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# Design, synthesis, and docking of highly hypolipidemic agents: *Schizosaccharomyces pombe* as a new model for evaluating α-asarone-based HMG-CoA reductase inhibitors

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ABSTRACT

A series of α-asarone-based analogues was designed by conducting docking experiments with published crystal structures of human HMG-CoA reductase. Indeed, synthesis and evaluation of this series showed a highly hypocholesterolemic in vivo activity in a murine model, as predicted by previous docking studies. In agreement with this model, the polar groups attached to the benzene ring could play a key role in the enzyme binding and probably also in its biological activity, mimicking the HMG-moiety of the natural substrate. The hypolipidemic action mechanism of these compounds was investigated by developing a simple, efficient, and novel model for determining HMG-CoA reductase inhibition. The partial purification of the enzyme from *Schizosaccharomyces pombe* allowed for testing of α-asarone- and fibrate-based analogues, resulting in positive and significant inhibitory activity.



## 1. Introduction

It is generally recognized that hypercholesterolemia and high levels of serum LDL-cholesterol contribute significantly to the progression of atherosclerosis,[1,2] which is the leading cause of cardiovascular diseases.[3,4] Liver enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) catalyzes the formation of mevalonate, the key step in the biosynthesis of cholesterol and isoprenoids.[5] Therefore, inhibition of this enzyme has proven to be the most efficient therapy for hyperlipidemia.[6]

Statins, which possess an HMG-like moiety linked to a hydrophobic decalin core, are the most effective hypocholesterolemic drugs for clinical use today.[7] Synthetic statin-like compounds that include an HMG-like moiety have shown significant hypocholesterolemic activity.[8] There are other agents without a structural HMG-like moiety that are known to inhibit HMGR, such as cholestin,[9] diosgenin,[10] ketanserin tartrate,[11] lanosterol analogues,[12] β-sitosterols,[13] and tunicamycin,[14] among many others.[15] This is also the case of α-asarone (**1**) (Fig. 1), which is the active metabolite of the Yucatan peninsula tree called Elemuy (*Mosannona depressa* (Baill.) Chatrou).[16,17] It has exhibited a potent in vivo hypolipidemic activity,[18] and inhibits hepatic HMGR.[19] By using an automated docking approach, we have reported a binding model of **1** with HMGR, concluding that the three methoxy groups of the substituted benzene bind to the enzyme active site like an HMG-moiety.[20]

In order to determine the pharmacophoric groups that give rise to the activity of **1**, and also to improve the latter and its pharmacological profile, numerous synthetic analogues have been prepared,[21] revealing the importance of the polar oxygen atoms and the hydrocarbon side-chain. Among the most active analogues are two series of compounds, one carrying a polar substituent in the C-4 carbon of the benzene ring, such as halogens, an amino or a nitro group, **2a–d** (Fig. 1),[21a] and the other in which the position of the double bond of the side-chain is changed.[21b] The series of hydroxyl analogues **3a–c**, which are the synthetic precursors of compounds **1** and **2**, exhibited significant hypocholesterolemic activity.[21a] Those compounds in which the characteristic three

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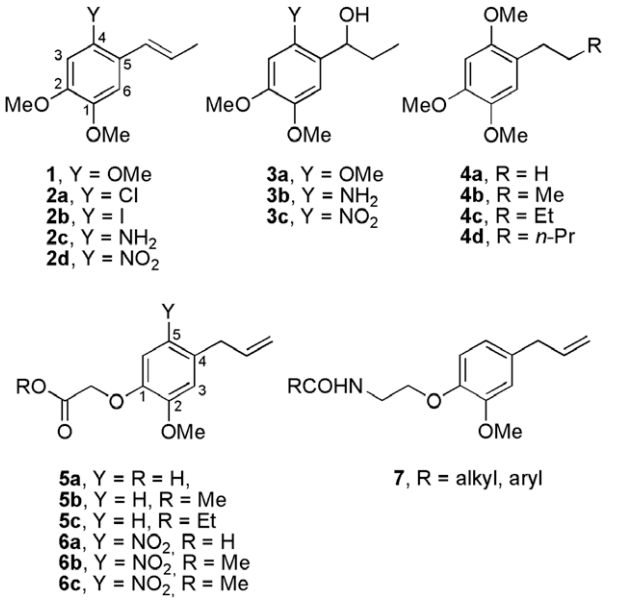



**Figure 1.** α-Asarone (**1**) and fibrate-based analogues that exhibit hypolipidemic activity.

methoxy groups of **1** were maintained, but the propenyl side-chain was saturated and homologated, **4a**–**d**, or just homologated, also showed a potent hypocholesterolemic activity (Fig. 1).[21c,d]

By maintaining a fairly close structural analogy with the α-asarone (**1**) framework, fibrate-related mimetic esters and amides **5**–**7** have also been designed and synthesized, and they display a high hypolipidemic activity (Fig. 1).[22] This activity is likely due to the fact that a phenoxyacetic acid moiety was incorporated to the frame, as this is a group that can potentially bind with the enzyme (vide infra).

In summary, the in vivo hypolipidemic evaluation and preliminary docking analysis of these new potent hypolipidemic agents provided valuable information about the possible pharmacophores: (1) the methoxy groups at the C-1 and C-2 carbons of the benzene ring, the polar functional group in C-4, and the C-5 hydrocarbon side-chain for the α-asarone (**1**) analogues; (2) the phenoxy acetic acid moiety, the polar functional group at C-5, and the C-4 hydrocarbon side-chain for the fibrate analogues.

The structure of the catalytic portion of human HMG-CoA reductase (HMGRh) consists of a proteinic tetramer, containing four actives sites formed by residues of two monomers.[23] This active site is characterized by the so-called *cis-loop* (residues 682–694),[24] which is in part involved in the reduction of the substrate HMG-CoA.[25]

Although fungi do not synthesize cholesterol, the close structural similarity between cholesterol and the fungal sterol ergosterol causes fungi to be a useful model for understanding sterol biosynthesis and regulation.[26] Many antifungal therapies have been proposed that target the ergosterol pathway yeasts.[27]

The fission yeast *Schizosaccharomyces pombe* is a unicellular eukaryote that has been found to contain a single HMGR gene. Even though the aminoacid sequences of this yeast and the human HMGR (HMGRh) proteins are divergent in the membrane domains, they are extensively conserved in the catalytic domains.[28] Completion of the *S. pombe* genome project revealed that fission yeasts, unlike budding yeast *Saccharomyces cerevisiae*, contain many sterol biosynthetic pathway genes homologous to the mammalian HMGR biosynthetic genes.[29] Hence, *S. pombe* has been proposed as a model to study aspects of regulation of sterol biosynthesis that have been difficult to address in other organisms (e.g., humans and rats), and could serve as a test organism to identify novel therapies that model cholesterol synthesis.[28]

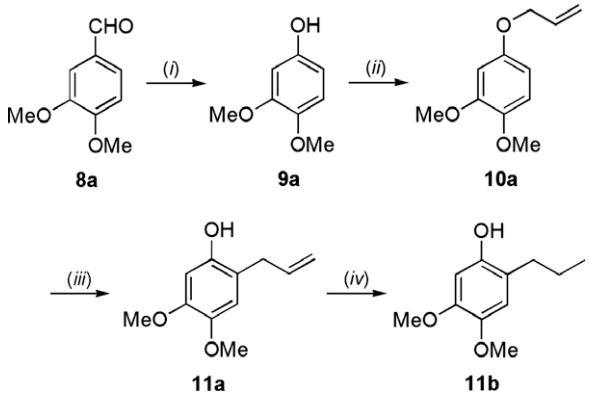
Although it has been established that the hypolipidemic action mechanism of α-asarone (**1**) has an inhibitory effect on hepatic HMGR in a rat model,[19] this methodology has disadvantages such as the dependence on the hormonal diurnal cycle of rats and the variation of results with each animal (vide infra). Hence, in order to determine the action mechanism of our synthetic α-asarone-like compound series, which could be similar to that of statins and α-asarone (**1**), it was useful to develop an easier and a more accurate method. Owing to the fact that the active site domain of the HMGR of *Schizosaccharomyces pombe* is quite similar to that of HMGRh, we developed a new approach by using the fungal HMGR (HMGRf) as a straightforward in vitro test for measuring inhibitory activity of the test compounds.

