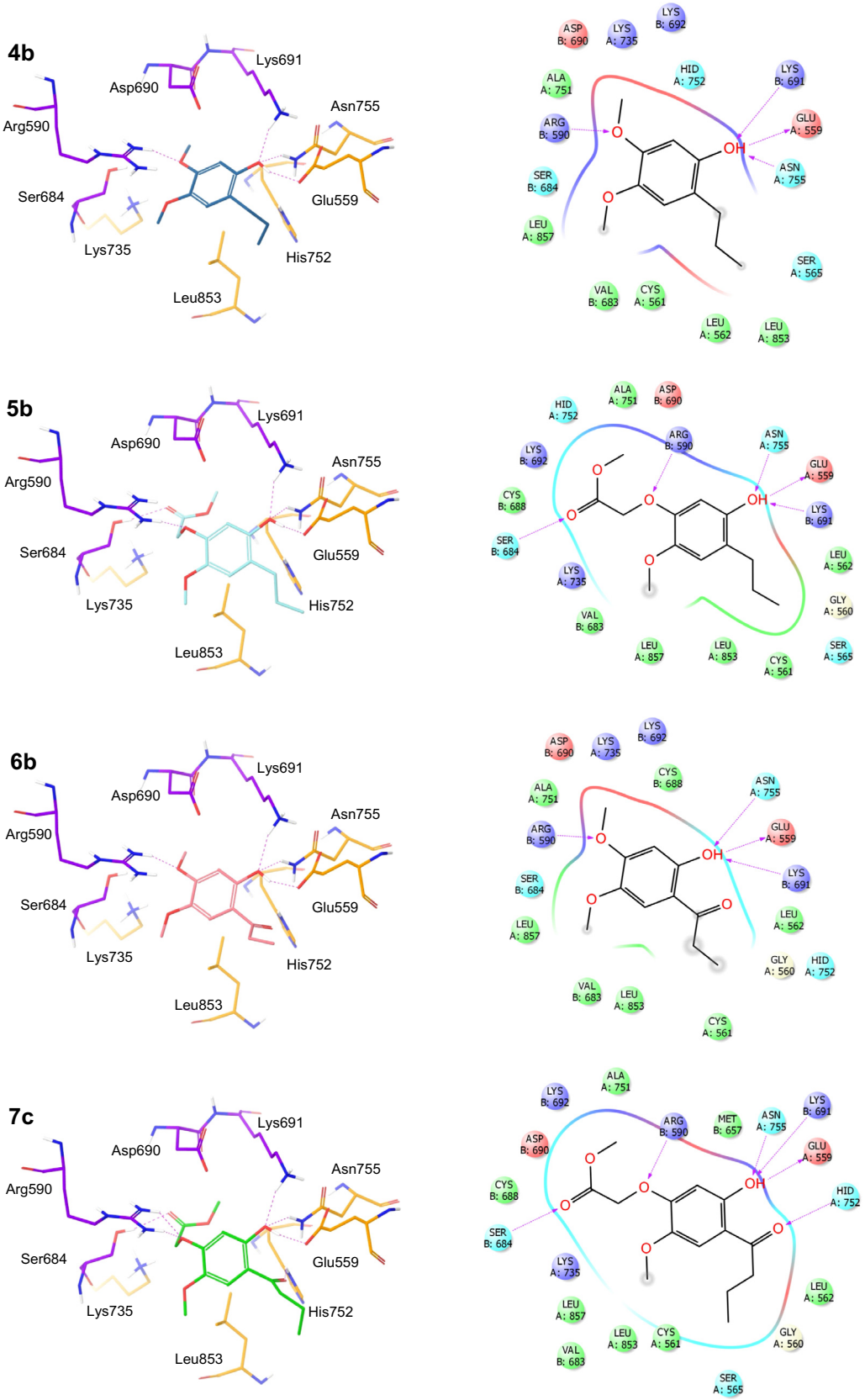


Figure 3. Predicted binding mode of the newly synthesized and tested compounds **4b**, **5b**, **6b** and **7c**, with the catalytic portion of HMGR using the program Glide XP. The 3D models show selected amino acid residues within 4.5 Å of the ligands. Carbon atoms of residues from one monomer are orange and those from the other monomer are purple. Non-polar hydrogens are omitted for clarity. The 2D interaction diagrams show all amino acid residues within 4.5 Å of the ligands and are represented as follows: hydrophobic in green, polar in cyan, negatively charge in red, positively charge in blue and glycine in white.

2.5. Docking of the analogs 4–7 with human HMGR

The α-asarone analogue compounds tested in the in vitro assay (**4b**, **5b**, **6b** and **7c**) were docked with the crystal structure of HMGRh in complex with simvastatin (PDB: 1HW9)[61] using the Glide Extra Precision (XP) program. Previous to this simulation, the protocol was validated by docking the co-crystal ligand. The docking protocol (see Section 4) was able to reproduce the experimental binding orientation of simvastatin (**11**) with a root mean square deviation (RMSD) of 0.60 (Fig. S30 in Supporting data). We previously obtained a similar low RSMD value for **11** with another docking program.[32] The Glide XP docking score for **11** was −11.44 kcal/mol.

Table 4 summarizes the docking results of the four analogues tested with the crystal of HMGRh. The docking results of simvastatin (**11**) and α-asarone (**1**) are shown as a reference. We did not observe a reasonable correlation between the Glide XP scores and the IC_{50} values. This can be attributed to the well-known issues of some scoring functions due, in part, to the substantial amount of approximations in automated docking.[62]

The binding mode of **4b**, **5b**, **6b** and **7c** as well as protein–ligand interaction diagrams are depicted in Figure 3. For the sake of clarity, and in accordance with a common practice in the visualization of protein–ligand interactions,[63] the three-dimensional representations of the binding models (Fig. 3) show only selected residues of the binding pocket. In contrast, the interaction diagrams (e.g., the two-dimensional representations of the binding models) display all the binding residues in the pocket. All compounds adopt a very similar binding orientation, a result that is further illustrated by the overlay of the four analogues (Fig. 4A).

