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ARTICLE

On the Inhibitor Effects of Bergamot Juice Flavonoids Binding to the 3-Hydroxy-3-methylglutaryl-CoA Reductase (HMGR) Enzyme

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Density functional theory was applied to study the binding mode of new flavonoids as possible inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), an enzyme that catalyzes the four-electron reduction of HMGCoA to mevalonate, the committed step in the biosynthesis of sterols. The investigated flavonoid conjugates brutieridin and melitidin were recently quantified in the bergamot fruit extracts and identified to be structural analogues of statins, lipids concentration lowering drugs that inhibit HMGR. Computations allowed us to perform a detailed analysis of the geometrical and electronic features affecting the binding of these compounds, as well as that of the excellent simvastatin drug, to the active site of the enzyme and to give better insight into the inhibition process.

KEYWORDS: HMGR; statin-like principles; enzyme inhibition; DFT

INTRODUCTION

Bergamot (*Citrus bergamia* Risso), a hybrid between orange and lemon plants, is a typical fruit of the southern provinces of Italy. It is mostly used for the extraction of its essential oil from the peel. Because of its unique fragrance and freshness, bergamot oil is widely used in the cosmetic and food industries (*1*). Furthermore, due to its antiseptic and antibacterial properties, the essence is also used in the pharmaceutical industry (*2*).

In the past few years, following the growing interest in antioxidant bioactive compounds and their dietary sources, bergamot juice, as well as its peel, has attracted some attention because of its remarkable content of flavonoids (*3*−*9*). In particular, it has been identified and quantified (*10*) that together with the most abundant neoeriocitrin, naringin and neohesperidin, also *C*-glucosides (lucenin-2, vicenin-2, stellarin-2, lucenin-2−4′-methyl ether, scoparin, and orientin 4′-methyl ether), flavone *O*-glycosides (rhoifolin 4′-*O*-glucoside, chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside, rhoifolin, chrysoeriol 7-*O*-neohesperidoside, and neodiosmin), and flavanone *O*-glycosides (eriocitrin) are present.

Many epidemiological and biochemical studies (*11*) have demonstrated flavonoids to possess beneficial effects on human health because of their established antioxidant activity in scavenging harmful free radicals. Recently (*12*), a detailed analysis of bergamot fruit extractions, carried out by LC-MS, MS/MS, and NMR techniques, has led to the isolation of two new flavonoid conjugates, named brutieridin (hesperetin 7-(2′′-α-rhamnosyl-6′′-(3′′′′-hydroxy-3′′′′-methylglutaryl)-β-glucoside)) and melitidin (naringenin 7-(2′′-α-rhamnosyl-6′′-(3′′′′-hydroxy-3′′′′-methylglutaryl)-β-glucoside)) (1 and 2 in **Scheme 1**). They are present in bergamot fruit in concentration ranges of approximately 300−500 and 150−300 ppm, respectively, and are found either in the juice or in the albedo and flavedo layers of bergamot.

Their structure resembles that of cholesterol inhibitor drugs statins (*13*). Like statins, the new active principles contain the hydroxyl mevalonate moiety. Mevalonate is a precursor of isoprenoids, a class of compounds involved in several cellular functions such as cholesterol synthesis and growth control. Within cells, the concentration of mevalonate and therefore that of its metabolic products is tightly controlled through the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), an enzyme that catalyzes the four-electron reduction of HMGCoA to mevalonate (*14*).

Elevated serum-blood cholesterol levels are associated with a high risk of heart coronary artery diseases (*15*). Inhibition of HMGR enzyme is reported to significantly decrease cholesterol levels and to reduce the risks of stroke (*16*). Statins are potent HMGR inhibitors since they bind to the active site where also the natural substrate HMG binds, and are prescribed widely in the treatment of hypercholesterolemia. They are thought to bind competitively with the natural substrate in the active site of HMGR (*17*, *18*). However, adverse experiences have been associated with statins, both in monotherapy and in combination therapy with other agents (*19*, *20*).

Both the wide variety of different types of flavonoids molecules and the fact that some of these compounds are present in good amounts (150−500 ppm) make possible the future applications of bergamot juice in health care, also considering the recommended statins dosage ranging from 5 mg to 80 mg, depending on the particular drug and cholesterol levels.

