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New Ergostane-Type Sterol Produced by an Endophytic Fungus *Fusarium phaseoli* Isolated from *Chisocheton macrophyllus* (Meliaceae)

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Abstract: A new ergostane-type sterol, ergost-5,22*E*-dien-3*β*-oleate-20-ol (**1**), along with four known steroids, cerevisterol (**2**), atroside (**3**), ergosterol (**4**), and ergosterol peroxide (**5**) were isolated from the solid brown rice culture of the endophytic fungus *Fusarium phaseoli*, derived from the root of *Chisocheton macrophyllus*. The structures were determined by spectroscopic methods including 2D NMR techniques, MS, and chemical derivatization. Compounds **1**-**5** were evaluated their antibacterial activities against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229, the result showed no activities.

Keywords: *Fusarium phaseoli; Chisocheton macrophyllus;* steroid; antibacterial activity;

1. Introduction

Endophytic fungi are microorganism lives in colony inside the internal tissue of plant without causing palpable damage to the host plant [1,2]. Moreover, endophytic fungi provide benefits to the

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host plant including supply of energy, nutrients [1], plant adaptation towards biotic and abiotic stress, protection against pathogen [3] and also enhanced the growth of the host plant by producing secondary metabolite required by the host plant [4,5]. Endophytic fungi known as a source of novel bioactive metabolites exhibiting potent biological activities such as antiviral, antioxidant, antibacterial, anticancer, cytotoxic, antidiabetic, immunosuppressant, and antifungal activity [6-8]. Over the past 50 years, several medicinal approved drugs were obtained from fungi [9].

Some of bioactive secondary metabolites isolated from *Fusarium* species have frequently caught the attention and attracted the interest of pharmacologist due to their ability to produce distinctive structure of secondary metabolites including alkaloid, terpenoid, limonoid, quinone, polyketide, pyranone and steroid [10,11]. Several types of steroids have been previously reported from *Fusarium* species [12,13].

Chisocheton macrophyllus is a higher tropical plant growing in the northern part of Sulawesi Island, Indonesia [14,15]. In Sulawesi, Ma-aa is known as local name of this plant species and the seed oil are used for lighting [15]. Previously the stembark and the fruit of *C. macrophyllus* yielded 13 identified limonoids [16,17]. In our continuous search for bioactive compounds from *C. macrophyllus,* a phytochemical investigation of endophytic fungus *Fusarium phaseoli* collected from the roots of *C. macrophyllus* was carried out, and a new ergostane-type steroid, ergost-5,22*E*-dien-3*β*-oleate-20-ol (**1**), along with four known steroids **2-5** was isolated. Therefore, we report the isolation and structure elucidation of compounds **1-5** along with their antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*.

2. Materials and Methods

2.1. General

IR spectra was recorded using Thermo Scientific Nicolet Summit FTIR (Thermo Fisher Scientific, Madison, WI, USA), while optical rotations were measured using an ATAGO AP-300 automatic polarimeter (ATAGO, Japan). High-resolution of mass spectra (HR-TOFMS) were determined on a Waters Xevo Q-TOF direct probe/MS system, utilizing ESI mode and micro channel plates MCPs detector (Milford, MA, USA). NMR spectra were recorded on a Bruker Topspin spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C using TMS as an internal standard (Ettligen, Germany). Moreover, column chromatography (CC) was conducted on silica gel 60 (70-230 and 230-400 mesh, Merck, Darmstadt, Germany) and octa desylsilane (Chromatorex® C_{18} DM1020 M, 200-400 mesh, Fuji Sylisia, Tokyo, Japan). Meanwhile, thin-layer chromatography (TLC) plates were precoated with silica gel GF_{254} (0.25 mm, Merck, Darmstadt, Germany) and spot detection was obtained by spraying with 10% H_2SO_4 in EtOH, followed by heating.

2.2. Fungal Material

The plant material was collected in Bogor Botanical Garden, Bogor, West Java Province, Indonesia in August 2020. The plant was identified by the staff of the Bogoriense Herbarium, Bogor, Indonesia. A voucher specimen (No. Bo-1295453) has been deposited at the herbarium. *Fusarium phaseoli* was isolated from the internal tissue of *C. macrophyllus* roots. The inner root tissues were surface-sterilized successively following method previously reported [18]. The fungus was identified by molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA by Genetika Science Indonesia (No. GMS-1264). The strain was identified as *Fusarium phaseoli* (99% similarity). For large-scale fermentation, the fresh mycelia of *Fusarium phaseoli* were cultivated on sterilized unpolished brown rice (total 6kg, 30g/flask x 200) at 28 ± 2 °C for 6 weeks