For all four molecules, hydrogen bond interactions are predicted between the hydroxyl group (in the equivalent C-4 position of α-asarone (**1**)) and the side-chains of Glu559, Lys691 and Asn755. The same hydrogen bonds are observed for the O5-hydroxyl group of simvastatin (**11**) (Fig. S31 in Supporting data). Similar interactions are also reported in the crystallographic structures of compactin, fluvastatin, cerivastatin, atorvastatin and rosuvastatin.[61] These results support our previous hypothesis that replacing the C-4 methoxy group of **1** by a hydroxyl group would lead to the formation of a hydrogen bond network. Surprisingly, only two compounds, **4b** (IC_{50} = 4.79 μM) and **6b** (IC_{50} = 4.95 μM) showed improved enzymatic inhibitory activity compared to **1** (IC_{50} = 5.86 μM) under the assay conditions used in this study. In fact, **4b** and **6b** have a very similar structure as well as a comparable predicted binding mode and similar docking scores. The carbonyl group at the C-5 position in **6b** does not have a major effect on the binding orientation.

A second common interaction between all docked compounds and the catalytic portion of HMGRh is a hydrogen bond between the C-2 oxygen and the side-chain of Arg590. The same hydrogen bond interaction with this residue was previously predicted for the C-2 methoxy group of α-asarone (**1**). The O3-hydroxyl group of simvastatin (**11**) (Fig. S31) also forms a hydrogen bond with the side-chain of Arg590.

Compounds **5b** and 7**c** have very similar structures and also similar binding modes, making equivalent protein–ligand interaction contacts (with comparable docking energies). A carbonyl oxygen of **7c** forms an additional hydrogen bond contact with the side-chain of His752. However, this structural difference does not have a significant impact on the enzymatic activity, as indicated by the comparable IC_{50} between **5b** and **7c**. This contrasts with the in vivo hypolipidemic activity, which was found to be improved in **7c**. This can be ascribed to the lipophilic efficiency (log*P*) of the latter compound due to the polar-indice effect of the carbonyl group in the chain, which improves the absorption or excretion process of **7c** and thus its pharmacological profile.

As expected from our previous docking studies with α-asarone (**1**)[32] and phenoxyacetic analogues,[30] the acetylated phenoxyacetic group at the C-2 position of **5b** and **7c** occupy the same pocket cavity as the carboxylate group of simvastatin (**11**). Notably, the binding position of the carboxylate group of **11** is similar to that of the terminal carboxylate of the HMG moiety of other statins.[61] The carbonyl oxygen of the ester of **5b** and **7c** makes a hydrogen bond with the side-chain of Ser684, similar to the carbonyl oxygen of **11** (Fig. S31). Despite the stability shown by compounds **4**–**7** under the enzymatic conditions, the ester group in **5b** and **7c** is likely to be hydrolyzed in vivo. As extensively discussed above, the ester moiety that is exposed in the in vitro inhibition assay with HMGRh is probably different from the group that is exposed in the in vivo assay. Therefore, there is not necessarily a direct relationship between the in vitro and in vivo activity of **5b**, **7c** and other compounds.

Similar to the conclusions reported for the docking of α-asarone (**1**) and other analogues, the binding mode of **4b**, **5b**, **6b** and **7c** (Fig. 4B) has significant structure-binding contact similarities when compared to the binding mode of sinvastatin (**11**) and other

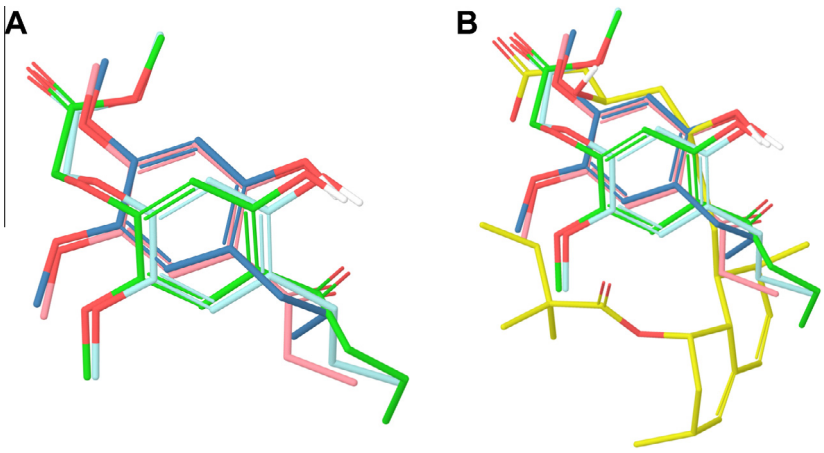


Figure 4. (A) Overlay of the docking poses of **4b** (carbon atoms in dark blue), **5b** (light blue), **6b** (pink) and **7c** (green). (B) Comparison of the binding modes with the co-crystal position of simvastatin (**11**) (carbon atoms in yellow).

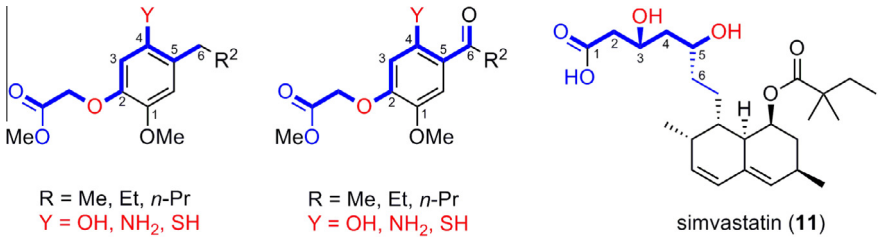


Figure 5. Structure-binding contact similarities of test compounds and simvastatin (**11**) (in blue bold) and the catalytic portion of HMGRh, and specific structural requirements for a future plausible design of more active compounds (in blue and red colors).

statins (Fig. 5). Hence, from Figure 4B and the discussion of Figure 3, it can be appreciated that:

(1) The C-4 hydroxyl group of the α-asarone analogues occupies a similar binding region as the O5-hydroxyl group of statins.
(2) The carbonyl oxygen of the group at the C-2 position of **5b** and **7c** has similar interactions as the terminal carboxylate group of the HMG moiety of the statins.
(3) The C-2 oxygen of the α-asarone analogues makes similar protein–ligand contacts as the O3-hydroxyl group of **11**.