The aim of this computational study is to investigate the interaction of the two new compounds brutieridin and melitidin with the active site of the human HMGR enzyme in order to have insights on their possible inhibitory effect. For the purpose of

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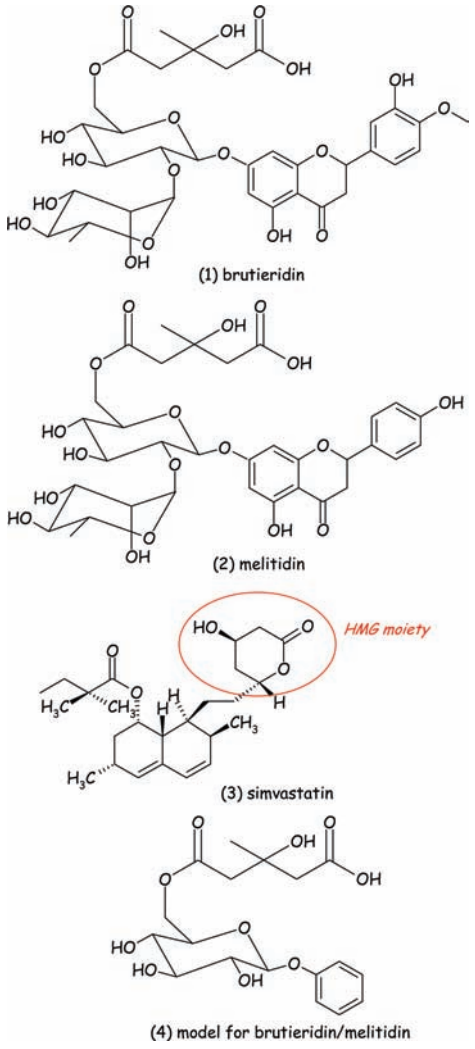
Scheme 1. Representation of (1) Brutieridin, (2) Melitidin, (3) Simvastatin, and (4) the Model Used for Brutieridin/Melitidin



comparison, the behavior of the excellent HMGR inhibitor simvastatin (3 in scheme **1**) is also investigated.

MATERIALS AND METHODS

The model cluster used to simulate the active site of the HMGR enzyme was built starting from the 2.33 Å X-ray structure of human HMGR in complex with the simvastatin inhibitor (pdb code 1HW9) (*21*).

In the case of the simvastatin−enzyme complex, the cluster was built by simply cutting the active site with selected residues and the drug from the available X-ray structure. For the brutieridin/melitidin−HMGR complex, since no experimental X-ray structures exist, we rebuilt the 3D structure by deleting the non-HMG moiety of simvastatin and substituting it with the remaining part of the brutieridin/melitidin. To get the reasonable structure for quantum mechanical investigation, molecular dynamics (MD) calculations were performed. These computations were carried out by means of the Gromacs 3.2 program (*22*). The gromacs force field was applied, and the force field parameter of the brutieridin/melitidin drug was generated by the PRODRG server (*23*) to build a Gromacs 87 topology for the drug. The solvent SPC water model implemented in the Gromacs package and an octahedral box, setting the box edge at a distance of 5.0 nm from the target, were used. Na^+ counterions were added to satisfy the electroneutrality condition.

First of all, the energy minimization was performed, applying the steepest descent method to remove local strain and bad van der Waals contacts. Then we ran a 20 ps position-restrained dynamics simulation, restraining the atom positions of the macromolecule while letting the solvent and the drug relax into the protein. The simulation was performed at constant temperature and pressure by coupling the system to a Berendsen bath (*22*) at 300 K and 1 atm. Finally, a 100 ps MD simulation was performed for the system. Root-mean-square deviation (rmsd) time series (Figure S1 in the Supporting Information) suggest that the system was well equilibrated after about 50 ps.

Enzymes are proteins with a high molecular weight so that they cannot be entirely studied without introducing significant approximations or using chemical models to represent the active region of the enzymes. Results must be interpreted in light of the above-mentioned computational limitations.