## 2. Results and discussion

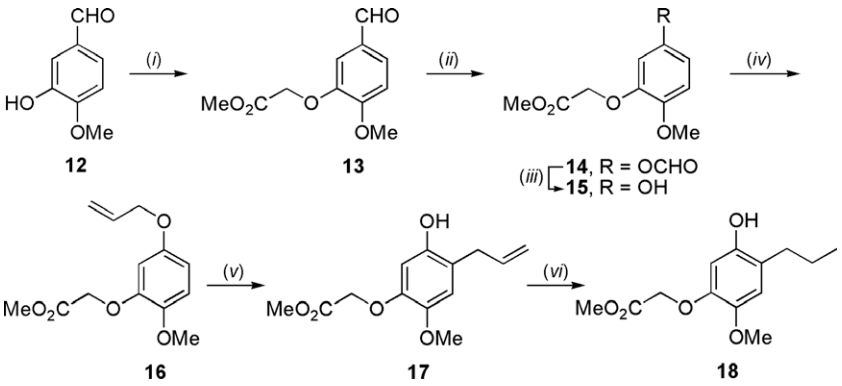
### 2.1. Design and synthesis of α-asarone (1) and fibrate analogues 11a–b, 17, 18, and 20a–d

With the preliminary docking results for α-asarone (**1**),[20] new structural features and functionalization requirements were proposed for the basic α-asarone scaffold that could increase affinity with HMGRh and consequently improve the hypolipidemic activity (vide infra).[20] Among these requirements, the modification of the C-4 methoxy group in the benzene ring by a more polar substituent, such as a hydroxy or an amino group, was expected to increase the activity.[20] These interesting results prompted us to prepare the α-asarone analogues **11a**–**b**, in which a hydroxy group was incorporated in C-4 of the benzene ring, and the double bond of the propenyl side-chain was deconjugated (**11a**) or saturated (**11b**) (Scheme 1). Thus, the Baeyer–Villiger reaction of the vanillin-derived **8a**, by treatment with *m*-chloroperbenzoic acid (MCPBA) followed by basic hydrolysis, yielded phenol **9a**. The latter was in turn protected by the acid-promoted etherification with allyl bromide to give the corresponding allyl ether **10**, which was submitted to 220 °C to lead to the Claisen rearrangement compound **11a**. Upon hydrogenation catalyzed by Pd/C of **11a**, the desired compound **11b** was afforded in good yield.

Assuming a similar effect on the activity of the framework of fibrate-based analogues **5** and **6**, we designed the preparation of the phenoxyacetic derivative **18**, through an analogous synthetic approach (Scheme 2) as that depicted in Scheme 1. Starting from functionalization of isovanillin (**12**) with methyl bromoacetate under basic conditions, aldehyde **13** was obtained in high yield (91%).




**Scheme 1.** Reagents and conditions: (i) (a) MCPBA, $CH_2Cl_2$, 20 °C, 6 h, 85%; (b) MeOH, HCl (6 N), 20 °C, 1 h, 89%; (ii) $CH_2$=$CHCH_2Br$, $K_2CO_3$, acetone, 60 °C, 6 h, 71%; (iii) decaline, 220 °C, 12 h, 86%; (iv) $H_2$, Pd/C, EtOH, 20 °C, 12 h, 81%.




**Scheme 2.** Reagents and conditions: (i) $BrCH_2CO_2Me$, $K_2CO_3$, acetone, 60 °C, 6 h, 91%; (ii) MCPBA, $CH_2Cl_2$, 20 °C, 3 h, 65%; (iii) MeOH, HCl (6 N), 20 °C, 1 h, 89%; (iv) $CH_2{=}CHCH_2Br$, $K_2CO_3$, acetone, 60 °C, 3 h, 87%; (v) Decaline, 220 °C, 12 h, 83%; (vi) $H_2$, Pd/C, EtOH, 20 °C, 12 h, 60%.

Oxidation of the latter with MCPBA, to give **14**, was followed by acid hydrolysis affording phenol **15** in 58% yield (for the two steps). The propenyl side-chain of **17** was introduced by Claisen rearrangement of the allyl ether precursor **16**, which was in turn prepared by base-promoted allylation of phenol **15** (Scheme 2), to give **17** in 72% yield (for the two steps). The latter was hydrogenated under mild conditions to afford the saturated side-chain analogue **18** in modest yield.

With the aim of introducing a nitro or an amino group into the C-5 benzene ring of the phenoxyacetic skeleton, we carried out the functionalization of the eugenol analogue with a saturated side-chain **19** (Scheme 3). Treatment of the latter with sodium chloroacetate in the presence of NaOH yielded **20a**, which was nitrated by using a mixture of sulfuric and nitric acid to afford **20b** in 76% yield. Acid-catalyzed esterification of **20b** with methyl alcohol provided **20c** in high yield (92%), which was hydrogenated in good yield (80%), leading to the amino derivative **20d**.

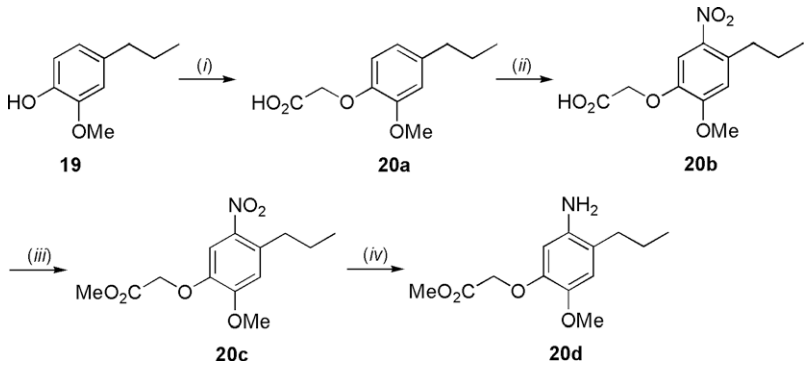
## 2.2. Docking of the analogues 11a–b, 17, 18, and 20a–d with human HMGR

The newly designed compounds described above were docked with a crystal structure of HMGRh. We employed the same validated docking protocol we described previously to conduct the docking studies of α-asarone.[20] Details of the docking methodology are presented in the Section 4, and the results are summarized in Table 1. Compound **1** is included in Table 1 as a reference. A three-dimensional representation of the optimized docked model of a representative compound, **20b**, in the active site of HMGRh is depicted in Figure 2. The corresponding two-dimensional representation of the docked model (Fig. 3) shows amino acid residues within 4.5 Å of **20b**. Docking energies for all compounds in Table 1 were better than the docking energy calculated for α-asarone (**1**). In particular, the docking energies calculated for **20a** and **20b** were the most favorable, due to the interactions of the carboxylate group with the polar side-chains of Ser684, Lys692 and Lys735 (Figs. 2 and 3). Similar interactions are observed for the C-1 carboxylate group of both the HMGR substrate and the statins.[23,24] To note, the carboxylate group of **20b** in this binding model forms hydrogen bonds with the side-chains of Ser684 and Lys692 of one monomer, and with the side-chain of Lys735 of the second monomer. An additional hydrogen bond is predicted to be formed between the C-1 oxygen of **20b** and the side-chain of Arg590 (Fig. 2). According to the docking model, the carboxylic group of **20a** and **20b** seem to be pharmacophoric. However, as it will be discussed below, there are other factors that influence the activity of these molecules in vivo.

**Table 1**
Docking results of compounds **11a–b**, **17**, **18**, and **20a–d** with HMGRh

| Compound | Docked energy (kcal/mol) | ΔG (kcal/mol) |
|---|---|---|
| **1** | −6.25 | −5.66 |
| **11a** | −6.82 | −6.11 |
| **11b** | −6.91 | −6.36 |
| **17** | −8.93 | −6.98 |
| **18** | −9.39 | −7.40 |
| **20a** | −10.23 | −8.54 |
| **20b** | −10.94 | −8.77 |
| **20c** | −8.92 | −6.58 |
| **20d** | −9.09 | −7.20 |

Docking results of **1** are included as reference.




**Scheme 3.** Reagents and conditions: (i) $ClCH_2CO_2Na$, NaOH, $H_2O$, 60 °C, 24 h, 69%; (ii) $H_2SO_4$, $HNO_3$, 20 °C, 24 h, 76%; (iii) MeOH, *p*-TsOH, 60 °C, 2 h, 92%; (iv) $H_2$ (30 psi), Pd/C, EtOAc, 20 °C, 24 h, 80%.

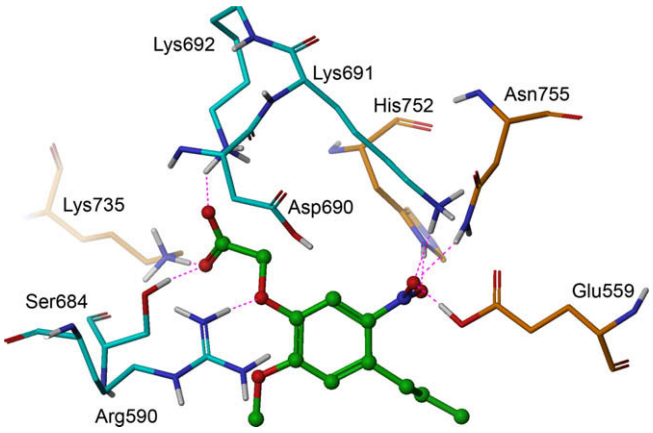



**Figure 2.** Optimized docking model for compound **20b** with the catalytic portion of human HMGR. Hydrogen bonds are indicated with magenta dashes. Representative amino acid residues within 4.5 Å of **20b** are shown. Carbon atoms of residues from one monomer are orange and those from the other monomer are cyan. Non-polar hydrogens are omitted for clarity.