The four compounds did not show great differences in potency (Table 3), which is in agreement with a relatively flat SAR with respect to the in vitro activity of **4b**, **5b**, **6b** and **7c**.

3. Conclusions

The four series of acyl and alkyl phenols herein synthesized showed high hypolipidemic activity. The lowest dose (25 mg/kg/day) of the acyl phenols caused a reduction of more than 80% in total cholesterol, LDL-cholesterol and triglycerides in serum. Actually, series **4**–**7** proved to be the most active α-asarone- and fibrate-based analogues ever reported from our laboratories. Four selected compounds, **4b**, **5b**, **6b** and **7c**, displayed in vitro inhibition of the human HMG-CoA reductase (responsible for cholesterol biosynthesis). Docking studies with this enzyme established multiple molecular modeling interactions between the active site and the different polar and non-polar functional groups of the derivatives. These results provide a good SAR image of the structural requirements of more active compounds to be used in future drug design. Like the highly active derivatives **5a**–**c** and **7a**–**c**, the new lead compounds should include polar groups (hydroxyl, amino and thiol groups) separated at a distance similar to that between the carboxylic group and both the C-3 and C-5 hydroxyl groups, as established in the active side-chain of **11** and other statins (Fig. 5). It is also necessary to include the hydrophobic moiety, which should not be larger than the decalin skeleton of statins, but instead a saturated side-chain. The benzene ring can be a good and versatile scaffold to obtain such a setting of substituents, as shown for the series herein tested.

Evidence from studies carried out with fibrates suggests that other mechanisms may be responsible for the reduction of triglycerides, such as the peroxisome proliferator-activated receptor (PPAR) pathway. PPARs are a subfamily of nuclear hormone receptors, which are ligand-activated transcription factors regulating the metabolism of dietary fats.[64] These receptors (particularly PPARα) reduce apoC-III expression and thus result in increased lipolysis.[65–70] Compounds **5** and **7**, structurally related to fibrates, probably reduce lipid levels through a similar mechanism of action.[71] Experimental and computational studies on this mechanism are currently underway and the results will be reported in due course.

4. Experimental

4.1. Synthesis

Melting points were determined on an Electrothermal apparatus and are uncorrected. Infrared spectra (IR) were recorded on a FT-IR 2000 Perkin–Elmer spectrometer. ^{1}H (300 or 500 MHz) and ^{13}C (75 or 125 MHz) NMR spectra were recorded on Varian Mercury-300 or Varian V NMR System instruments, with TMS as internal standard; chemical shifts (δ) are reported in ppm. Mass Spectra were recorded on a Polaris Q-Trace GC Ultra (Finnigan Co.). High-resolution mass spectra (HRMS), in electron impact mode, were obtained on Jeol JSM-GCMateII. Elemental analyses were performed on a CE-440 Exeter Analytical instrument. A Multi-Therm Benchmark, Model H5000-HC was used for enzymatic stability assays as a heating and cooling shaker. Commercial reagents were used as received from Aldrich and anhydrous solvents were obtained by a distillation process. Thin layer chromatography was performed on precoated silica gel plates (Merck $60F_{254}$). Silica gel (230–400 mesh) was used for column chromatography. The preparation of compounds **8** and **9** has been previously described.[30]

4.1.1. General procedure for the synthesis of 2-acyl phenols 6 and 7

BF_3Et_2O (1.0 mol equiv) was added to a solution of phenol **8** or **9** (1.0 mol equiv) and acetic anhydride or the corresponding acyl chloride (2.0 mol equiv) under nitrogen atmosphere at 0 °C. The mixture was stirred at 80 °C for 3 h. The residue was poured into ice water (20 mL), adjusted to neutral pH with an aqueous saturated solution of $NaHCO_3$, and extracted with EtOAc (3 × 20 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc, ca. 9:1).

4.1.1.1. 1-(2-Hydroxy-4,5-dimethoxyphenyl)ethanone (6a). Following the general procedure, a mixture of **8** (1.500 g, 9.74 mmol), acetic anhydride (1.987 g, 19.48 mmol) and BF_3Et_2O (1.383 g, 9.74 mmol) afforded **6a** (1.57 g, 82%) as a colorless solid. R_f 0.20 (hexane/EtOAc, 8:2); mp 107–108 °C [Lit.[72] 112 °C]. IR (KBr): 3449, 2947, 1637, 1510, 1265, 1204, 1062, 838, 808 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.56 (s, 3H, CH_3CO), 3.87 (s, 3H, CH_3O-C5), 3.92 (s, 3H, CH_3O-C4), 6.46 (s, 1H, H-3), 7.06 (s, 1H, H-6), 12.65 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 26.3 (CH_3CO), 56.1 (CH_3O-C4), 56.6 (CH_3O-C5), 100.5 (C-3), 111.3 (C-6), 111.5 (C-1), 141.9 (C-5), 156.8 (C-4), 160.1 (C-2), 202.0 (CH_3CO). MS (70 eV) m/z 196 (M^+, 59), 181 (100), 153 (13), 135 (20), 125 (32), 110 (18), 95 (12), 43 (18).

4.1.1.2. 1-(2-Hydroxy-4,5-dimethoxyphenyl)propan-1-one (6b). Following the general procedure, a mixture of **8** (1.500 g, 9.74 mmol), propanoyl chloride (1.802 g, 19.48 mmol) and BF_3Et_2O (1.383 g, 9.74 mmol) afforded **6b** (1.91 g, 93%) as a colorless solid.