The model cluster was reproduced by considering the 7 Glu559, Cys561, Leu562, Lys735, His752, Asn755, and Leu853 residues and the 10 Arg590, Asn658, Ser684, Gly685, Asn686, Cys688, Thr689, Asp690, Lys691, and Lys692 residues belonging to the chains A and B of the protein, respectively. Following a consolidated procedure (*24*−*36*), simple models for these amino acids were employed, i.e., CH_3COO^- for Asp and Glu, $CH_3NHC(NH_2)_2^+$ for Arg, $CH_3NH_3^+$ for Lys, CH_3OH for Ser, CH_3CH_2-CH_3 for Leu, CH_3CONH_2 for Asn, CH_3SH for Cys, methylimidazole for His, and CH_3CONH- for Gly and Thr residues. This practice is widely applied in the studies regarding enzymatic proteins and in many cases has given very reliable results (*24*−*36*). The built up cluster model for the enzyme active site resulted in having a total charge of +3 and to be made up by 150 atoms, all described at the quantum mechanical level. Another simple model (4 in **Scheme 1**) was used to simulate the brutieridin/melitidin statin-like molecule. It consists of a phenyl ring for the flavonone moiety and only one sugar molecule, so as to reduce the computational efforts. Simvastatin was considered as such. Both brutieridin/melitidin and simvastatin have a charge of −1.

An H atom of each amino acid residue coming from the protein was kept frozen at its crystallographic position in order to mimic the steric effects produced by the surrounding protein and to avoid an unrealistic expansion of the cluster during the optimization procedure (*27*, *28*). No constraints were imposed to simvastatin and brutieridin/melitidin. All of the quantum chemical computations were performed with the Gaussian 03, revision C02, code (*37*).

The hybrid B3LYP functional was employed to perform geometry optimization, and the 6-31G* basis set was chosen to describe the C and H atoms, while for O and N atoms, the 6-31+G* orbital set was used, as implemented in Gaussian 03. Frequency calculations were performed at the B3LYP level on all of the stationary points so as to establish their character of minima. Zero point energy (ZPE) corrections, obtained by vibrational analysis, were then included in all relative energies. The basis set superposition error (BSSE), computed according to the counterpoise method of Boys and Bernardi, as implemented in Gaussian 03, was always less than 1 kcal/mol.

To account for the polarization effects caused by the part of the surrounding enzyme that is not explicitly included in the quantum model, solvent effects were introduced as B3LYP/6-311++G** single point computations on the optimized gas phase structures, in the framework of Self Consistent Reaction Field Polarizable Continuum Model (SCRF-PCM) using the IEF-PCM model, as implemented in Gaussian 03, in which the cavity is created via a series of overlapping spheres. This is a simple model for treating long-range solvent effects and considers the solvent as a macroscopic continuum with a dielectric constant and the solute as filling a cavity in this continuous medium. United Atom Topological (UA0) Model applied on atomic radii of the UFF force field was used to build the cavity, in the gas-phase equilibrium geometry. The dielectric constant of the protein is the main empirical parameter of the model, and it was chosen to be equal to 4, in line with previous suggestions for proteins (*24*−*36*). This value corresponds to a dielectric constant of about 3 for the protein itself and of 80 for the water medium surrounding the protein.

The binding energies (BE) were computed as in protein-like environment B3LYP/6-311++G** single point energy computations on the optimized geometries of the inhibitor (brutieridin/melitidin and simvastatin), of the model active site, and of both inhibitor−enzyme complexes:

$$BE = E(\text{complex}) - [E(\text{inhibitor}) + E(\text{active site})]$$

RESULTS AND DISCUSSION

Simvastatin−HMGR Complex. Crystal structures of HMGR in complex with statins show that the orientation and bonding

