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Molecular docking, synthesis and biological evaluation of ergosteryl-ferulate as a HMG-CoA reductase inhibitor

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Abstract: In the present study, ergosteryl-ferulate (**5**), oryzanol analog was evaluated for its possibility as the inhibitor of HMG-CoA reductase (HMGR), through *in silico* and *in vitro* approach. Firstly, the study was conducted through molecular docking simulation using AutoDock Tools software to predict the interaction of **5** in complexes with HMGR. In addition, four major compounds of oryzanol (**1**-**4**) were employed as a comparison. Secondly, **5** was synthesized through esterification using thionyl chloride as an activator. Lastly, **5** was evaluated for its capacity to inhibit HMGR activity using HMGR assay kit. Molecular docking simulation results suggest that oryzanol (**1**-**4**) and **5** exhibited a binding affinity against HMGR. The activity of **5** was predicted to be the best among the oryzanol compounds (**1**-**4**), in which, the free binding energy and inhibition constant were -4.17 kcal/mol and 0.88mM. The *in vitro* assay showed that **5** had inhibitory activity against HMGR 1.93 times higher than oryzanol. In summary, **5** has more potential candidates for HMGR inhibitor than oryzanol.

Keywords: Docking, ergosteryl-ferulate, esterification, HMG-CoA reductase, oryzanol.

INTRODUCTION

Oryzanol is an attractive substance isolated from the outer layer of rice, also known as rice bran. At the beginning of the discovery, oryzanol was presumed as a single compound. Later, by the development of the separation technique, it was known to be a mixture of ferulate esters and phytosterols or triterpene alcohols (Patel and Naik, 2004). The oryzanol fraction which has been identified consists of at least 10 compounds (Xu and Godber, 1999). Four of them are ferulic acid esters with 24-methylenecycloartanol, cycloartenol, campesterol, and sitosterol, which are the major components with the proportion of 37.6%, 31.5%, 17.0% and 8.4%, respectively (Mäkynen *et al.*, 2012).

Oryzanol has been recognized to have several biological activities, such as inhibit tumor promotion (Yusukawa *et al.*, 1998), reduce total serum cholesterol (Wilson *et al.*, 2007), prevent cardiorenal metabolic syndrome (Francisqueti *et al.*, 2017), neuroprotection and improvement cognitive behavior (Jha and Panchal, 2017), anxiolytic effects (Akter *et al.*, 2019), inhibit hepatic fat accumulation and anti-inflammation (Wang *et al.*, 2015). Previous reports have proposed several mechanisms of oryzanol in reducing total serum cholesterol (Bhaskaragoud *et al.*, 2016; Mäkynen *et al.*, 2012). One of which is the inhibitory activity against HMGR, an important enzyme controlling the early steps of cholesterol biosynthesis and the molecular target of statins.

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Unfortunately, oryzanol is present very low level in rice bran. Its presence in crude rice bran oil varies between 1.7 -2.1% (Patel and Naik, 2004). These conditions encourage the researchers to obtain a single compound of oryzanol or its analogs in large quantity through enzymatic (Schar and Nystrom, 2016) and chemical synthesis (Begum *et al.*, 2016; Winkler-moser *et al.*, 2015; Condo *et al.*, 2001). Most of the works reported were related to their antioxidant activities. Therefore, we are interested to synthesize analog of oryzanol by utilizing the steroid compound that is relatively easy to obtain at large quantity and to test their hypocholesterolemic activity. The present study was carried out by evaluating the possibility of the inhibitory role of ergosteryl-ferulate (**5**) against HMGR, through *in silico* and *in vitro* approaches.