R_f 0.34 (hexane/EtOAc, 8:2); mp 124–125 °C [Lit.[72] 125 °C]. IR (KBr): 3349, 2986, 1615, 1509, 1427, 1205, 1156, 1025, 816 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.24 (t, J = 7.5 Hz, 3H, CH_3CH_2CO), 2.95 (q, J = 7.5 Hz, 2H, CH_3CH_2CO), 3.86 (s, 3H, CH_3O-C5), 3.91 (s, 3H, CH_3O-C4), 6.46 (s, 1H, H-3), 7.10 (s, 1H, H-6), 12.76 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 8.3 (CH_3CH_2CO), 31.3 (CH_3CH_2CO), 56.1 (CH_3O-C4), 56.7 (CH_3O-C5), 100.6 (C-3), 110.9 (C-6), 111.1 (C-1), 141.8 (C-5), 156.5 (C-4), 160.0 (C-2), 204.7 (CH_3CO). MS (70 eV) m/z 210 (M^+, 23), 182 (12), 181 (100), 149 (4), 138 (9), 125 (72), 121 (9), 110 (28), 93 (7), 91 (8).

4.1.1.3. 1-(2-Hydroxy-4,5-dimethoxyphenyl)butan-1-one (6c). Following the general procedure, a mixture of **8** (1.500 g, 9.74 mmol), butanoyl chloride (2.075 g, 19.48 mmol) and BF_3Et_2O (1.383 g, 9.74 mmol) afforded **6c** (1.77 g, 81%) as a colorless solid. R_f 0.25 (hexane/EtOAc, 8:2); mp 76–77 °C [Lit.[72] 81 °C; 77–78 °C[73]]. IR (KBr): 3300, 2960, 2931, 1630, 1603, 1508, 1432, 1267, 1204, 1147, 1031, 833, 789 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.03 (t, J = 7.5 Hz, 3H, $CH_3CH_2CH_2CO$), 1.78 (sext, J = 7.5 Hz, 2H, $CH_3CH_2CH_2CO$), 2.88 (t, J = 7.5 Hz, 2H, $CH_3CH_2CH_2CO$), 3.87 (s, 3H, CH_3O-C5), 3.91 (s, 3H, CH_3O-C4), 6.45 (s, 1H, H-3), 7.10 (s, 1H, H-6), 12.79 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.8 ($CH_3CH_2CH_2CO$), 18.0 ($CH_3CH_2CH_2CO$), 39.9 ($CH_3CH_2CH_2CO$), 56.1 (CH_3O-C4), 56.7 (CH_3O-C5), 100.6 (C-3), 111.1 (C-6), 111.3 (C-1), 141.8 (C-5), 156.5 (C-4), 160.2 (C-2), 204.3 (CH_3CO). MS (70 eV) m/z 224 (M^+, 34), 193 (10), 181 (100), 125 (35), 110 (17), 95 (10).

4.1.1.4. Methyl 2-(4-acetyl-5-hydroxy-2-methoxyphenoxy)acetate (7a). Following the general procedure, a mixture of **9** (1.000 g, 4.72 mmol), Ac_2O (0.963 g, 9.44 mmol) and BF_3Et_2O (0.670 g, 4.72 mmol) afforded **7a** (1.08 g, 90%) as a white solid. R_f 0.30 (hexane/EtOAc, 6:4); mp 132–133 °C. IR (KBr): 2943, 1770, 1633, 1510, 1397, 1374, 1226, 1197, 1168, 1023, 832 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 2.58 (s, 3H, CH_3CO), 3.82 (s, 3H, CO_2CH_3), 3.89 (s, 3H, CH_3O-C2), 4.75 (s, 2H, OCH_2CO_2Me), 6.32 (s, 1H, H-6), 7.11 (s, 1H, H-3), 12.57 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 26.4 (CH_3CO), 52.5 (CO_2CH_3), 56.8 (CH_3O-C2), 65.3 (OCH_2CO_2Me), 101.4 (C-6), 112.5 (C-3), 112.6 (C-4), 141.8 (C-2), 154.8 (C-1), 159.4 (C-5), 168.0 (OCH_2CO_2Me), 202.3 (CH_3CO). MS (70 eV) m/z 254 (M^+, 89), 239 (100), 179 (49), 165 (13), 151 (24), 135 (19), 43 (16). HRMS (70 eV) Calculated for $C_{12}H_{14}O_6$: 254.0790; found: 254.0782. Anal. Calcd for $C_{12}H_{14}O_6$: C, 56.69; H, 5.55. Found: C, 56.70; H, 5.51.

4.1.1.5. Methyl 2-(5-hydroxy-2-methoxy-4-propionylphenoxy)acetate (7b). Following the general procedure, a mixture of **9** (1.00 g, 4.72 mmol), *n*-propanoyl chloride (0.873 g, 9.44 mmol) and BF_3Et_2O (0.670 g, 4.72 mmol) afforded **7b** (1.22 g, 97%) as a white solid. R_f 0.50 (hexane/EtOAc, 6:4); mp 119–120 °C. IR (KBr): 3101, 2978, 1754, 1639, 1511, 1453, 1390, 1257, 1223, 1168, 1081, 864, 803 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.24 (t, J = 7.5 Hz, 3H, CH_3CH_2CO), 2.95 (q, J = 7.5 Hz, 2H, CH_3CH_2CO), 3.81 (s, 3H, CO_2CH_3), 3.88 (s, 3H, CH_3O-C2), 4.74 (s, 2H, OCH_2CO_2Me), 6.33 (s, 1H, H-6), 7.16 (s, 1H, H-3), 12.62 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 8.3 (CH_3CH_2CO), 31.4 (CH_3CH_2CO), 52.4 (CO_2CH_3), 57.0 (CH_3O-C2), 65.4 (OCH_2CO_2Me), 101.7 (C-6), 112.1 (C-3), 112.2 (C-4), 141.9 (C-2), 154.5 (C-1), 159.5 (C-5), 168.1 (OCH_2CO_2Me), 205.0 (CH_3CH_2CO). MS (70 eV) m/z 268 (M^+, 39), 240 (14), 239 (100), 138 (19). HRMS (70 eV) Calculated for $C_{13}H_{16}O_6$: 268.0947; found: 268.0955. Anal. Calcd for $C_{13}H_{16}O_6$: C, 58.20; H, 6.01. Found: C, 58.20; H, 6.03.