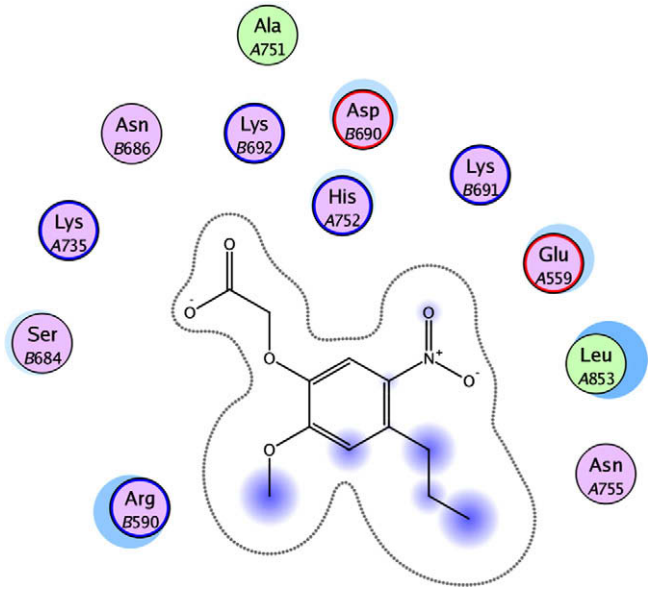



**Figure 3.** Two-dimensional representation of the optimized docking model of **20b** with the catalytic portion of human HMGR. Amino acid residues within 4.5 Å of **20b** are shown. The ligand proximity contour is depicted with a dotted line. The ligand solvent exposure is represented with blue circles; larger and darker circles on ligand atoms indicate more solvent exposure. The receptor solvent exposure differences in the presence and absence of the ligand are represented by the size and intensity of the turquoise disks surrounding the residues; larger and larger disks indicate residues highly exposed to solvent in the active site when the ligand is absent. Figure created with the Ligand Interactions application of MOE.[46]

In agreement with previous docking studies, the docked analogues interact with two monomers of the HMGRh tetramer and the polar groups OH, $NH_2$, and $NO_2$ are predicted to play an important role in binding.[20] As illustrated in Figure 2, the $NO_2$ group of **20b** is very close to the polar side-chains of Glu559, Lys691, His752, and Asn755, making an extensive hydrogen bond network (Fig. 2). As previously reported, this is the same binding region of the C-5 carbonyl oxygen of the HMGR substrate.[20] Notably, Glu559 of one monomer and Lys691 of the second monomer seem to participate directly in the catalysis of the reduction of HMG-CoA.[25] According to this mechanism, Glu559 could be protonated under physiological conditions.[25] Similar interactions with the side-chains of Glu559 and Lys691 were predicted previously for the C-4 oxygen of the methoxy group of α-asarone (**1**).[20] Although there are several factors that affect the biological activity of a compound in vivo beyond the predicted docking interactions and energies with a particular target (vide infra), the above docking results with HMGRh supported the structure-based design of the new analogues.

### 2.3. In vivo hypolipidemic activity of the analogues 11a–b, 17, 18, and 20a–d

A hypolipidemic screening of the prepared derivative series **11a–b**, **18**, and **20a–d** was carried out, and the data on the lipid-lowering activity of the four parameters evaluated are summarized in Tables 2 and 3.

Treatment of ICR mice with Triton WR 1339 (Tyloxapol) resulted in a significant elevation in total serum cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides (Table 2). A significant difference was observed for the whole series of tested compounds, causing a large decrease in total cholesterol. Although not in the same proportion, a significant decrease of LDL-cholesterol is also observed for all the cases. Among the whole series, compound **11b** registered the most important effect (−76.85%) for total cholesterol and for LDL-cholesterol (−66.67%), as well as the highest decrease in the triglyceride levels (−60.69%). Most of the compounds induced no significant decrease in the levels of HDL-cholesterol. In some derivatives, there was no relationship between dose and effect. Comparable effects were observed for those mice treated with a high cholesterol diet (Table 3).

It is well accepted that the in vivo activity is related to pharmacodynamic and pharmacokinetic effects. Therefore establishing structure–in vivo activity relationships is a challenging task. In an effort to explore such relationships we observed the following trends in the data. Overall compound **11b** was more efficient than **11a** (Tables 2 and 3). Fibrate-like analogues **18** and **20b–d** were as potent as compounds **11a–b**, also at both treatments. In particular, derivatives **11a–b**, **18**, and **20b–d**, which have the C-4 or C-5 substituents with the polar groups OH, $NO_2$, and $NH_2$, respectively, proved to be the most active compounds in vivo among the whole series, thus supporting the docking analysis, which revealed that the polar groups in these positions on the benzene ring increase the binding interaction energy by hydrogen bonding with the amino acid residues of the HMGR active site (vide supra).[20] It is interesting that the data for compounds **18** and **20b–d** reveal an important role for the acetate moiety in increasing such effect, since these derivatives were equally active with respect to compounds **11a** and **11b**, which bear instead a methyl group in the same position in the benzene ring.

Evaluating the effect of the side-chain pharmacophore of compounds **11a** and **11b**, it appears that the saturated propanyl side-chain exhibited a better hypolipidemic profile. Although we cannot establish a similar comparison for the **18** and **20a–d** analogues, since all of them have a saturated side-chain, it is likely that the presence of the alkyl chain has a synergetic effect with respect to the phenoxyacetic frame. This frame, judging by the current and previous studies of SAR,[22] seems to be the main hypocholesterolemic pharmacophore, either as the free phenoxyacetic acid or as the ester form. It is possible that the lower hydrophilic effect of the ester form is associated with the more efficient membrane transport and pharmacokinetic effects of **20c** than **20b**, the latter of which has an acetic group.

In spite of the unexpected fact most of the series of compounds only reduced the HDL-cholesterol levels non-significantly, there was a general significant trend to reduce triglyceride levels in both protocols.

**Table 2**
Percentages of hypolipidemic effects produced by the compounds **11a–b**, **18**, and **20a–d** in ICR male mice treated with tyloxapol[a]

| Compound | Dose mg/kg/day | Total cholesterol | LDL-cholesterol | HDL-cholesterol | Triglycerides |
|---|---|---|---|---|---|
| Normal diet | — | −75.60 ± 5.76* | −40.70 ± 0.10* | −28.33 ± 1.11* | −56.33 ± 2.95* |
| Tyloxapol | — | 100 ± 8.27[b] | 100 ± 6.29[c] | 100 ± 3.06[d] | 100 ± 6.2[e] |
| **11a** + Tyloxapol | 50 | −49.79 ± 3.91* | −42.98 ± 0.76* | −39.67 ± 0.79* | −23.20 ± 1.85* |
| | 100 | −54.11 ± 3.42* | −52.07 ± 0.99* | −41.77 ± 0.76* | −6.83 ± 2.64 |
| **11b** + Tyloxapol | 50 | −76.85 ± 0.94* | −66.67 ± 0.79* | −38.67 ± 2.29* | −60.69 ± 0.95* |
| | 100 | −55.75 ± 2.71* | −35.05 ± 1.85* | −37.36 ± 1.24* | −14.84 ± 1.32* |
| **18** + Tyloxapol | 50 | −68.32 ± 2.13* | −28.94 ± 0.14* | −29.15 ± 1.09 | −38.63 ± 1.42* |
| | 100 | −68.42 ± 2.26* | −23.15 ± 0.15* | −25.03 ± 1.36 | −47.07 ± 1.93* |
| **20a** + Tyloxapol | 50 | −21.28 ± 12.32* | −32.56 ± 2.34 | −18.23 ± 3.5 | −32.46 ± 5.67 |
| | 100 | −22.59 ± 8.97 | −39.12 ± 6.2* | −15.32 ± 2.8 | −29.89 ± 8.12 |
| **20b** + Tyloxapol | 50 | −51.59 ± 5.07* | −40.34 ± 0.07* | −21.86 ± 2.64 | −10.09 ± 2.96 |
| | 100 | −59.67 ± 4.88* | −44.61 ± 0.04* | −20.86 ± 4.01 | −25.11 ± 5.28 |
| **20c** + Tyloxapol | 50 | −68.64 ± 5.97* | −45.69 ± 0.09* | −23.15 ± 2.43 | −11.99 ± 4.09 |
| | 100 | −60.10 ± 4.12* | −33.33 ± 0.04* | −25.81 ± 2.57 | −19.91 ± 3.40 |
| **20d** + Tyloxapol | 50 | −69.10 ± 4.57* | −24.22 ± 0.11 | −11.21 ± 2.58 | −15.45 ± 4.70* |
| | 100 | −59.67 ± 5.39* | −37.31 ± 0.05* | −26.23 ± 1.70 | −22.14 ± 5.26* |

[a] Expressed as percentage of the group treated with tyloxapol (mean ± standard error); $n = 6$.
[b] 162.16 mmol/L.
[c] 95.07 mmol/L.
[d] 81.94 mmol/L.
[e] 30.25 mmol/L. Tyloxapol group was compared with the normal diet group.
* Significantly different from the Tyloxapol group, $P < 0.05$.