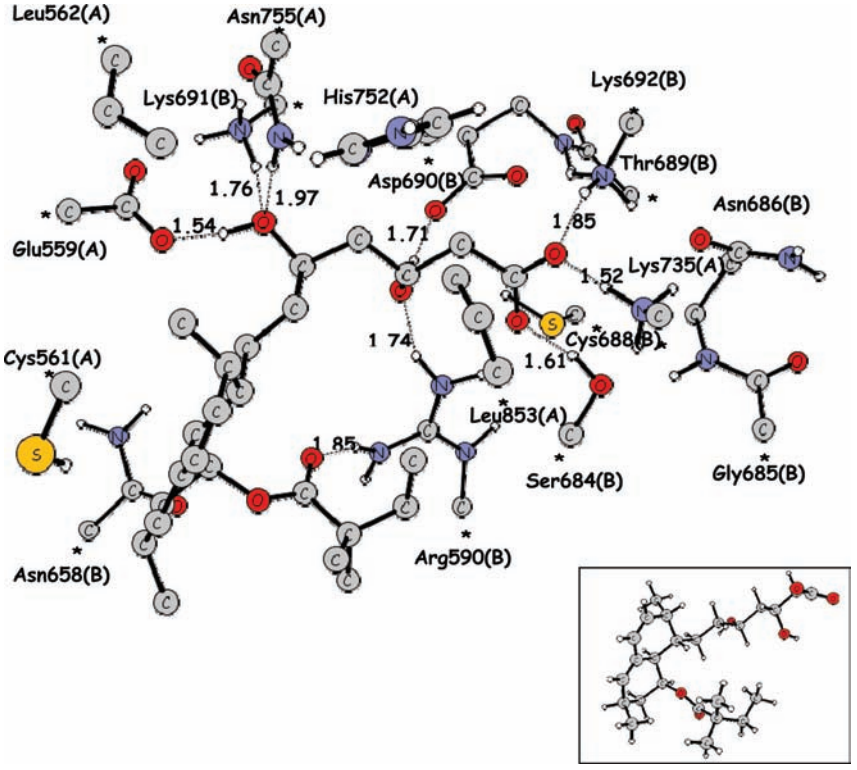


Figure 1. B3LYP optimized geometry of the simvastatin−HMGR complex. For clarity, unimportant hydrogen atoms are omitted. Inset: the geometry of simvastatin.

interactions of the HMG moieties of the inhibitors resemble those of the HMGCoA substrate (*38*). Crystal structures clearly indicate that the linear forms of statins are predisposed to bind to the enzyme. The hydroxy acid side chain is the most flexible part of the drug molecules. Because of the structural similarity to the HMG moiety, it is mainly responsible for the competitive inhibition of human HMGR.

Several dipole/dipole interactions and hydrogen bonds are used by statins to bind to the active site of the enzyme, focusing on residues in the cis loop (Ser684, Asp690, Lys691, and Lys692), with minimal involvement of the hydrophobic interactions. Lys691 is suggested to be involved in a hydrogen bond with the O5-hydroxyl of the statins, while the terminal carboxylate of the HMG moiety seems to establish a salt bridge to Lys735. Hydrophobic side chains of the enzyme involved in contact with statins were identified to be Leu562, Val683, Leu853, Ala856, and Leu857 residues (*38*).

The B3LYP optimized structure of the complex between simvastatin and the HMGR active site model cluster is illustrated in the **Figure 1**. The minimum energy structure is characterized by several hydrogen bonds as well as hydrophobic interactions.

The rigid hydrophobic naphthalene ring of simvastatin rearranges to maximize nonpolar interactions which are established with Cys561 and Leu562, and with Leu853 also through the methyl butyrate ester moiety. The binding mode of this portion of the molecule is also characterized by the presence of a H-bond (1.85 Å) between the positive NH_2 group of Arg590 and the carbonyl oxygen atom of the simvastatin ester moiety.

The terminal carboxylic group of simvastatin gives rise to strong hydrogen bonds. One carboxylic oxygen of the drug is involved in a H-bond with the −OH of Ser684 (1.61 Å). The other oxygen establishes two strong hydrogen bonds, with the amino groups from Lys735 (1.52 Å) and Lys692 (1.85 Å). The original salt bridge (*21*) involving the negative COO^- of simvastatin and the NH_3^+ of the lysine residue is retained during the optimization procedure.

The hydroxyl attached on C_3 in simvastatin forms two hydrogen bonds with the oxygen atom coming from Asp690 (1.71 Å) and with the NH_2 group of Arg590 (1.74 Å). The C_5-OH, replacing the thio-ester oxygen atom found in the HMG-CoA substrate, acts as a H-bond donor with Glu559 (1.54 Å) and as an acceptor with Lys691 (1.76 Å) and Asn755 (1.97 Å).

This bonding network obtained through B3LYP computations is in good agreement with that observed in the experimental crystal structure of the simvastatin−HMGR adduct (*21*). The statin is positioned approximately in the HMG-CoA binding site, thus not occupying the NADP(H) binding pocket. In particular, the H-bonds involving the hydroxyls attached to C3 and C5 as well as those involving the carboxylic moiety in simvastatin are quite well reproduced, with a deviation of ∼0.3 Å.