4.1.1.6. Methyl 2-(4-butanoyl-5-hydroxy-2-methoxyphenoxy)acetate (7c). Following the general procedure, a mixture of **9** (1.00 g, 4.72 mmol), *n*-butanoyl chloride (1.005 g, 9.44 mmol) and BF_3Et_2O (0.670 g, 4.72 mmol) afforded **7c** (1.26 g, 95%) as a white solid. R_f 0.50 (hexane/EtOAc, 6:4); mp 122–123 °C. IR (KBr): 3100, 2967, 1772, 1631, 1510, 1384, 1250, 1219, 1193, 1165, 1025, 856, 830 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.02 (t, J = 7.5 Hz, 3H, $CH_3CH_2CH_2CO$), 1.78 (sext, J = 7.5 Hz, 2H, $CH_3CH_2CH_2CO$), 2.88 (t, J = 7.5 Hz, 2H, $CH_3CH_2CH_2CO$), 3.81 (s, 3H, CO_2CH_3), 3.89 (s, 3H, CH_3O-C2), 4.74 (s, 2H, OCH_2CO_2Me), 6.32 (s, 1H, H-6), 7.16 (s, 1H, H-3), 12.68 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.8 ($CH_3CH_2CH_2CO$), 17.9 ($CH_3CH_2CH_2CO$), 40.0 ($CH_3CH_2CH_2CO$), 52.4 (CO_2CH_3), 57.0 (CH_3O-C2), 65.4 (OCH_2CO_2Me), 101.7 (C-6), 112.3 (C-4), 112.4 (C-3), 141.9 (C-2), 154.5 (C-1), 159.6 (C-5), 168.1 (OCH_2CO_2Me), 204.5 (*n*-PrCO). MS (70 eV) m/z 282 (M^+, 37), 239 (100), 207 (10), 179 (10), 138 (20). HRMS (70 eV) Calculated for $C_{14}H_{18}O_6$: 282.1103; found: 282.1105. Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.57; H, 6.43. Found: C, 59.54; H, 6.39.

4.1.2. General procedure for the synthesis of 2-alkyl phenols 4 and 5

A mixture of Zn–Hg (10.0 mol equiv) and conc. aqueous solution of HCl (36%) (6.0 mL) was heated to 65 °C, and a solution of the corresponding acyl phenol **6a–c** or **7a–c** (1.0 mol equiv) in MeOH (2 mL) and HCl (36%; 1.0 mL) was added dropwise. The mixture was stirred for 4 h and extracted with EtOAc (5 × 10 mL). The organic layer was washed with a 5% aqueous solution of $NaHCO_3$ until neutral, dried (Na_2SO_4), and the solvent was removed under vacuum. The residue was purified by column chromatography over silica gel (20 g, hexane/EtOAc, ca. 9:1) to give the respective alkyl phenols **4a–c** or **5a–c**.

4.1.2.1. 2-Ethyl-4,5-dimethoxyphenol (4a).[74] 2-Ethyl-5-methoxycyclohexa-2,5-diene-1,4-dione (10a). Following the general procedure, a mixture of **6a** (1.200 g, 6.12 mmol), Zn–Hg (16.28 g, 61.2 mmol) and HCl (16 mL) afforded **4a** (0.92 g, 83%) as a pale yellow solid, and **10a** (0.04 g, 4%) as a yellow solid. Data of **4a**: R_f 0.63 (hexane/EtOAc, 7:3); mp 43–44 °C. IR (KBr): 3276, 2959, 1618, 1528, 1451, 1410, 1204, 1170, 1106, 990, 828 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.22 (t, J = 7.5 Hz, 3H, CH_3CH_2), 2.56 (q, J = 7.5 Hz, 2H, CH_3CH_2), 3.81 (s, 3H, CH_3O-C5), 3.83 (s, 3H, CH_3O-C4), 4.53 (br s, 1H, OH), 6.43 (s, 1H, H-6), 6.66 (s, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.4 (CH_3CH_2), 22.6 (CH_3CH_2), 56.0 (CH_3O-C5), 56.6 (CH_3O-C4), 100.9 (C-6), 113.1 (C-3), 120.6 (C-2), 143.1 (C-4), 147.0 (C-1), 147.8 (C-5). MS (70 eV) m/z 182 (M^+, 79), 167 (100), 139 (33), 111 (58), 93 (15), 77 (25). HRMS (70 eV) Calculated for $C_{10}H_{14}O_3$: 182.0943; found: 182.0949.

Data of **10a**: R_f 0.65 (hexane/EtOAc, 7:3); mp 155–156 °C [Lit.[75] 57–58 °C]. ^1H NMR (500 MHz, $CDCl_3$): δ 1.14 (t, J = 7.5 Hz, 3H, CH_3CH_2), 2.48 (qd, J = 7.5, 1.5 Hz, 2H, CH_3CH_2), 3.82 (s, 3H, CH_3O), 5.92 (s, 1H, H-6), 6.50 (t, J = 1.5 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.7 (CH_3CH_2), 22.0 (CH_3CH_2), 56.2 (CH_3O), 107.7 (C-6), 129.6 (C-3), 151.9 (C-2), 158.6 (C-5), 182.4 (C-4), 187.4 (C-1). HRMS (70 eV) Calculated for $C_9H_{10}O_3$: 166.0630; found: 166.0632.