**Table 3**
Percentages of hypolipidemic effects produced by the compounds **11a–b**, **18**, and **20a–d** in ICR male mice fed with a high cholesterol diet[a]

| Compound | Dose mg/kg/day | Total cholesterol | LDL-cholesterol | HDL-cholesterol | Triglycerides |
|---|---|---|---|---|---|
| Normal diet | — | −81.34 ± 1.69* | −76.53 ± 0.49* | −83.10 ± 1.07* | −73.48 ± 5.51* |
| Cholesterol diet (CD) | — | 100 ± 8.2[b] | 100 ± 6.29[c] | 100 ± 3.06[d] | 100 ± 6.29[e] |
| **11a** + CD | 50 | −33.45 ± 2.47* | 26.49 ± 8.99 | −40.08 ± 6.86 | −37.78 ± 4.45 |
| | 100 | −40. 67 ± 4.25* | −44.22 ± 8.71* | −35.96 ± 2.39 | −42.39 ± 4.90* |
| **11b** + CD | 50 | −59.76 ± 3.52* | −60.81 ± 4.03* | −72.01 ± 1.27 | −62.42 ± 3.88* |
| | 100 | −24.12 ± 4.88* | −22.77 ± 2.10 | −23.07 ± 1.66 | −38.38 ± 5.01 |
| **18** + CD | 50 | −53.34 ± 25.76* | −28.88 ± 3.48* | −18.89 ± 21.39 | −68.42 ± 3.87* |
| | 100 | −74.14 ± 22.55* | −41.81 ± 1.79* | −13.45 ± 22.73 | −55.91 ± 3.53* |
| **20a** + CD | 50 | −32.83 ± 4.54* | −33.54 ± 22.3 | −15.66 ± 4.3 | −38.38 ± 5.43 |
| | 100 | −25.66 ± 5.60 | −44.51 ± 3.4* | −19.33 ± 3.2 | −27.55 ± 2.34* |
| **20b** + CD | 50 | −47.12 ± 19.52* | −41.10 ± 3.20* | −79.36 ± 8.17 | −49.21 ± 8.01* |
| | 100 | −50.22 ± 23.06* | −28.88 ± 2.16 | −55.62 ± 15.42 | −47.72 ± 8.52 |
| **20c** + CD | 50 | −48.64 ± 24.10* | −21.13 ± 2.80 | −11.36 ± 18.42 | −41.98 ± 6.24* |
| | 100 | −39.51 ± 21.30* | −16.76 ± 2.92* | −11.43 ± 23.09 | −28.78 ± 11.14 |
| **20d** + CD | 50 | −41.40 ± 28.05* | −41.10 ± 2.94* | −36.04 ± 18.30 | −12.22 ± 21.06 |
| | 100 | −49.47 ± 15.94* | −31.54 ± 3.07* | −39.58 ± 13.50 | −24.98 ± 14.98 |

[a] Expressed as percentage of the cholesterol diet group (mean ± standard error); $n = 6$.
[b] 153.28 mmol/L.
[c] 121.34 mmol/L.
[d] 83.21 mmol/L.
[e] 43.97 mmol/L. Cholesterol diet group was compared with the normal diet group.
* Significantly different from the cholesterol diet group, $P < 0.05$.

### 2.4. Development of a novel and simple protocol to determine the HMG-CoA reductase inhibition

The current biological models to test the inhibition of the HMGR enzyme mainly use liver enzymatic extracts from rats,[30] which present several disadvantages such as the dependence on the hormonal diurnal cycle of rats, the delay in carrying out the test, and the variation of results with each animal. Taking into account that HMGR is an enzyme that prevails in several organisms including bacteria, human beings, and even yeasts, we developed a simple and accurate protocol to evaluate the inhibition of such enzyme, and to isolate a partially purified fraction of the latter from *Schizosaccharomyces pombe*, and we designed a methodology for evaluating the specific enzymatic activity. HMGR from *S. pombe* was obtained by differential centrifugation, protein precipitation, and membranal solubilization by modifying the Qureshi's methodology.[31] We designed a spectrophotometric method to measure the NADPH+ oxidation by HMG-CoA reductase, with the aim of evaluating the specific enzymatic activity in presence or absence of the newly designed HMGR inhibitors. These results were compared with the activity of simvastatin, which was used as the control drug.[32]

### 2.5. Evaluation of the HMG-CoA reductase inhibition of the α-asarone and fibrate analogues

Once having established the protocol for the partial purification of the enzyme and for the determination of the inhibition of HMGR from the yeasts, the activity of selected derivatives of the series of α-asarone (**1**) and fibrate analogues (**11**, **18**, and **20**) was evaluated. Table 4 summarizes the results of this evaluation, including the activity of **1**. Although the latter was able to inhibit HMGRf, its activity was lower than that of simvastatin (Table 4, entries 1 and 2). However, the activity displayed by all the analogues was higher than that shown by **1**. Furthermore, in some cases (**20b** and **20d**), the activity was comparable (Table 4, entries 6 and 7)

**Table 4**
Inhibitory concentration 50% ($IC_{50}$) of the activity of the HMGR enzyme obtained from *S. pombe*

| Entry | Compound | $IC_{50}$ (mM) |
|---|---|---|
| 1 | Simvastatin | 0.0952 ± 0.00159[†] |
| 2 | **1** | 2.4880 ± 0.039 |
| 3 | **11a** | 0.0788 ± 0.0002[†] |
| 4 | **11b** | 0.0736 ± 0.0256[*,†] |
| 5 | **18** | 0.0665 ± 0.0018[*,†] |
| 6 | **20b** | 0.1350 ± 0.0004[†] |
| 7 | **20d** | 0.0929 ± 0.0083[†] |

[†] Significantly different from the α-asarone group ($P$ <0.05).
[*] Significantly different from the simvastatin group ($P$ <0.05).

or even higher (**11a**, **11b**, and **18**) (Table 4, entries 3–5) than that of the statin. It is likely that those derivatives not evaluated, **17**, **20a**, and **20c**, may also exhibit significant inhibitory activity. Although molecular modeling studies towards understanding the inhibition of HMGR from the yeast are warranted, no crystallographic structural information is available for HMGRf. However, our research group is working on the development of a validated homology model that will be reported in a separate work.

With the aim of gathering evidence on the mode of action for the series of analogues that we have previously reported, we carried out the evaluation of representative derivatives, as summarized in Table 5. Therefore, the compounds structurally related with α-asarone (**1**) **2a**, **2c**, **3b**,[21a] **4c**, **4d**,[21c] and **5a**, **5c**, **6a**,[22a] and related with fibrates **7a**, **7b**,[22c] and **21a**–**g**,[22b,d] (Fig. 4) were tested following the same protocol.

With the exception of compound **21f**, which did not show any inhibition, the results summarized in Table 5 reveal that all the analogues were more active than α-asarone (**1**). Certainly, this inhibitory activity does not completely correlate with the in vivo hypolipidemic activity,[21a,c,22] since **1** was almost the most active agent of the series. It is well known that factors other than the inhibition of the enzyme, such as absorption, distribution, and elimination, are also responsible for the action of a drug.[33] Nevertheless, not only did all these series share the same action mechanism as that of **1** and the statins, but also some of them exhibited a higher activity on the HMGRf enzyme than simvastatin. For instance, compounds **2a**, **5a**, and **21a** display significant inhibition activity, which can be related to the fact that they have not only a polar group at C-5 but also a carboxylate group, respectively, as considered by the docking study as the more significant pharmacophore groups. This correlation is also supported by the fact that compounds **18** and **20d** (Table 4), which have both groups, were among the most active compounds. It is noteworthy that the hypolipidemic activity is also due to parallel mechanisms, such as the stimulation of bile secretion, to which the cholelitholytic activity of α-asarone (**1**) is also associated.[19]

**Table 5**
Inhibitory concentration 50% ($IC_{50}$) of the activity of the HMGR enzyme obtained from *S. pombe*

| Entry | Compound | $IC_{50}$ (mM) |
|---|---|---|
| 1 | Simvastatin | 0.0952 ± 0.00159[†] |
| 2 | **1** | 2.488 ± 0.039 |
| 3 | **2a** | 0.0714 ± 0.00008[†] |
| 4 | **2c** | 0.1672 ± 0.0114[†] |
| 5 | **3b** | 0.1530 ± 0.0030[†] |
| 6 | **4c** | 0.2188 ± 0.0274[†] |
| 7 | **4d** | 0.0846 ± 0.0001[†] |
| 8 | **5a** | 0.0734 ± 0.0016[†] |
| 9 | **5c** | 0.1556 ± 0.0073[†] |
| 10 | **6a** | 0.1444 ± 0.0014[†] |
| 11 | **7a** | 0.2388 ± 0.0021[†] |
| 12 | **7b** | 0.1288 ± 0.0050[†] |
| 13 | **21a** | 0.0913 ± 0.0061[†] |
| 14 | **21b** | 0.5059 ± 0.0460[†] |
| 15 | **21c** | 1.3158 ± 0.01[†] |
| 16 | **21d** | 0.1818 ± 0.0019[†] |
| 17 | **21e** | 1.6152 ± 0.0622[†] |
| 18 | **21f** | No inhibition |
| 19 | **21g** | 0.1971 ± 0.0008[†] |

[†] Significantly different from the α-asarone group ($P$ <0.05).