MATERIAL AND METHODS

In Silico Study

Macromolecule and ligand preparation

The HMGR macromolecule was acquired from the data bank (www.rcsb.org), with PDB ID 1HW9. The X-ray crystal structure of HMGR was in complex form with simvastatin acid (native ligand), 2.33 Å resolution, which consists of four chains (chain A, B, C, and D). Auto Dock Tools 1.5.6 (ADT) software was used for macromolecule and ligands preparation. The macromolecule preparation was conducted by separating two chains (A and B) in the structure, removing H_2O and native ligands, adding polar hydrogen atoms and Kollman charges. Four oryzanol compounds and one oryzanol analog were used as the ligand in the present study (fig. 1). The Ligand structures were prepared by Chem Bio Draw Ultra 12.0 and

optimized by ChemBio3D Ultra 12.0 using MM2 and AM1. The optimized structures were prepared by addition of all hydrogen atoms, computing Gestiger charges and finally merging non-polar hydrogen atoms. The structure-based property prediction for the optimized structure was determined at www.chemicalize.com.

Molecular docking

The molecular docking calculation was performed by ADT software. All molecular docking was set at number of points dimension 60 x 60 x 60 with 0.375 Å spacing and center of grid box was set for x = 3.981; y = -9.204; and z = -11.326. The docking parameters were carried out by default parameters contained in ADT software. The calculation was performed using the Lamarckian Genetic Algorithm for 100 GA run. This process was iterated for each ligand. The interaction between ligand-macromolecule was analyzed and visualized by ADT software and Biovia Discovery Studio V.16.1.0.15350 software. Free binding energy (ΔG), inhibition constant, and amino acid residues were observed.

Synthesis of Ergosteryl-Ferulate

General materials

All chemicals and solvents used were an analytical grade, purchased from TCI, Merck, WAKO, and JT-Baker. The alcohol-free chloroform was prepared by washing chloroform with water and dried with sodium sulfate. Anhydrous solvents were dried by refluxing in the presence of sodium sulfate. Sodium methoxide solution was prepared by careful addition of sodium metal into methanol. All reaction steps were monitored by thin layer chromatography. The visualization was done under UV light and the specific spray reagent. Melting points were measured by Electrothermal (Thermo Fisher®) and the results were uncorrected. The NMR spectra of ^{1}H (500 MHz) and ^{13}C (125 MHz) were recorded on Agilent® series DD2 console spectrometer with a suitable solvent.

Acetylation of trans-ferulic acid

The hydroxyl group of *trans*-ferulic acid was protected by acetylation according to reported methods with slight modification (Condo *et al.*, 2001; Shimizu and Kojima, 1984). 0.50g *trans*-ferulic acid (2.60mmol) was dissolved in 5ml pyridine and 1g acetic anhydride (9.80mmol). Then the mixture was stirred and left for 24 h in the dark at room temperature. The solution was then poured into 200ml of cool water and crystallization was left to proceed overnight. The yellowish precipitate was filtered, rinsed with water, then dried. The *trans*-4-*O*-acetylferulic acid was obtained 0.55g (89.63%).

Yellowish solid, m.p. 194.4-198.0 °C. ^{1}H-NMR (DMSO-d_6, 500MHz) ppm: 7.48 (1H, d, *J*=1.5Hz, H-2); 7.12 (1H, d, *J*=8Hz, H-5); 7.26 (1H, dd, *J*=1.5 & 8 Hz, H-6); 7.58 (1H, d, *J*=16Hz, H-1'); 6.58 (1H, d, *J*=16Hz, H-2'); 3.82 (3H, s, H-4'); 2.26 (3H, s, H-6'); 12.38 (1H, s). ^{13}C-NMR (DMSO-d_6, 125MHz) ppm: 133.2 (C-1); 111.9 (C-2); 151.1 (C-3); 140.8 (C-4); 123.2 (C-5); 121.3 (C-6); 143.3 (C-1'); 119.5 (C-2'); 167.6 (C-3'); 55.9 (C-4'); 168.4 (C-5'); 20.4 (C-6'). The ^{1}H and ^{13}C NMR spectra were similar to the previously published structure (Ebenezer, 1991).

Preparation of trans-4-O-acetylferuloyl chloride

The *trans*-4-*O*-acetylferulic acid was activated by forming an acyl chloride according to the reported method with some modification (Shimizu and Kojima, 1984). 0.20g *trans*-4-*O*-acetylferulic acid (0.85mmol) was refluxed and stirred with 50ml thionyl chloride in dichloromethane (50mmol) on a silicone oil bath for 4 h. The excess of thionyl chloride was taken out by evaporation in a vacuum rotary evaporator to leave a yellow liquid which then crystallized in the fridge to produce 0.23g *trans*-4-*O*-acetylferuloyl chloride.