4.1.2.2. 4,5-Dimethoxy-2-propylphenol (4b). Following the general procedure, a mixture of **6b** (0.620 g, 2.95 mmol), Zn–Hg (7.85 g, 29.5 mmol) and HCl (3.0 mL) afforded **4b** (0.34 g, 60%) as a white solid. R_f 0.50 (hexane/EtOAc, 7:3); mp 75–76 °C [Lit.[30] 75–76 °C].

4.1.2.3. 2-Butyl-4,5-dimethoxyphenol (4c). 2-Butyl-5-methoxycyclohexa-2,5-diene-1,4-dione (10b). Following the general procedure, a mixture of **6c** (1.000 g, 4.46 mmol), Zn–Hg (11.875 g, 44.64 mmol) and HCl (5.0 mL) afforded **4c** (0.66 g, 70%) as a pale yellow solid, and **10b** (0.05 g, 6%) as a yellow solid. Data of **4c**: R_f 0.45 (hexane/EtOAc, 7:3); mp 54–55 °C. IR (KBr): 3325, 2948, 1619, 1527, 1457, 1417, 1198, 1116, 1006, 854 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 0.94 (t, J = 7.5 Hz, 3H, $CH_3(CH_2)_3$), 1.38 (sext, J = 7.5 Hz, 2H, $CH_3CH_2(CH_2)_2$), 1.57 (qu, J = 7.5 Hz, 2H, $CH_3CH_2CH_2CH_2$), 2.53 (t, J = 7.5 Hz, 2H, $CH_3(CH_2)_2CH_2$), 3.80 (s, 3H, CH_3O-C5),

3.82 (s, 3H, CH_3O-C4), 4.61 (s, 1H, OH), 6.42 (s, 1H, H-6), 6.64 (s, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.0 ($CH_3(CH_2)_3$), 22.5 ($CH_3CH_2(CH_2)_2$), 29.3 ($CH_3(CH_2)_2CH_2$), 32.3 ($CH_3CH_2CH_2CH_2$), 55.9 (CH_3O-C5), 56.6 (CH_3O-C4), 100.9 (C-6), 113.8 (C-3), 119.3 (C-2), 142.9 (C-4), 147.2 (C-1), 147.8 (C-5). MS (70 eV) *m/z* 210 (M^+, 32), 167 (100), 139 (21), 79 (12). HRMS (70 eV) Calculated for $C_{12}H_{18}O_3$: 210.1256; found: 210.1256. Anal. Calcd for $C_{12}H_{18}O_3$: C, 68.54; H, 8.63. Found: C, 68.51; H, 8.58.

Data of **10b**: R_f 0.47 (hexane/EtOAc, 7:3); mp 128–129 °C [Lit. [76] 129–130 °C]. ^1H NMR (500 MHz, $CDCl_3$): δ 0.93 (t, J = 7.5 Hz, 3H, $CH_3(CH_2)_3$), 1.38 (sext, J = 7.5 Hz, 2H, $CH_3CH_2(CH_2)_2$), 1.45–1.52 (m, 2H, $CH_3CH_2CH_2CH_2$), 2.43 (td, J = 7.5, 1.5 Hz, 2H, $CH_3(CH_2)_2CH_2$), 3.82 (s, 3H, CH_3O), 5.92 (s, 1H, H-6), 6.50 (t, J = 1.5 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.8 ($CH_3(CH_2)_3$), 22.4 ($CH_3CH_2(CH_2)_2$), 28.6 ($CH_3(CH_2)_2CH_2$), 30.0 ($CH_3CH_2CH_2CH_2$), 56.2 (CH_3O), 107.8 (C-6), 130.4 (C-3), 150.7 (C-2), 158.5 (C-5), 182.4 (C-4), 187.5 (C-1). HRMS (70 eV) Calculated for $C_{11}H_{14}O_3$: 194.0943; found: 194.0939.

4.1.2.4. Methyl 2-(4-ethyl-5-hydroxy-2-methoxyphenoxy)acetate (5a). Following the general procedure, a mixture of **7a** (1.200 g, 4.72 mmol), Zn–Hg (12.56 g, 47.2 mmol) and HCl (12 mL) afforded **5a** (0.60 g, 52%) as a white solid. R_f 0.25 (hexane/EtOAc, 7:3); mp 55–56 °C. IR (KBr): 3585, 3252, 2962, 1748, 1616, 1522, 1420, 1203, 1120, 1019, 845 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.19 (t, J = 7.5 Hz, 3H, CH_3CH_2), 2.55 (q, J = 7.5 Hz, 2H, CH_3CH_2), 3.77 (s, 3H, CO_2CH_3), 3.82 (s, 3H, CH_3O-C2), 4.62 (s, 2H, OCH_2CO_2Me), 4.97 (br s, 1H, OH), 6.40 (s, 1H, H-6), 6.68 (s, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.2 (CH_3CH_2), 22.6 (CH_3CH_2), 52.2 (CO_2CH_3), 56.8 (CH_3O), 66.8 (OCH_2CO_2Me), 104.0 (C-6), 114.1 (C-3), 123.3 (C-4), 143.6 (C-2), 145.7 (C-1), 147.1 (C-5), 169.8 (CO_2Me). MS (70 eV) *m/z* 240 (M^+, 80), 225 (41), 167 (72), 165 (40), 151 (13), 139 (44), 121 (14), 111 (100), 107 (22), 93 (22), 79 (23), 77 (24). HRMS (70 eV). Calculated for $C_{12}H_{16}O_5$: 240.0998; found: 240.0998. Anal. Calcd for $C_{12}H_{16}O_5$: C, 59.99; H, 6.71. Found: C, 59.96; H, 6.68.

4.1.2.5. Methyl 2-(5-hydroxy-2-methoxy-4-propylphenoxy)acetate (5b). Following the general procedure, a mixture of **7b** (1.000 g, 3.73 mmol), Zn–Hg (9.93 g, 37.3 mmol) and HCl (5.0 mL) afforded **5b** (0.63 g, 67%) as a white solid. R_f 0.38 (hexane/EtOAc, 7:3); mp 57–58 °C [Lit.[30] 57–58 °C].