The interactions between the HMG-like moieties of the simvastatin and the active site of the enzyme are mainly electrostatic involving numerous hydrogen binding connections similar to those formed with the HMG-CoA substrate. The bulky hydrophobic part, indeed, occupies the HMG-binding pocket and part of the binding surface for CoA so that the access of the substrate HMG-CoA to HMGR is blocked when the statin is bound.

The binding energy in a protein-like environment is computed to be 101.1 kcal/mol, indicating that the binding of the drug is thermodynamically favored, further contributing to the inhibition process.

Brutieridin/Melitidin−HMGR Complex. The B3LYP optimized structure of the complex between brutieridin/melitidin and the HMGR active site model cluster is depicted in **Figure 2**. Most of the noncovalent bonds found for the binding of simvastatin to the HMGR active site are involved in the formation of the complex between brutieridin/melitidin and the HMGR enzyme. However, the starting geometry of the brutieridin/melitidin−HMGR complex for the QM computations is slightly different with respect to the simvastatin−enzyme one as far as the Ser684 residue is concerned. Particularly, upon MD simulations, the

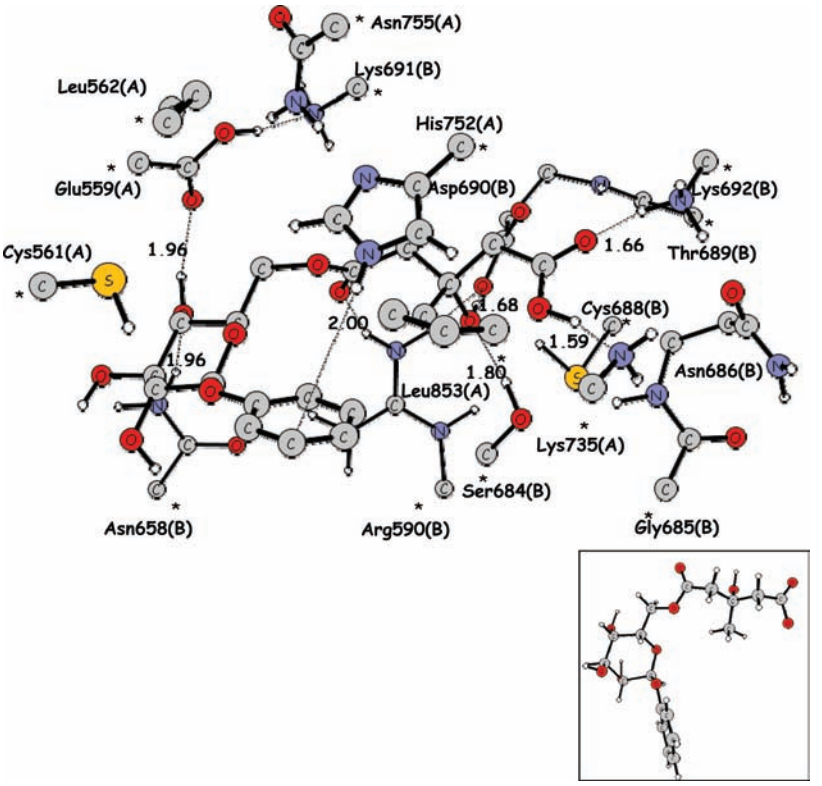


Figure 2. B3LYP optimized geometry of the brutieridin/melitidin−HMGR complex. For clarity, unimportant hydrogen atoms are omitted. Inset: the geometry of brutieridin/melitidin.

Ser684 OH points toward the hydroxyl at the C3 and not to the carboxylic moiety of the statin-like molecule. This will cause some differences in the binding of the brutieridin/melitidin molecule in the HMGR active site when compared to the behavior of simvastatin.

Lys735 and Lys692 form two H-bonds with the terminal carboxylic group of the brutieridin/melitidin molecule (1.59 and 1.66 Å, respectively). It is worth noting that during the optimization procedure, the salt bridge involving the negative COO^- of brutieridin/melitidin and the NH_3^+ of Lys735 is removed, leading to both neutral COOH and NH_2 groups. It is possible, however, that this proton transfer from Lys735 is an artifact of the relative smallness of the employed HMGR model and that it might not occur when more parts are included that can provide proper solvation to the COO^-−Lys^+ ion pair. In the former simvastatin−enzyme complex, the COO^-−Lys^+ couple remains unprotonated probably because of the presence of the Ser684 hydroxyl that stabilizes the negative charge on the carboxylate. The C_3-OH interacts with the oxygen coming from Asp690 (1.68 Å) and with the OH group coming from Ser684 (1.80 Å).