Conjugation of trans-4-O-acetylferuloyl chloride with ergosterol

The method for conjugation was described by Sukrasno (1991) with several modifications. 0.23g *trans*-4-*O*-acetylferuloyl chloride was dissolved in 20ml chloroform containing 4ml pyridine, then into the mixture, about 0.70g ergosterol (1.77mmol) was added and stirred for two days at room temperature. The mixture was subsequently washed with equal volumes of sulfuric acid 2N, sodium bicarbonate 2N and water. The chloroform fraction was collected and evaporated in a vacuum rotary evaporator. The fraction was purified using radial chromatography with silica gel GF_{254} and gradient elution using hexane-chloroform to give 52mg the product (9.41%).

White solid, m.p. 166.0-171.3 °C. ^{1}H-NMR ($CDCl_3$, 500MHz) ppm: *trans*-4-*O*-acetylferuloyl moiety 7.11 (1H, d, *J*=2Hz, H-2); 7.05 (1H, d, *J*=8Hz, H-5); 7.12 (1H, d, *J*=7.5Hz, H-6); 7.63 (1H, d, *J*=16Hz, H-1'); 6.38 (1H, d, *J*=16Hz, H-2'); 3.86 (3H, s, H-4'); 2.32 (3H, s, H-6'); ergosterol moiety 5.60 (1H, d, *J*=3.5Hz, H-6); 5.40 (1H, qui, H-7); 0.64 (3H, s, H-18); 0.98 (3H, s, H-19); 1.04 (3H, d, *J*= 6.5Hz, H-21); 5.18 (1H, dd, *J*=7.5 & 15 Hz, H-22); 5,23 (1H, dd, *J*=7 & 15.5 Hz, H-23); 0.92 (3H, d, *J*=7Hz, H-24'); 0.83 (3H, d, *J*=7Hz, H-26); 0.84 (3H, d, *J*=7Hz, H-27); 1.28-2.59 overlap. ^{13}C-NMR ($CDCl_3$, 125 MHz) ppm: *trans*-4-*O*-acetylferuloyl moiety 133.6 (C-1); 111.3 (C-2); 151.5 (C-3); 141.5 (C-4); 123.5 (C-5); 121.3 (C-6); 143.9 (C-1'); 119.0 (C-2'); 166.3 (C-3'); 56.0 (C-4'); 168.9 (C-5'); 20.8 (C-6'); ergosterol moiety 38.1 (C-1); 28.4 (C-2); 73.2 (C-3); 36.9 (C-4); 138.6 (C-5); 120.4 (C-6); 116.5 (C-7); 141.5 (C-8); 46.2 (C-9); 37.3 (C-10); 21.2 (C-11); 39.2 (C-12); 43.0 (C-13 and C-24); 54.7 (C-14); 23.1 (C-15); 28.4 (C-16); 55.9 (C-17); 12.2 (C-18); 16.4 (C-19); 40.6 (C-20); 21.3 (C-21); 135.7 (C-22); 132.1 (C-23); 17.8 (C-24'); 33.2 (C-25); 19.8 (C-26); 20.1 (C-27). The ^{1}H and ^{13}C NMR spectra were found to be identical with the reported structure (Begum *et al.*, 2016).