4.1.2.6. Methyl 2-(4-butyl-5-hydroxy-2-methoxyphenoxy)acetate (5c). Following the general procedure, a mixture of **7c** (1.000 g, 3.55 mmol), Zn–Hg (9.44 g, 35.5 mmol) and HCl (5.0 mL) afforded **5c** (0.73 g, 77%) as a white solid. R_f 0.41 (hexane/EtOAc, 7:3); mp 74–75 °C. IR (KBr): 3581, 3457, 3123, 2952, 1771, 1625, 1528, 1422, 1217, 1128, 1021, 856 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 0.93 (t, J = 7.5 Hz, 3H, $CH_3(CH_2)_3$), 1.37 (sext, J = 7.5 Hz, 2H, $CH_3CH_2(CH_2)_2$), 1.55 (qu, J = 7.8 Hz, 2H, $CH_3CH_2CH_2CH_2$), 2.52 (t, J = 7.5 Hz, 2H, $CH_3(CH_2)_2CH_2$), 3.78 (s, 3H, CO_2CH_3), 3.82 (s, 3H, CH_3O-C2), 4.63 (s, 2H, OCH_2CO_2Me), 4.79 (br s, 1H, OH), 6.40 (s, 1H, H-6), 6.66 (s, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.9 ($CH_3(CH_2)_3$), 22.5 ($CH_3CH_2(CH_2)_2$), 29.4 ($CH_3(CH_2)_2CH_2$), 32.2 ($CH_3CH_2CH_2CH_2$), 52.2 (CO_2CH_3), 56.8 (CH_3O-C2), 66.8 (OCH_2CO_2Me), 103.9 (C-6), 114.7 (C-3), 121.9 (C-4), 143.6 (C-2), 145.8 (C-1), 147.1 (C-5), 169.8 (CO_2Me). MS (70 eV) *m/z* 268 (M^+, 34), 225 (100), 195 (19), 167 (17), 137 (21), 111 (16). HRMS (70 eV) calculated for $C_{14}H_{20}O_5$: 268.1311; found: 268.1311. Anal. Calcd for $C_{14}H_{20}O_5$: C, 62.67; H, 7.51. Found: C, 62.67; H, 7.46.

4.1.3. Single-crystal X-ray crystallography

A single crystal of compound **7b**, obtained from recrystallization of EtOAc/CH_2Cl_2, 9:1, was mounted on a glass fiber. Crystallographic measurements were performed using Mo$K\alpha$ radiation (graphite crystal monochromator, λ = 71073 Å) at 19 °C. Two standard reflections, which were monitored periodically, showed no change during data collection. Unit cell parameters were obtained from least-squares refinement of 3005 reflections in the range of $2 < 2\Theta < 20°$. Intensities were corrected for Lorentz and polarization effects. Multi-scan absorption correction was applied. Anisotropic temperature factors were introduced for all non-hydrogen atoms. Hydrogen atoms were placed in idealized positions and their atomic coordinates refined. Unit weights were used in the refinement. Details of data collection and refinement for this crystal are listed in Tables S1–S5 (Supplementary data), which include bond distances and angles, atomic coordinates, and anisotropic thermal parameters. The structure was solved using the SHELXS97[77] program as implemented in the WinGX suite,[78] and refined using SHELXL97[79] within WinGX, on a personal computer. In all cases ORTEP and packing diagrams were made with ORTEP-3.[80] The structure was submitted to Cambridge Crystallographic Data Centre: **7b**, CCDC No. 977212.

4.2. Hypolipidemic activity

Hypolipidemic activity was studied in (ICR) male mice weighing 25–30 g (Birmex, S.A. de C.V., Mexico City). All animals were housed in hanging metal cages and maintained at 24 ± 2 °C and 50 ± 10% relative humidity, with 12 h light/dark cycle (lights on at 8:00 a.m.). They were fed on standard pellet diets (Rodent Diet 5001, PMI Nutrition International, Inc., Brenwood, MO) and drinking water was freely available. All animals appeared healthy throughout the dosing period, maintaining normal food intake and weight gain. At the time of sacrifice, no gross abnormalities were observed in any treated mice. All animals were treated in accordance with ethical principles and regulations specified by the Bioethics Committee of our Institution and the Standards of the National Institutes of Health of Mexico.

An aqueous solution of Tyloxapol was given ip to mice (400 mg/kg) and one hour later the test compounds (25, 50 and 100 mg/kg), dissolved in saline or saline-tween were ip administered. Simvastatin (**11**) (17 mg/kg) was used as a positive control group. After 24 h, blood was taken from the retro-orbital puncture and centrifuged at 13,000 rpm for the determination of serum levels of total cholesterol (TC), LDL-cholesterol (LDL-C) and triglyceride (TG), using a Wiener Lab Selectra 2 instrument. Serum LDL-cholesterol levels were calculated using the Friedewald equation.[81] Levels of serum lipids were determined in duplicate and values represent the mean from 6 mice (per compound). All data were statistically analyzed by Student–Newman–Keuls (SNK) test. *P* values less than 0.05 were considered as indicative of significance.

4.3. Enzymatic evaluation

4.3.1. In vitro digestion of compounds 4a, 4c, 5a, 5c, 6a, 6c, 7a and 7c by pepsin and trypsin/α-chymotrypsin

In an Eppendorf tube, compounds were dissolved at 10 mM (1 mL) in 10 mM HCl (pH 2.0) and pepsin was added at an enzyme/substrate (E/S) ratio of 1:200 (w/w). The solution was centrifuged at 37 °C. After 12 h of hydrolysis by pepsin, an aliquot of 500 μL was taken to assess the process. The peptic reaction was stopped in the aliquot by adding 500 μL of sodium phosphate 100 mM buffer (pH 8.3), and the mixture was extracted with CH_2Cl_2 (3 × 200 μL). The solvent was removed under vacuum and the residue kept at 5 °C until further analysis.