The ester moiety of brutieridin/melitidin, which replaces the C_5-OH of simvastatin, is responsible for a different arrangement of this compound with respect to simvastatin. The carbonyl oxygen receives an H-bond from Arg590 (2.00 Å), but no interaction with Glu559 occurs. The latter in fact establishes a strong polar interaction with the amino group of Lys691 so that a spontaneous proton shift from the positive NH_3^+ to the negative COO^- takes place.

The sugar moiety rearranges itself to establish H-bonds through its hydroxyl groups with polar residues in the active site. Particularly, the hydroxyl attached to the C3′ in the sugar ring donates a H-bond to the oxygen of Glu559 (1.96 Å) while receiving one from the side chain of Asn658 (1.96 Å).

The aromatic ring of brutieridin/melitidin establishes van der Waals interactions with the nonpolar Leu562 and Leu853. The imidazole ring of His752 approaches the phenyl ring of the brutieridin/melitidin molecule in such a way that one hydrogen of the imidazole ring points toward the π electrons of the aromatic ring (see **Figure 2**).

One has to remember that the phenyl ring is just a crude representation of the flavanone moiety of brutieridin/melitidin, which thanks to the presence of multiple OH groups could establish H-bonds with the active site of the enzyme. This binding mode is absent in statins, which possess rigid hydrophobic substituents that presumably should mimic the nicotinamide ring of NADP(H) (*39*).

The B3LYP binding energy in the protein-like medium is computed to be 90.8 kcal/mol. This value is comparable to the one exhibited by simvastatin and may indicate that brutieridin/melitidin is a good candidate in performing the binding to the active site of HMGR.

Brutieridin/melitidin seems to invade the HMG-binding site and partially occupies the site accommodating the CoA part of the substrate, like that occurring in the simvastatin binding mode. Electrostatic interactions are predominant in brutieridin/melitidin binding to the HMGR active site, while in the case of the simvastatin−HMGR complex, a certain amount of van der Waals bonds is also present as far as the hydrophobic decalin ring is concerned.

In conclusion, we have investigated by means of density functional theory the binding of new bergamot fruit containing flavonoids conjugates, brutieridin and melitidin, to a simplified model of the active site of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Their structure closely resembles that of cholesterol inhibitor drugs, statins. The calculations have allowed us to investigate the geometrical features affecting the bond to the active site and to compute the protein-like binding energy. The behavior of the excellent HMGR inhibitor simvastatin has been also studied.

The binding mode of the bergamot fruit containing statin-like molecules to the HMGR active site involves the Arg590, Ser684, Asp690, Lys692, and Lys735 residues, as well as the nonpolar aminoacids. The terminal carboxylic group of the brutieridin/melitidin molecule is involved in two H-bonds with the amino groups of Lys735 and Lys692. The C_3-OH interacts with the oxygen coming from Asp690 and with the OH group coming from Ser684 (1.80 Å).

The ester moiety of brutieridin/melitidin, which replaces the C_5-OH of simvastatin and resembles the thio-ester of the CoA substrate, receives an H-bond from Arg590, while no interactions with Glu559 are found. The aromatic ring of brutieridin/melitidin establishes van der Waals interactions with the nonpolar Leu562 and Leu853, and a H-bond-like interaction with the hydrogen atom coming from the imidazole ring of His752. This binding mode is absent in statins, which possess rigid hydrophobic substituents.

Consistent with the presence of the HMG-like moiety, brutieridin and melitidin compounds seem to be competitive inhibitors of HMG-CoA reductase with respect to the binding of HMG-CoA. Computations of binding energies in the protein-like medium indicate that brutieridin and melitidin compounds are good candidates to perform the inhibition of HMG-CoA reductase in vivo.

These findings support the experienced anticholesterolemic activity of the new drug candidates (*40*) and also the recent results about the in vitro inhibition of HMG-CoA reductase performed by isoflavones (*41*). Light on the prescription of local folk medicine indicating bergamot juice as a natural product that lowers blood cholesterol levels is thrown. This study may be the starting point for bergamot exploitation in drug industry applications.

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Supporting Information Available: Root-mean-square deviation (rmsd) of the protein structure during MD simulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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