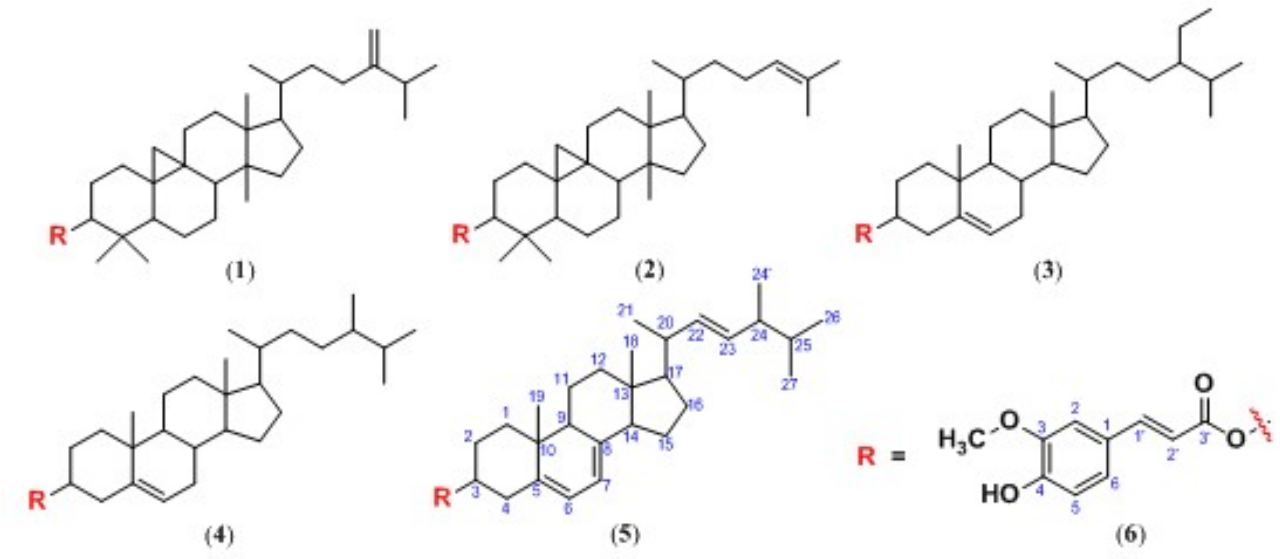

Fig. 1: The ligand structures. Structures of oryzanol compounds: 24-methylenecycloartanyl-ferulate (**1**), cycloartenyl-ferulate (**2**), sitosteryl-ferulate (**3**), campesteryl-ferulate (**4**), and the structure of an oryzanol analog: ergosteryl-ferulate (**5**). R = *trans*-ferulic acid (**6**).