To the acidic pepsin solution containing the corresponding compound, one volume (500 μL) of the buffer (sodium phosphate, 100 mM) was added and then a mixture of trypsin and α-chymotrypsin at an E/S ratio of 1:50 (w/w) was added. The mixture was maintained at 37 °C for 24 h, then heated to 90 °C for 15 min to

inactivate the enzymes. The pH was adjusted to 7.0 with 1.0 M HCl and the mixture was extracted with CH_2Cl_2 (3 × 200 μL). The solvent was removed under vacuum and the residue kept at 5 °C for further analysis.

In an Eppendorf tube, a mixture of **4a** (18.2 mg) (or of **4c** (21.0 mg)) with 1 mL of HCl 10 mM (pH 2.0) was agitated at 300 rpm for 12 h at 37 °C. Afterwards, an aliquot (0.5 mL) was taken and a 100 mM aqueous solution of NaOH was added until neutral, and then extracted with CH_2Cl_2 (3 × 200 μL). The solvent was removed under vacuum and the residue kept at 5 °C until further analysis.

To the remaining centrifuged mixture, a 1.0 M aqueous solution of NaOH was added to adjust pH to 8.3, and the solution was maintained in agitation at 300 rpm for 24 h at 37 °C. The pH was adjusted to 7.0 with 1.0 M HCl and the mixture was extracted with CH_2Cl_2 (3 × 200 μL). The solvent was removed under vacuum and the residue kept at 5 °C until further analysis.

Reaction products were analyzed by HPLC under gradient conditions using a Zorbax Eclipse Plus HT C18 column (4.6 mm × 100 mm, 3.5 μm). Samples were dissolved in methanol and filtered through a 0.22 μm syringe filter, and then 10 μL was injected into the column. Elution was carried out at 40 °C. The flow rate was 1.0 mL/min with a linear gradient of solvent B (acetonitrile) in solvent A (aqueous solution of formic acid, 0.2%) from 20% to 50% for 18 min, and an isocratic stage with 50% of solvent B for 2 min. Absorbance peaks were monitored at 254 nm by a DAD detector. Calibration curves of pure compounds **4a**, **4c**, **5a**, **5c**, **6a**, **6c**, **7a** and **7c** were obtained under the same chromatographic conditions at concentrations of 1.0, 2.5, 5.0, 7.5, and 10.0 mM.

4.3.2. Assay of human HMG-CoA reductase activity

NADPH, human HMG-CoA reductase (HMGRh), simvastatin (**11**), α-asarone (**1**), and dimethyl sulfoxide (DMSO) were purchased from Sigma (Sigma, Saint Louis, MO, USA). The human HMG-CoA reductase (HMGRh) activity and inhibition assays were performed using the HMGRh Assay Kit from Sigma–Aldrich (Sigma CS-1090, Saint Louis, MO, USA), according to the manufacturer's instructions, using HMGRh, NADPH and Tris–HCl, pH 7.5. The oxidation of NADPH was spectrophotometrically monitored at 340 nm in a BioSpectometer-Kinetic from Eppendorff. HMG-CoA reductase activity was assayed at least six times. The reaction mixture contained: 0.13 mM HMG-CoA, 1 μL of HMGRh with or without inhibitor, and 50 mM Tris–HCl, pH 7.5, to a final volume of 100 μL. After 15 min incubation at 37 °C, the reaction was started with the addition of 0.13 mM NADPH, and monitored for 10 min. For all six reactions, 1 unit of enzyme activity is defined as the amount of enzyme required to catalyze the oxidation of 1 mmol of NADPH per min (1 μU catalyzes 1 μM of NADPH, in 1 min), modified from Bischoff and Rodwell[82] and Argüelles.[30]

4.3.3. Effect of synthetic compounds on HMGRh activity

The HMGRh (HMG-CoA Reductase Assay Kit from Sigma–Aldrich) enzyme was pre-incubated with the respective compounds for 30 min at 37 °C, followed by the standard enzyme assay. The synthetic compounds, **4b**, **5b**, **6b** and **7c**, and positive controls of inhibition, α-asarone (**1**) and simvastatin (**11**), were prepared in DMSO at the final concentrations of 2.5, 5.0, 10.0 and 15.0 μM. DMSO alone was tested with the enzyme to exclude the possibility that this solvent may have an inhibitory effect. After measuring HMGR inhibition, the mean IC_{50} values were compared to each other and evaluated by the Tukey post hoc test with the two-way ANOVA. Significant differences were determined with a value of p <0.05. Statistical analysis was carried out using the SigmaStat software.

4.4. Docking

The crystallographic structure of human HMGR in complex with simvastatin (**11**) was retrieved from the Protein Data Bank (PDB), with the code 1HW9.[61] The protein was prepared using the Protein Preparation Wizard implemented in Maestro 9.3,[83] which optimizes H-bond networks and flip orientations/tautomeric states of Gln, Asn and His residues. Geometry optimization was performed to a maximum root mean square deviation (RMSD) of 0.3 Å with the OPLS2005 force field. We recently employed a similar procedure to prepare the structure of other proteins.[84] During the protein preparation all water and adenosine-5′-diphosphate molecules were removed from the original PBD file. The binding site was established by grids with a default rectangular box centered on the co-crystal ligand simvastatin. XP descriptors were generated to obtain atom-level energy terms for the docking run, such as hydrogen bond interaction, electrostatic interaction, hydrophobic enclosure and π–π stacking interaction. The structure of the ligands was generated with Molecular Operating Environment (MOE), version 2011.1,[85] by starting from the coordinates of the crystallographic structure of compound **7b**. Docking was performed with the program Glide Extra Precision (XP).[86,87] Up to ten binding poses were generated per molecule and the top ranked binding pose was selected for analysis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.09.022.

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