The activation of the peroxisome proliferator-activated receptor-α (PPARα), which promotes the elevation of HDL-cholesterol in the blood, has been established as the mechanism for fibrate drugs.[34] Although this mechanism cannot be completely ruled out for some of the series that we previously evaluated,[22c] the results herein presented support the inhibition of the HMG-CoA reductase as the mode of action for most of the α-asarone-like and fibrate-like analogues that we have prepared and demonstrated as potential hypocholesterolemic agents.

From a biochemical point of view, one of the interesting findings of this study was to verify that the fibrate-like phenoxyacetic derivatives, which were among the most active compounds, are capable of inhibiting the HMGRf enzyme as their molecular target. Therefore, the HMGR enzyme of *S. pombe* proved to be a suitable model for studies of inhibition by compounds structurally related to fibrates. Although yeasts synthesize ergosterol instead of cholesterol, *S. pombe* shares many of the enzymes involved in the sterol synthesis in mammals, including HMGR.[29]

## 3. Conclusions

A new series of compounds has been designed and prepared on the basis of experimental SAR and a docking study, thereby resulting in a high hypolipidemic activity that was ascribed to the presence of polar hydroxy and amino substituents in the C-4 or C-5 positions of the benzene ring of the α-asarone (**1**)- and fibrate-based analogues, respectively. The crucial role that these groups, along with the key pharmacophores such as the C-1 and C-2 methoxy groups and the phenoxyacetic moiety, play in the biological activity has been supported by the strong binding interactions with key amino acid residues inside the active site of HMG-CoA reductase. For the first time, an accurate and simple model has been developed for evaluating the inhibition of this enzyme by using the yeast extract of *S. pombe*, thus avoiding cholesterol biosynthesis. This model allows us to ascertain the mechanism of action not only of the new potential hypolipidemic agents, whose preparation is herein described, but also of the agents that we have previously reported. These SAR and docking results, as well as the protocol for evaluating the active site of the enzyme, are certainly valuable tools for suggesting novel modifications to the pharmacophore frames and for synthesizing further analogues to improve the HMGR inhibitory activity through structure-based design.

## 4. Experimental

### 4.1. Synthesis

Melting points (uncorrected) were determined with an Electrothermal capillary melting point apparatus. IR spectra were recorded on a Perkin Elmer 2000 spectrophotometer. $^{1}H$ and $^{13}C$ NMR spectra were obtained on a Varian Mercury-300 (300 MHz), with $CDCl_3$ as solvent and TMS as internal standard. Mass spectra (MS) were taken, in electron impact mode, on Hewlett–Packard 5971A and Thermo-Finnigan Polaris Q spectrometers. High-resolution mass spectra (HRMS), in electron impact mode, were obtained on Jeol JSM-GCMateII. Microanalyses were performed by M-H-W Laboratories (Phoenix, AZ). All air moisture sensitive reactions were carried out under nitrogen using oven-dried glassware. All re-

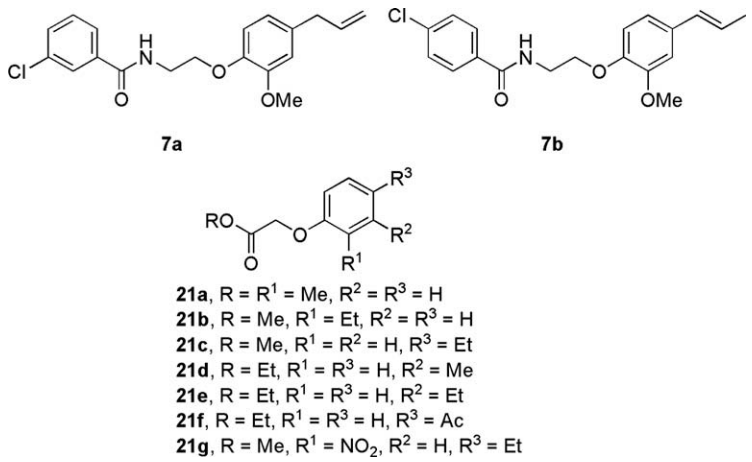



**Figure 4.** α-Asarone (**1**) and fibrate analogues tested for the inhibition of HMGR from *S. pombe*.

agents were used without further purification. Compounds **10a** and **19** were prepared according to the reported methods.[35,36]

#### 4.1.1. 2-Allyl-4,5-dimethoxyphenol (11a)

A mixture of **10a** (5.0 g, 25.8 mmol) and decaline (50 mL) was heated to 220 °C for 12 h. The residue was purified by column chromatography over silica gel (hexane/EtOAc, 9:1), to give 4.29 g (86%) of **10a** as a white solid. $R_f$ 0.20 (hexane/EtOAc, 8:2). Mp 40–41 °C (Lit.[37] 42–42.5 °C).

#### 4.1.2. 4,5-Dimethoxy-2-propylphenol (11b)

A mixture of **11a** (3.7 g, 0.019 mol) and Pd/C (5%) (0.185 g, 0.089 mmol) in EtOH (10 mL) under $H_2$ atmosphere (20 psi) was stirred at 20 °C for 12 h. The mixture was filtered on Celite, the solvent was removed under vacuum, and the residue was purified by column chromatography over silica gel (hexane/EtOAc, 8:2), to give 3.05 g (81%) of **11b** as a white solid. $R_f$ 0.22 (hexane/EtOAc, 8:2). Mp 75–76 °C (Lit.[38] 70.5–72.0 °C). IR (KBr) 3445, 1619, 1521, 1454, 1416, 1202, 1115, 997 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 0.95 (t, $J$ = 7.5 Hz, 3H, $CH_3CH_2CH_2$), 1.53–1.67 (m, 2H, $CH_3CH_2CH_2$), 2.51 (t, $J$ = 7.5 Hz, 2H, $CH_3CH_2CH_2$), 3.72 (s, 3H, OMe), 3.81 (s, 3H, OMe), 5.36 (s, 1H, OH), 6.40 (s, 1H, ArH), 6.64 (s, 1H, ArH). $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 13.8 ($CH_3CH_2CH_2$), 23.2 ($CH_3CH_2CH_2$), 31.5 ($CH_3CH_2CH_2$), 55.7 ($OCH_3$), 56.5 ($OCH_3$), 100.8 (ArH), 113.8 (ArH), 119.4 (Ar), 142.4 (Ar), 147.3 (Ar), 147.4 (Ar). MS (70 eV) $m/z$ 196 ($M^+$, 31), 182 (5), 168 (11), 167 (100), 153 (5), 139 (27), 111 (45), 109 (17), 93 (10). Anal. Calcd for $C_{11}H_{16}O_3$: C, 67.32; H, 8.22. Found: C, 67.40; H, 8.42.

#### 4.1.3. Methyl 2-(5-formyl-2-methoxyphenoxy)acetate (13)[39]

A mixture of **12** (5.0 g, 0.032 mol) and $K_2CO_3$ (5.4 g, 0.039 mol) in dry acetone (30 mL) was stirred to 20 °C for 30 min, and methyl bromoacetate (5.33 g, 0.0349 mmol) was added, and the mixture heated to 60 °C for 3 h. The mixture was filtered and the solvent removed under vacuum. The residue was purified by column chromatography over silica gel (hexane/EtOAc, 7:3), to give 6.7 g (91%) of **13** as a white solid. $R_f$ 0.48 (hexane/EtOAc, 6:4). Mp 71–73 °C. IR ($CH_2Cl_2$) 1757, 1684, 1587, 1512, 1437, 1273, 1138, 1019 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 3.81 (s, 3H, $CO_2CH_3$), 3.97 (s, 3H, OMe), 4.77 (s, 2H, $OCH_2$), 7.02 (d, $J$ = 8.1 Hz, 1H, ArH), 7.32 (d, $J$ = 2.1 Hz, 1H, ArH), 7.53 (dd, $J$ = 8.1, 2.1 Hz, 1H, ArH), 9.82 (s, 1H, CHO). $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 52.1 ($CO_2CH_3$), 55.9 ($OCH_3$), 65.4 ($OCH_2$), 110.8 (ArH), 112.7 (ArH), 127.4 (ArH), 129.5 (Ar), 147.4 (Ar), 154.4 (Ar), 168.5 ($CO_2CH_3$), 190.3 (CHO). MS (70 eV) $m/z$ 224 ($M^+$, 40), 223 (7), 192 (7), 165 (100), 151 (57), 137 (34), 135 (61), 109 (25), 95 (65).