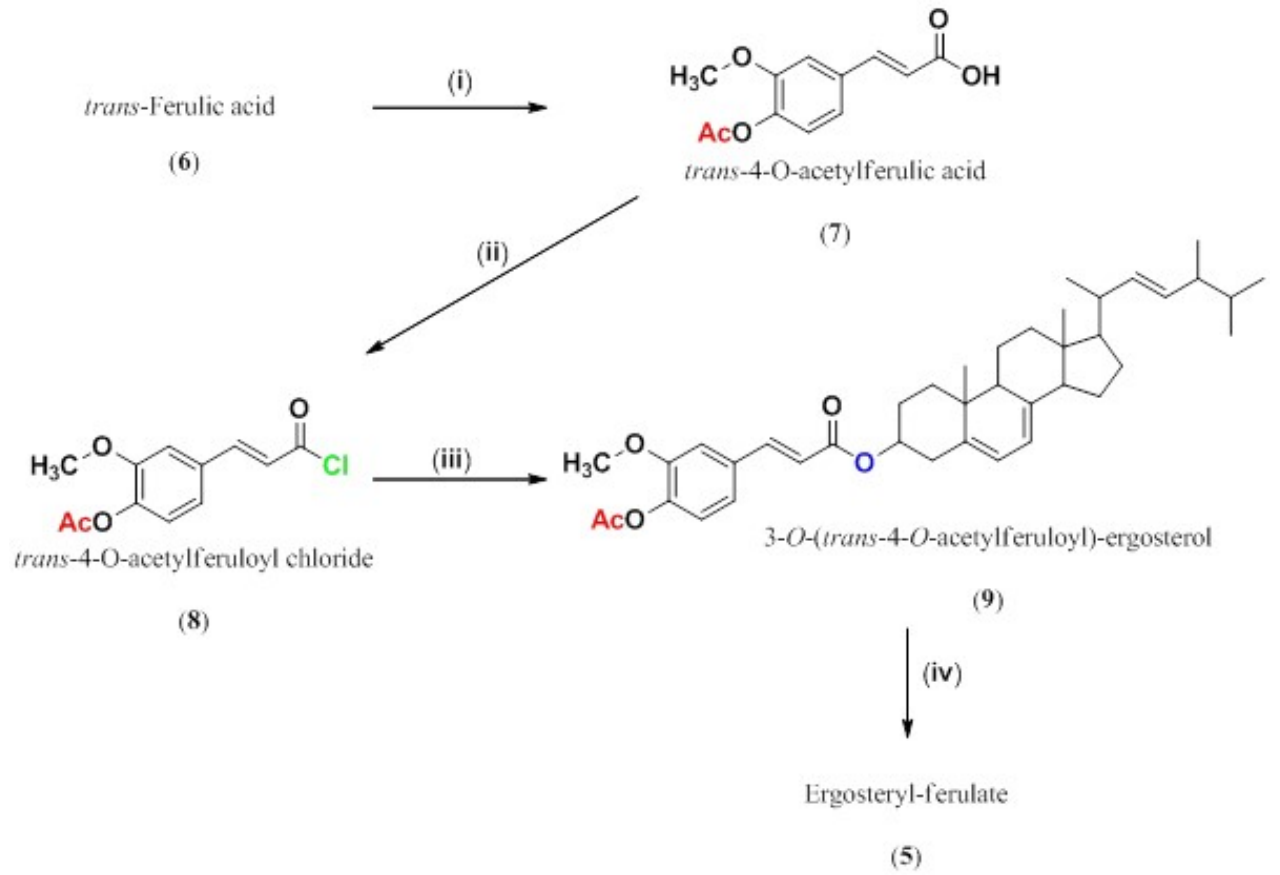

Fig. 2: The synthetic approaches of ergosteryl-ferulate. (**i**) Acetylation of *trans*-ferulic acid, (**ii**) Preparation of *trans*-4-*O*-acetylferuloyl chloride, (**iii**) Conjugation of *trans*-4-*O*-acetylferuloyl chloride with ergosterol, and (**iv**) Deacetylation of 3-*O*-(*trans*-4-*O*-acetylferuloyl)-ergosterol.

Table 1: Compositions of the HMGR assay (in µl)

Group	Assay buffer	Pravastatin	Sample	NADPH	HMG-CoA	HMGR
Blank	184	-	-	4	12	-
Activity	182	-	-	4	12	2
Inhibition	181	1	-	4	12	2
Experiment	181	-	1	4	12	2

Table 2: Chemical properties of optimized structures

Ligand	Molecular formula	Mass (g/mol)	Hydrogen bond donor count	Hydrogen bond acceptor count	Log *P*
1	$C_{41}H_{60}O_4$	616.93	1	3	10.47
2	$C_{40}H_{58}O_4$	602.90	1	3	10.12
3	$C_{39}H_{58}O_4$	590.89	1	3	10.42
4	$C_{38}H_{56}O_4$	576.86	1	3	9.97
5	$C_{38}H_{52}O_4$	572.83	1	3	9.21

Table 3: The results of molecular docking

Macromolecule	Ligand	ΔG (kcal/mol)	Inhibition constant (mM)	Amino acid residues (Hydrogen bond)
HMGR (1HW9) Chain A & B	**1**	-3.45	2.97	Glu559, Arg590, **Lys691**, His752, Asn755
	2	-3.63	2.18	Arg590, **Asp690**, Ala751
	3	-3.47	2.88	Glu559, Arg590, **Ser684**, Lys735
	4	-3.53	2.60	**Lys691**, **Lys692**, Lys735, Ala751, His752, Asn755
	5	-4.17	0.88	**Ser684**, **Asp690**, **Lys691**, Ala751, His752, Asn755
	Native ligand	-6.37	0.02	Glu559, Arg590, **Ser684**, **Asp690**, **Lys691**, **Lys692**, Lys735, Asn755

Table 4: The relative inhibition activity rate of HMGR

Sample	% Relative Inhibition Activity
Ergosteryl-ferulate (**5**)	16.17 ± 5.84
Oryzanol (TCI)	8.38 ± 3.95
Pravastatin	78.61 ± 4.56

Deacetylation of 3-O-(trans-4-O-acetylferuloyl)-ergosterol

Acetyl group was removed from ester 3-*O*-(*trans*-4-*O*-acetylferuloyl)-ergosterol according to the Sukrasno (1991) methods with several modifications. The ester of 3-*O*-(*trans*-4-*O*-acetylferuloyl)-ergosterol (52mg) was dissolved in 5ml methanol and cooled in an ice bath. 5ml of 0.1N sodium methoxide was added into the mixture and stirred for 60 minutes. Amberlite IR 120 was added as necessary whilst stirred to neutralize the solution and the solution was subsequently evaporated in a vacuum rotary evaporator. The residue was re-suspended in 10ml water, followed by three times of extraction with equal volumes of chloroform. The chloroform fraction was collected and evaporated in a vacuum rotary evaporator to give 46mg the product (94.61%).

Yellowish solid, m.p. 166.4-170.9 °C. ^{1}H-NMR ($CDCl_3$, 500MHz) ppm: *trans*-4-*O*-feruloyl moiety 7.03 (1H, d, *J*=1.5Hz, H-2); 6.91 (1H, d, *J*=8.5Hz, H-5); 7.07 (1H, dd, *J*=1.5 & 8 Hz, H-6); 7.61 (1H, d, *J*=16Hz, H-1'); 6.28 (1H, d, *J*=16Hz, H-2'); 3.92 (3H, s, H-4'); ergosterol moiety 4.84 (1H, m, H-3); 2.44 & 2.58 (2H, m, H-4); 5.59 (1H, dd, *J*=2 & 5.5 Hz, H-6); 5.40 (1H, qui, H-7); 0.64 (3H, s, H-18); 0.98 (3H, s, H-19); 1.04 (3H, d, *J*=7Hz, H-21); 5.20 (1H, dd, *J*=7.5 & 15.5 Hz, H-22); 5,22 (1H, dd, *J*=7 & 15.5 Hz, H-23); 0.92 (3H, d, *J*=7Hz, H-24'); 0.83 (3H, d, *J*=7Hz, H-26); 0.84 (3H, d, *J*=7.5Hz, H-27); 1.27-2.58 overlap. ^{13}C-NMR ($CDCl_3$, 125MHz) ppm: *trans*-4-*O*-feruloyl moiety 127.2 (C-1); 109.4 (C-2); 146.9 (C-3); 148.0 (C-4); 114.8 (C-5); 123.2 (C-6); 144.7 (C-1'); 116.1 (C-2'); 166.8 (C-3'); 56.1 (C-4'); ergosterol moiety 38.1 (C-1); 28.4 (C-2); 72.9 (C-3); 37.0 (C-4); 138.8 (C-5);

120.4 (C-6); 116.5 (C-7); 141.7 (C-8); 46.2 (C-9); 37.3 (C-10); 21.2 (C-11); 39.2 (C-12); 43.0 (C-13 and C-24); 54.7 (C-14); 23.2 (C-15); 28.3 (C-16); 55.9 (C-17); 12.2 (C-18); 16.4 (C-19); 40.6 (C-20); 21.3 (C-21); 135.7 (C-22); 132.1 (C-23); 17.8 (C-24'); 33.2 (C-25); 19.8 (C-26); 20.1 (C-27). The ^{1}H and ^{13}C NMR spectra were identic with the previously reported (Begum *et al.*, 2016).

The HMGR Assay

The HMGR assay kit was obtained from Sigma-Aldrich (CS1090), which consisted of assay buffer, NADPH, a substrate solution (HMG-CoA), HMGR and inhibitor solution (pravastatin). The test compounds were **5** and oryzanol (TCI) solubilized in a suitable solvent contains dimethyl sulfoxide and pyridine, in which, the final concentration of each was 50µg/ml (correspond to 87.41 µM and 83.06µM, respectively). The concentration of pravastatin as a standard solution was 0.5µM. The protocol assay was conducted in accordance with the technical product bulletin as shown in table 1. The absorbance was determined by Infinite® M200 spectrophotometer, with a kinetic program at 37°C and 340nm, every 20 seconds for up to 10 minutes.