#### 4.1.4. Methyl 2-(5-(formyloxy)-2-methoxyphenoxy)acetate (14)

A mixture of **13** (5.1 g, 0.022 mol) and MCPBA (5.6 g, 0.032 mol) in $CH_2Cl_2$ (50 mL) was stirred at 20 °C for 3 h, then filtered, and the solvent was removed under vacuum. The residue was purified by column chromatography over silica gel (hexane/EtOAc, 8:2), to give 3.5 g (65%) of **14** as a white solid. $R_f$ 0.50 (hexane/EtOAc, 6:4). Mp 68–69 °C. IR ($CH_2Cl_2$): 1766, 1729, 1601, 1513, 1440, 1228, 1159, 1090, 1016 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 3.80 (s, 3H, $CO_2CH_3$), 3.89 (s, 3H, $OCH_3$), 4.69 (s, 2H, $OCH_2$), 6.65 (d, $J$ = 2.7 Hz, 1H, ArH), 6.77 (dd, $J$ = 8.7, 2.7 Hz, 1H, ArH), 6.90 (d, $J$ = 8.7 Hz, 1H, ArH), 8.26 (s, 1H, OCHO). $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 52.3 ($CO_2CH_3$), 56.2 ($OCH_3$), 66.4 ($OCH_2$), 108.1 (ArH), 112.0 (ArH), 114.3 (ArH), 143.1 (Ar), 147.5 (Ar), 147.8 (Ar), 159.4 (OCHO), 168.9 ($CO_2Me$). MS (70 eV) $m/z$ 240 ($M^+$, 12), 212 (27), 197 (5), 153 (5), 139 (30), 137 (83), 112 (9), 111 (100), 109 (9), 93 (27). HRMS ($EI^+$) calcd for $C_{11}H_{12}O_6$ ($M^+$): 240.0634; found: 240.0635.

#### 4.1.5. Methyl 2-(5-hydroxy-2-methoxyphenoxy)acetate (15)

A mixture of **14** (3.5 g, 0.0145 mol) and 6 N HCl (1 mL) in MeOH (10 mL) was stirred at 20 °C for 1 h, and the solvent was removed under vacuum. The residue was extracted with EtOAc (3 × 25 mL), and the organic layers were dried ($Na_2SO_4$), and the solvent removed under vacuum. The residue was purified by column chromatography over silica gel (hexane/EtOAc, 6:4), to give 2.75 g (89%) of **15** as a white solid. $R_f$ 0.43 (hexane/EtOAc, 6:4). Mp 104–106 °C. IR ($CH_2Cl_2$): 3566, 3178, 1770, 1609, 1515, 1438, 1215, 1172, 1130, 1019 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 3.76 (s, 3H, $CO_2CH_3$), 3.79 (s, 3H, $OCH_3$), 4.63 (s, 2H, $OCH_2$), 6.42 (d, $J$ = 2.7 Hz, 1H, ArH), 6.44 (dd, $J$ = 8.7, 2.7 Hz, 1H, ArH), 6.73 (d, $J$ = 8.7 Hz, 1H, ArH), 6.74 (s, 1H, OH). $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 52.2 ($CO_2CH_3$), 56.5 ($OCH_3$), 66.0 ($OCH_2$), 102.8 (ArH), 107.9 (ArH), 113.3 (ArH), 143.1 (Ar), 147.6 (Ar), 150.1 (Ar), 169.8 ($CO_2Me$). MS (70 eV) $m/z$ 212 ($M^+$, 17), 211 (4), 197 (4), 153 (5), 139 (32), 137 (85), 111 (100), 97 (5), 93 (30). HRMS ($EI^+$) calcd for $C_{10}H_{12}O_5$ ($M^+$): 212.0685; found: 212.0685.

#### 4.1.6. Methyl 2-(5-(allyloxy)-2-methoxyphenoxy)acetate (16)

A mixture of **15** (2.7 g, 0.013 mol), allyl bromide (2.38 g, 0.02 mol), and $K_2CO_3$ (3.6 g, 0.026 mol) in dry acetone (50 mL) was heated to 60 °C for 3 h. The solvent was removed under vacuum, and the residue purified by column chromatography over silica gel (hexane/EtOAc, 6:4), to give 2.8 g (87%) of **16** as a white solid. $R_f$ 0.23 (hexane/EtOAc, 8:2). Mp 75–76 °C. IR ($CH_2Cl_2$) 1741, 1649, 1264, 1147 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 3.78 (s, 3H, $CO_2CH_3$), 3.83 (s, 3H, $OCH_3$), 4.43–4.48 (m, 2H, $CH_2CH$=), 4.67 (s, 2H, $OCH_2$), 5.24–5.30 (m, 1H, $CH_2$=), 5.34–5.43 (m, 1H, $CH_2$=),

5.96–6.10 (m, 1H, CH=), 6.44–6.48 (m, 2H, ArH), 6.80 (d, $J$ = 9.0 Hz, 1H, ArH). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 52.1 ($CO_2CH_3$), 56.4 ($OCH_3$), 66.1 ($OCH_2$), 69.2 ($CH_2CH$=), 103.2 (ArH), 106.1 (ArH), 112.5 (ArH), 117.6 ($CH_2$=), 133.2 (CH=), 143.9 (Ar), 147.7 (Ar), 152.7 (Ar), 169.2 ($CO_2$Me). MS (70 eV) $m/z$ 252 ($M^+$, 6), 237 (8), 179 (29), 177 (53), 163 (23), 149 (26), 147 (23), 133 (9), 123 (100), 121 (16), 103 (9), 95 (4), 91 (22). HRMS ($EI^+$) calcd for $C_{13}H_{16}O_5$ ($M^+$): 252.0998; found: 252.1000.

#### 4.1.7. Methyl 2-(4-allyl-5-hydroxy-2-methoxyphenoxy)acetate (17)

A mixture of **16** (3.95 g, 15.8 mmol) and decaline (40 mL) was heated to 220 °C for 12 h. The residue was purified by column chromatography over silica gel (hexane/EtOAc, 8:2), to give 3.3 g (83%) of **17** as a pale yellow powder. $R_f$ 0.21 (hexane/EtOAc, 8:2). Mp 48–49 °C. IR ($CH_2Cl_2$) 3461, 3078, 1746, 1617, 1521, 1203, 1120, 1007 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 3.30 (br d, $J$ = 6.6 Hz, $CH_2$CH=), 3.71 (s, 3H, $CO_2CH_3$), 3.77 (s, 3H, $OCH_3$), 4.56 (s, 2H, $OCH_2$), 5.00–5.10 (m, 2H, $CH_2$=), 5.87–6.02 (m, 1H, CH=), 6.38 (br s, 1H, OH), 6.43 (s, 1H, ArH), 6.65 (s, 1H, ArH). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 33.6 ($CH_2$CH=), 51.9 ($CO_2CH_3$), 56.3 ($OCH_3$), 66.0 ($OCH_2$), 103.3 (ArH), 114.3 (Ar), 115.4 (ArH), 118.8 ($CH_2$=), 136.3 (CH=), 142.7 (Ar), 145.6 (Ar), 147.5 (Ar), 169.7 ($CO_2$Me). MS (70 eV) $m/z$ 252 ($M^+$, 13), 237 (14), 179 (36), 177 (49), 163 (30), 151 (18), 149 (14), 133 (10), 123 (100), 121 (13), 105 (60), 91 (13). HRMS ($EI^+$) calcd for $C_{13}H_{16}O_5$ ($M^+$): 252.0998; found: 252.0998.

#### 4.1.8. Methyl 2-(5-hydroxy-2-methoxy-4-propylphenoxy) acetate (18)

A mixture of **17** (2.0 g, 7.93 mmol) and Pd/C (5%) (0.10 g, 0.048 mmol) in EtOH (10 mL) under $H_2$ atmosphere (20 psi) was stirred at 20 °C for 12 h. The mixture was filtered on Celite, the solvent was removed under vacuum, and the residue was purified by column chromatography over silica gel (hexane/EtOAc, 8:2), to give 1.2 g (60%) of **18** as a white solid. $R_f$ 0.42 (hexane/EtOAc, 6:4). Mp 57–58 °C. IR (KBr) 3462, 1747, 1616, 1520, 1447, 1201, 1123, 1009 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.94 (t, $J$ = 7.5 Hz, 3H, $CH_3CH_2CH_2$), 1.56–1.65 (m, 2H, $CH_3CH_2CH_2$), 2.51 (t, $J$ = 7.5 Hz, 2H, $CH_3CH_2CH_2$), 3.75 (s, 3H, $CO_2$Me), 3.80 (s, 3H, OMe), 4.60 (s, 2H, $OCH_2$), 5.64 (br s, 1H, OH), 6.41 (s, 1H, ArH), 6.65 (s, 1H, ArH). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 13.8 ($CH_3CH_2CH_2$), 23.0 ($CH_3CH_2CH_2$), 31.6 ($CH_3CH_2CH_2$), 52.2 ($CO_2CH_3$), 56.6 ($OCH_3$), 66.5 ($OCH_2$), 103.6 (ArH), 114.6 (ArH), 121.8 (Ar), 143.0 (Ar), 145.4 (Ar), 147.3 (Ar), 170.0 ($CO_2$Me). MS (70 eV) $m/z$ 254 ($M^+$, 18), 226 (15), 225 (100), 181 (17), 179 (17), 167 (28), 139 (36), 137 (42), 123 (15), 111 (59), 109 (12), 93 (8). HRMS ($EI^+$) calcd for $C_{13}H_{16}O_5$ ($M^+$): 254.1154; found: 254.1151.

#### 4.1.9. 2-(2-Methoxy-4-propylphenoxy)acetic acid (20a)

A mixture of NaOH (0.74 g, 19.0 mmol) in 10 mL of $H_2O$ and **19** (2.0 g, 12.0 mmol) was stirred at room temperature for 30 min. Then, sodium chloroacetate (1.71 g, 14.7 mmol) was added, and the mixture was heated to 60 °C for 24 h under $N_2$ atmosphere. At 0 °C, HCl (36%) was added until pH 1, and the mixture was stirred for 30 min. A precipitate was formed, which was filtered, washed with $H_2O$ (2 × 5 mL), and dissolved with EtOAc (50 mL). The organic layer was dried ($Na_2SO_4$) and the solvent removed under vacuum. The residue was purified by recrystallization (hexane/EtOAc, 8:2), to give 1.86 g (69%) of **20a** as a white solid. $R_f$ 0.60 (hexane/EtOAc/AcOH, 8:2:0.1). Mp 88–90 °C [Lit.[40] 110–112 °C]. IR (film) 3750–2400, 1734, 1516, 1457, 1259, 1229, 1145, 1033, 813 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.93 (t, $J$ = 7.5 Hz, 3H, $CH_3CH_2CH_2$), 1.61 (sx, $J$ = 7.5 Hz, 2H, $CH_3CH_2CH_2$), 2.53 (t, $J$ = 7.5 Hz, 2H, $CH_3CH_2CH_2$), 3.87 (s, 3H, $OCH_3$), 4.66 (s, 2H, $OCH_2$), 5.95 (br, 1H, OH), 6.70 (dd, $J$ = 7.8, 1.7 Hz, 1H, ArH), 6.73 (d, $J$ = 1.7 Hz, 1H, ArH), 6.81 (d, $J$ = 7.8 Hz, 1H, ArH), 7.93 (br s, 1H, $CO_2H$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 13.7 ($CH_3CH_2CH_2$), 24.5 ($CH_3CH_2CH_2$), 37.6 ($CH_3CH_2CH_2$), 55.7 ($OCH_3$), 67.1 ($OCH_2$), 112.4 (ArH), 115.4 (ArH), 120.5 (ArH), 138.0 (Ar), 144.9 (Ar), 149.2 (Ar), 173.0 (CO). MS (70 eV) $m/z$ 224 ($M^+$, 13), 195 (32), 165 (41), 151 (9), 137 (100), 95 (41).

#### 4.1.10. 2-(2-Methoxy-5-nitro-4-propylphenoxy)acetic acid (20b)

A mixture of $H_2SO_4$ (5.0 mL) and $HNO_3$ (10 mL) in 5 mL of $H_2O$ was stirred at 20 °C for 1 h, then, at 0 °C, **20a** (5.03 g, 22.0 mmol) was added, and the mixture was stirred at 20 °C for 24 h under $N_2$ atmosphere. The precipitate was filtered, dissolved with EtOAc (25 mL), and dried ($Na_2SO_4$), then the solvent was removed under vacuum. The residue was purified by recrystallization (hexane/EtOAc, 6:4), to give 4.59 g (76%) of **20b** as a pale yellow solid. $R_f$ 0.60 (hexane/EtOAc, 1:1). Mp 98–100 °C. IR (film) 3200–2400, 1725, 1585, 1528, 1504, 1460, 1332, 1265, 1228, 1207, 1049, 866, 800 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 7.5 Hz, 3H, $CH_3CH_2CH_2$), 1.64–1.71 (m, 2H, $CH_3CH_2CH_2$), 2.86–2.94 (m, 2H, $CH_3CH_2CH_2$), 3.97 (s, 3H, $OCH_3$), 4.78 (s, 2H, $OCH_2$), 6.76 (s, 1H, ArH), 7.59 (s, 1H, ArH), 9.69 (br s, 1H, $CO_2H$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.1 ($CH_3CH_2CH_2$), 23.9 ($CH_3CH_2CH_2$), 35.7 ($CH_3CH_2CH_2$), 56.4 ($OCH_3$), 65.9 ($OCH_2$), 111.4 (ArH), 113.9 (ArH), 135.1 (Ar), 140.8 (Ar), 144.6 (Ar), 153.3 (Ar), 173.4 (CO). MS (70 eV) $m/z$ 269 ($M^+$, 3), 266 (25), 238 (40), 206 (94), 191 (69), 165 (100), 150 (42), 136 (71), 124 (41), 120 (30), 91 (16). Anal. Calcd for $C_{12}H_{15}NO_6$: C, 53.53; H, 5.62; N, 5.20. Found: C, 53.71; H, 5.63; N, 5.01.

#### 4.1.11. Methyl 2-(2-methoxy-5-nitro-4-propylphenoxy)acetate (20c)

A mixture of **20b** (1.0 g, 3.7 mmol), dry MeOH (10 mL), and $p$-TsOH (0.1 g, 0.58 mmol) was stirred at 60 °C for 2 h. The solvent was removed under vacuum, and the residue was extracted with EtOAc (3 × 25 mL). The organic layer was washed with saturated aqueous solution of $NaHCO_3$ until neutral, the white precipitate was filtered and purified by recrystallization (hexane/EtOAc, 8:2), to give 0.97 g (92%) of **20c** as a pale yellow solid. $R_f$ 0.32 (hexane/EtOAc, 8:2 × 2). Mp 85–87 °C. IR (film) 1768, 1700, 1650, 1520, 1458, 1325, 1271, 1205, 1078, 870, 813 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 7.6 Hz, 3H, $CH_3CH_2CH_2$), 1.60–1.74 (m, 2H, $CH_3CH_2CH_2$), 2.85–2.94 (m, 2H, $CH_3CH_2CH_2$), 3.83 (s, 3H, $CO_2$Me), 3.97 (s, 3H, $OCH_3$), 4.74 (s, 2H, $OCH_2$), 6.75 (s, 1H, ArH), 7.53 (s, 1H, ArH). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.1 ($CH_3CH_2CH_2$), 23.9 ($CH_3CH_2CH_2$), 35.8 ($CH_3CH_2CH_2$), 52.4 ($CO_2CH_3$), 56.3 ($OCH_3$), 66.0 ($OCH_2$), 110.5 (ArH), 113.8 (ArH), 134.6 (Ar), 140.9 (Ar), 144.9 (Ar), 153.3 (Ar), 168.5 (CO). MS (70 eV) $m/z$ 283 ($M^+$, 15), 266 (68), 238 (44), 206 (100), 191 (27), 178 (22), 165 (67), 136 (20), 120 (13), 77 (13), 57 (42). Anal. Calcd for $C_{13}H_{17}NO_6$: C, 55.12; H, 6.05; N, 4.94. Found: C, 55.33; H, 6.13; N, 4.76.

#### 4.1.12. Methyl 2-(5-amino-2-methoxy-4-propylphenoxy)acetate (20d)

A mixture of **20c** (1.0 g, 6.9 mmol) and Pd/C (5%) (0.005 g, 0.0024 mmol) in EtOAc (10 mL) under $H_2$ atmosphere (30 psi) was stirred at 20 °C for 24 h. The mixture was filtered on Celite and washed with EtOAc (3 × 25 mL), then the solvent was removed under vacuum, and the residue was purified by column chromatography over silica gel (hexane/EtOAc, 8:2), to give 0.72 g (80%) of **20d** as a brown oil. $R_f$ 0.54 (hexane/EtOAc, 1:1). IR (film) 3748, 3366, 1752, 1623, 1517, 1457, 1287, 1201, 1132, 1027, 854 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.98 (t, $J$ = 7.3 Hz, 3H, $CH_3CH_2CH_2$), 1.53–1.68 (m, 2H, $CH_3CH_2CH_2$), 2.36–2.44 (m, 2H, $CH_3CH_2CH_2$), 3.40 (br s, 1H, NH), 3.78 (s, 3H, $CO_2$Me), 3.81 (s, 3H, $OCH_3$), 4.63 (s, 2H, $OCH_2$), 6.27 (s, 1H, ArH), 6.63 (s, 1H, ArH). $^{13}$C NMR

(75 MHz, $CDCl_3$) $\delta$ 14.0 ($CH_3CH_2CH_2$), 22.1 ($CH_3CH_2CH_2$), 33.0 ($CH_3CH_2CH_2$), 52.0 ($CO_2CH_3$), 56.7 ($OCH_3$), 66.7 ($OCH_2$), 103.8 (ArH), 114.9 (ArH), 120.4 (Ar), 137.7 (Ar), 142.3 (Ar), 146.0 (Ar), 169.7 (CO). MS (70 eV) *m*/*z* 253 ($M^+$, 34), 238 (24), 224 (68), 179 (22), 178 (100), 166 (30), 152 (50), 150 (72), 136 (27), 122 (72), 94 (19). Anal. Calcd for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56; N, 5.53. Found: C, 61.83; H, 7.69; N, 5.37.