The data were analyzed by plotting absorbances (at 340 nm) against time. The relative inhibition activity percentage was determined according to the following equation:

$$\% \text{ RI} = \frac{[\text{Ca} - \text{Cb}] - [\text{Ci} - \text{Cb}]}{[\text{Ca} - \text{Cb}]} \times 100\%$$

Where RI is the relative inhibition activity rate (%); Ca, Ci and Cb are the curve slope for the activity group, inhibition or experimental group and blank group, respectively.

STATISTICAL ANALYSIS

The data were expressed as mean ± standard deviation (n=3) using Microsoft Excel for Office 365 ProPlus.

RESULTS

In Silico Study

Table 2 showed the comparison of chemical properties of oryzanol structures (**1**-**4**) and ergosteryl-ferulate (**5**) with regard to Lipinski's rule, such as molecular mass, hydrogen donor, hydrogen acceptor, and partition coefficient (log *P*). Based on these data, it can be suggested that oryzanol (**1**-**4**) and **5** do not comply with the Lipinski's rule, especially for molecular mass and log *P*, since the value is above the criteria.

Prior to molecular docking calculation, the docking protocol was validated to ensure the accuracy and precision by re-docking the native ligand into the macromolecule. Simvastatin acid as the native ligand shows the root mean square deviation (RMSD) value between 0.90 to 1.07 Å with an average of 0.96 ± 0.09 Å. The RMSD value with less than or equal to 2 Å indicates that the macromolecule and method are acceptable.

The docking results (table 3) indicate that all ΔG scores of the ligands are smaller than zero and ranged from -3.45 to -4.17 kcal/mol. It means that oryzanol (**1**-**4**) and **5** have an affinity to the macromolecule and the interaction takes place spontaneously. Among the tested ligands, the best interaction energy (-4.17 kcal/mol) was exhibited by **5**. Each ligand showed the interaction with amino acid residues in cis loop area of HMGR as summarized in table 3 (shown as bold), in which, **5** interact with most member amino acid residues of HMGR compared to oryzanol (**1**-**4**).

Synthesis of Ergosteryl-Ferulate

In our study, an oryzanol analog was achieved in four steps as outlined in fig. 2. The structure of the intermediate and final target molecule was confirmed using ^{1}H, ^{13}C and 2D-NMR spectroscopy, as described in the experimental section.

The HMGR Assay

The HMGR inhibitory activity of oryzanol and synthesized compound **5** were proved in table 4. The inhibitory activity was assessed by spectrometric to measure the decrease of the absorbance, which represents the oxidation of reactant. Interestingly, **5** demonstrated 16.17 ± 5.84 % inhibition or 1.93 times higher inhibitory activity than oryzanol (TCI) with 8.38 ± 3.95 % inhibition.

DISCUSSION

In Silico Study

Theoretically, Lipinski made the criteria for a good drug, i.e., a hydrogen bond donor ≤5 a hydrogen bond acceptor ≤10, the molecular mass ≤50 and log *P*≤5 (Lipinski, 2004). Lipinski's rule stated that the above criteria are pivotal and recommended to ensure the fate of drugs in the human body. Consequently, a substance, if it has four proposed criteria of Lipinski's rule, then the process of absorption and distribution provided in orally will take place properly. Conversely, if a substance does not meet the Lipinski's rule, then it is not recommended for oral use. Based on physicochemical data properties (table 2), oryzanol (**1**-**4**) and **5** do not fulfill the rule, especially for molecular mass and partition coefficient. Therefore, oryzanol (**1**-**4**) and **5** are not suggested for orally because of Lipinski's rule. This prediction was also similar to proposed by Zhu *et al.*, (2015), who has reported that the permeation of oryzanol is very low in the intestines. On the other hand, this prediction should be supported by further research in order to improve the bioavailability.