### 4.2. Hypolipidemic evaluation

For hypolipidemic studies, compounds were suspended in a 1:100 tween 80:water solution, and administered orally by an intubation needle at doses of 0, 50 or 100 mg/kg. Doses were selected based on previous studies, where their effectiveness and that of other α-asarone analogues were tested in mice.[22a] The concentration of solutions was adjusted so that mice could be given 5 mL/kg. Animals in the control group received a similar volume of vehicle. Solutions were freshly prepared before each administration.

Hypolipidemic activity was studied in (ICR) male mice weighing 24–27 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 08:00 h). They were fed on standard pellet diets (Rodent Diet 5001, PMI Nutrition International, Inc., Brenwood, MO) and drinking water was freely available, unless experimental conditions required diet changes. All animals appeared healthy throughout the dosing period, maintaining normal food intake and weight gain. At sacrifice, no gross abnormalities were observed in any treated mice.

All animals were treated in accordance with ethical principles and regulations specified by the Animal Care and Use Committee of our Institution and the Standards of the National Institutes of Health of Mexico.

The mice were randomly divided into groups of six animals. Hyperlipidemia was induced in the mice by one of two methods. Firstly, ip administration of Tritón WR 1339 (Tyloxapol) was dissolved in water at 400 mg/kg. Mice were treated with the drugs 1 h before, and 22 and 48 h after the Tyloxapol injection. Animals receiving the vehicle were used as the non-cholesterol control group. Secondly, the animals were fed a high cholesterol diet ad libitum for 6 days, containing (wt/wt) cholesterol 1.0%, sodium cholate 0.5%, butter 5%, sucrose 30.0%, casein 10% and laboratory chow 53.5%, prepared from a powdered basal diet (5001 Lab Rodent Diet, PMI Nutrition International, Inc. Bienwood, MO, USA). Mice fed only with the same powered basal diet for the same duration as above were used as the non-cholesterol control groups.

For Tyloxapol-treated mice blood samples were taken 22 h after injection. On the other hand, animals receiving the high cholesterol diet were fasted for 12 h before sacrifice. Blood samples were collected by periorbital plexus bleeding and centrifuged at 3000 rpm for 15 min. Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides levels were determined in the serum, using a Wiener lab, Selectra II automatic analyzer. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation.[41]

All data are expressed as the percentage of the cholesterol diet group (the mean ± standard error) by using the bifactorial ANOVA and Newman-Keuls tests (Sigmastat 2.03 Program). P values less than 0.05 were considered significant.

### 4.3. Enzymatic evaluation

Chemicals: NADPH, HMG-CoA, and dimethyl sulfoxide (DMSO) from Sigma (Sigma, Saint Louis, MO, USA).

#### 4.3.1. Assay for protein

Protein assays were carried out by the method developed by Lowry et al.[42]

Medium for growth of yeast cells was either YPD (1% yeast extract, 2% peptone, 2% dextrose). For solid medium, agar was added to 2% final concentration. *S. pombe* 972 h was grown at 28 °C for 48 h, with shaking at 120 rpm in Fernbach flasks containing 1 L of YPD medium.[28]

#### 4.3.2. Partial purification of HMG-CoA reductase from *S. pombe*

Biomass from the culture medium was recovered by centrifugation at 8000g at 4 °C for 15 min. Cells were fragmented in Braun's mill using glass beads (0.5 mm diameter). The mixture contained 7.5 g washed acid beads, 12 mL 0.1 M Tris–HCl, pH 7.5 and 5 g cells. Total disintegration time was 20 min (20 series, 1 min each). The suspension was centrifuged at 2500g for 15 min to remove unbroken cells and debris. The supernatant solution was then centrifuged at 20,000g for 15 min. The pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7, containing 1 mM dithiothreitol (DTT) and 1 mM EDTA, and centrifuged at 100,000g for 90 min at 4 °C. The pellet was resuspended in the same buffer and, in order to dissolve HMG-CoA reductase, was treated with 0.02% triton X-100, modified from Qureshi et al.[31,43] The supernatant solution (from the above procedures) was heated in a water bath to a bath temperature of 65 °C for 1 h, and was immediately cooled on ice and centrifuged at 20,000g for 20 min at 4 °C. The supernatant was brought to 60% saturation with solid ammonium sulfate. The protein precipitate was dissolved in a minimal volume of 0.1 M potassium phosphate buffer, pH 7, containing 1 mM DTT and 1 mM EDTA. The ammonium sulfate was removed by ultrafiltration through a 50 kDa membrane. The retained protein was diluted in 25 mM potassium phosphate, containing 10 mM DTT and 1 mM EDTA.

#### 4.3.3. Calcium phosphate gel adsorption and ammonium sulfate precipitation

The solution was then mixed with calcium phosphate gel so that the weight ratio of protein to gel was 1:1 and centrifuged at 8000g for 5 min. The calcium phosphate gel was washed twice and centrifuged with 0.1 M phosphate buffer, pH 7, containing 10 mM DTT, and 1 mM EDTA. HMG-CoA reductase was removed from the gel by washing five times with 50 kDa membrane. The retained fraction was resuspended in the same buffer plus glycerol to a final concentration 25%, modified from Qureshi et al.[31]

#### 4.3.4. Assay of HMG-CoA reductase activities

The *S. pombe* HMG-CoA reductase activity was measured using HMG-CoA, NADPH and Tris–HCl, pH 7.5, according to Bischoff and Rodwell.[32] The oxidation of NADPH was spectrophotometrically monitored at 340 nm in a Varian UV–vis spectrophotometer, Model Cary 50. HMG-CoA reductase was assayed three times, and the enzyme was previously activated at 37 °C for 30 min. The reaction mixture contained: 0.13 mM HMG-CoA, 200 μg of partially purified enzyme (specific activity 406 μU/mg) with or without inhibitor and 50 mM Tris–HCl, pH 7.5, to a final volume of 375 μL. After 15 min incubation at 37 °C, the reaction was started with the addition of 0.13 mM NADPH addition and monitored for 2 min. For all three 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.[30,32]

#### 4.3.5. Effect of synthetic compounds on *S. pombe* HMGRp activity

The partial purified HMG-CoA reductase from *S. pombe* (obtained as described above) was pre-incubated with the respective

compounds for 30 min at 37 °C, followed by the standard enzyme assay. The synthetic compounds were prepared at 1, 2, 3, 4 and 5 mM in DMSO. A DMSO control was tested to exclude the inhibition of this solvent on the activity of the enzyme. After measuring HMGR inhibition, 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

A previously validated docking protocol we successfully used to explain the binding mode of α-asarone (**1**) with HMGR[20] was also employed in this work. Thus, automated docking with the program AUTODOCK, version 3.0.[44] was employed to locate the appropriate binding orientation and conformation of compounds **1**, **11a–b**, **17**, **18**, and **20a–d** with HMGR. Docking was performed using the structure of HMGR complexed with rosuvastatin (PDB code 1hwl)[23] obtained from the Protein Data Bank.[45] To note, in previous validation of the docking protocol, the root mean square deviation (RMSD) between the predicted conformation for rosuvastatin and the observed X-ray crystallographic conformation was 0.81.[20] Rosuvastatin was the statin for which the lowest RMSD value was obtained.[20] The structures of the ligands were prepared with the program Molecular Operating Environment (MOE), version 2008.10,[46] and visualizations were carried out with Maestro, version 9.0.[47] All water and adenosine-5′-diphosphate molecules were removed from the original Protein Data Bank file. Polar hydrogen atoms were added and Kollman charges,[48] atomic solvation parameters and fragmental volumes were assigned to the protein using AutoDock Tools (ADT). For docking calculations, Gasteiger-Marsili partial charges[49] were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The auxiliary program AutoGrid generated the grid maps. Each grid was centered at the crystal structure of rosuvastatin. The grid dimensions were $23 \times 23 \times 23$ Å$^3$ with points separated by 0.375 Å. Lennard-Jones parameters 12–10 and 12–6, supplied with the program, were used for modeling hydrogen bonds and van der Waals interactions, respectively. The distance-dependent dielectric permittivity of Mehler and Solmajer[50] was used for the calculation of the electrostatic grid maps. For all ligands, random starting positions, random orientations, and torsions were used. The translation, quaternion, and torsion steps were taken from default values in AutoDock. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters. The number of docking runs was 100. The complexes of the ligands with HMGR resulting from molecular docking were further structurally optimized with the MMFF94x force field implemented in MOE until the gradient 0.001 was reached. The default parameters implemented into the MOE's LigX[46] application were used as we have previously described for other systems.[51]

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