According to ΔG scores, **5** shows more potential than oryzanol (**1**-**4**) due to its ΔG score approached the ΔG score of the native ligand. According to Kartasamita *et al.*, (2009), the assessment of molecular docking was preferred to use ΔG score, since the ΔG score was contributed by the various effects of the bonds, other electronic effects, and regressions value that occurred during the binding process. In addition, compound **5** shows interaction with amino acids in the HMGR active site (cis loop area), such as Ser684, Asp690, and Lys691. According to Istvan and Deisenhofer (2001), the binding site of HMGR is characterized by a cis loop (Ser684, Asp690, Lys691, and Lys692), an amino acid that is thought to play essential role for the inhibitory activity of HMGR. From our point of view, **5** was proposed to have better inhibitory activity than oryzanol (**1**-**4**) as a result of ΔG score and member of interacting amino acid residues. Therefore, **5** needs to be further studied to prove this prediction.

Synthesis of Ergosteryl-Ferulate

In general, there are four steps in synthesizing of **5** in the present study. The first step is the acetylation at the hydroxyl group of *trans*-ferulic acid (**6**). Phenol-carboxylic acids (e.g. **6**) cannot be used for the direct esterification since the hydroxyl group on para- position is sensitive for the esterification via nucleophilic acyl substitution (Appendino *et al.*, 2002). The protection of phenolic hydroxyl **6** was required since the activated acid (acyl chloride) cannot distinguish between hydroxyl bound to cyclic (ergosterol) or to aromatic carbon (compound **6**). The protection of **6** with acetic anhydride was produced 89.63% conversion. These results are almost the same as the report from Winkler-moser *et al.* (2015) that yielded 91.60%. The second step is the activation of the carboxylic group with thionyl chloride. This formation was carried out prior to the esterification step and intended for better yield. It could increase the electrophilic properties of the carboxylic acid and eliminate the side reaction, and therefore the esterification will occur rapidly. Moreover, the product yielded will be easy to be purified (Vogel *et al.*, 2015). In this step, the formation of *trans*-4-*O*-acetylferuloyl chloride (**8**), an acyl chloride, was not purified and characterized due to the highly reactive intermediate compound, which can be easily degraded by the water that comes from the air. According to our result, even after two days reaction, the esterification step resulted in only 9.41% of 3-*O*-(*trans*-4-*O*-acetylferuloyl)-ergosterol (**9**). This was very contrast to the previous result reported by Sukrasno (1991). Theoretically, the formation of acyl chlorides should increase the yield of product synthesis. However, our result was very contradictive by the fact that only a small amount of the conversion yielded. This condition is probably due to the influence of alcohol type from the ergosterol structure as it is a secondary alcohol. As reported by Vogel *et al.* (2015), the esterification of secondary and tertiary alcohols with acyl chloride gave only a small yield. The final step is the removal of acyl groups from the esterification product by Zemplen method using sodium methoxide (Ren *et al.*, 2015). The experimental results suggest that there was no significant problem at this step since the conversion rate to **5** is 95%.

The HMGR Assay

Based on molecular docking and biological studies, it was observed that oryzanol analog with a substituted steroid/triterpenoid structure showed better activity. In our opinion, the inhibitory activity of oryzanol on HMGR can be increased by replacing a steroid/triterpenoid structure of oryzanol with ergosterol. On the other hand, as reported by Begum *et al.* (2016), the antioxidant property of **5** was found to be better than oryzanol. This biological activity is indirectly related to the HMGR inhibitory activity since the principle of the assay is based on the oxidation of coenzyme (NADPH) by HMGR in the presence of the substrate.

As proposed by Mäkynen *et al.* (2012), the hypocholesterolemic mechanisms of oryzanol are partly due to the inhibition of cholesterol uptake and the inhibition of HMGR. Our experiment indicated that oryzanol and **5** had poor inhibitory activity against HMGR. It seems that oryzanol and **5** might not act as the inhibitors of HMGR, but probably with other mechanisms.

CONCLUSION

Based on molecular docking experiments, **5** had higher activity compared to the major of oryzanol compounds i.e. 24-methylenecycloartanyl, cycloartenyl, sitosteryl, and campesteryl-ferulate. Consistent with the *in silico* study, *in vitro* experiments on HMGR inhibitory activity, **5** also demonstrated higher inhibitory activity compared to oryzanol. In conclusion, **5** has more potential candidates for HMGR inhibitor than oryzanol.

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