2.3. Extraction and Isolation

The rice culture was extracted with ethyl acetate (EtOAc) and concentrated under vacuum to yield EtOAc extract (242 g). The concentrated extract was mixed with 500 mL distilled H_2O and partitioned with *n*-hexane and EtOAc. The total of *n*-hexane extract (137 g) was subjected to vacuum liquid

chromatography (VLC) using a gradient elution of *n*-hexane, EtOAc and MeOH, which were separately concentrated to give eight fractions (A-H) combined according to TLC results. Fraction B (43.29 g), so was chromatographed on silica gel with a gradient elution of *n*-hexane-EtOAc (10:0 – 1:1, stepwise 2.5%) to obtain seven subfractions (B1-B7) based on TLC control. Sub-fraction B4 (452.8 mg) was then chromatographed on a column of silica gel, eluted successively with gradient of *n*-hexane-EtOAc (10:1 – 7:3, stepwise 2%) to produce six subfractions (B4A-B4F). Subfraction B4E (103.2 mg) was chromatographed on a column of silica gel, eluted with *n*-hexane:CH_2Cl_2:EtOAC (8:1:1) to yield **4** (24.2 mg), **5** (18.3 mg) and two subfractions (B4E1-B4E2). Subfraction B4E2 (58.2 mg) was then separated by column chromatography on ODS (200-400 mesh) using a solvent of MeOH:acetone (10:1) to give **1** (9.5 mg). Fraction G (2.6 g) was column chromatographed of silica gel and eluted successively with a gradient of *n*-hexane:EtOAc (5:5-0:10, stepwise 2.5%) to give ten subfractions (G1-G10). Subfraction G4 (88.3 mg) was subjected to column chromatography on ODS using MeOH:acetone (6:4) to yield **3** (7.3 mg). Subfraction G9 (736 mg) was separated by column chromatography on silica gel with a gradient elution of *n*-hexane:acetone (10:0 – 8:2, stepwise 1%) to give **2** (58.2 mg).

2.3.1. Ergost-5,22E-dien-3β-oleate-20-ol **(1)**

Pale yellow oil. $[\alpha]_D^{20}$ -28 (*c* 0.02, MeOH); IR (KBr) ν_{max} 3427, 2926, 2854 1738, 1456, 1374 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) see Table 1; HR-TOFMS (positive ion mode) *m/z* 679.6030 $[M+H]^+$ (calcd. for $C_{46}H_{79}O_3^+$, *m/z* 679.6029).

2.3.2. Steroid Moiety of **1**

White amorphorus. $[\alpha]_D^{20}$ -27 (*c* 0.02, MeOH); IR (KBr) ν_{max} 3371, 2925, 2868, 1625, 1456, 1370 cm^{-1}; HR-TOFMS (positive ion mode) *m/z* 415.3569 (calcd. for $C_{28}H_{47}O_2^+$, *m/z* 415.3576).

2.4. Transesterification of Fatty Acid Ester of Steroid

The transesterification of fatty acid esters was a modified method according to the previously reported procedures [19]. The fatty acid ester of **1** (4 mg) was respectively stirred on magnetic stirrer in dry MeOH (0.5 mL) with sodium methoxide (0.9 mg) for 5 h. The reaction product was extracted with H_2O and $CHCl_3$. The organic phase was separated, dried over Na_2SO_4 and concentrated under vacuum to obtain methyl ester of fatty acid moiety. Addition of HCl (1%) to the aqueous phase followed by extraction with $CHCl_3$ yielded steroid moiety. Transesterification of **1** yielded a fatty acid methyl ester of oleic acid (0.9 mg) and steroid alcohol moiety of **1** (0.85 mg). The steroid moiety of **1** was then purified using column chromatography to have steroid moiety (0.40 mg). The mass spectrum recorded of the transesterification product of methyl ester showed $[M+H]^+$ *m/z* 297. 2779 (calcd. for $C_{19}H_{37}O_2^+$, *m/z* 297.2794) which corresponded to the peak of methyl oleate. Meanwhile, the mass spectrum of steroid alcohol moiety of transesterification product showed $[M+H]^+$ *m/z* 415.3569 (calcd. for $C_{28}H_{47}O_2^+$, *m/z* 415.3576).

2.5. Antibacterial Activity

The *in vitro* antibacterial activities of compounds **1-5** were tested against the Gram-positive bacteria *S. aureus* ATCC 6538 and Gram-negative bacteria *E. coli* ATCC 11229 according to the procedures previously reported [20]. Bacterial culture was cultivated in agar medium (Mueller Hinton agar) by inoculating bacterial colony, continued with incubation at 37°C for 24 h. Bacterial culture was suspended in liquid media (Mueller Hinton Broth) and then incubated at 37°C for 24 h. The suspended bacterial culture was compared equal to 0.5 Mc Farland standard (2×10^8 CFU mL^{-1}). The culture was diluted into the concentration of 5×10^5 CFU mL^{-1}. Five compounds with a concentration of 1 μg/mL were dissolved in DMSO 2%. The percentage of microbial inhibition and cell death was used to determine the MIC.

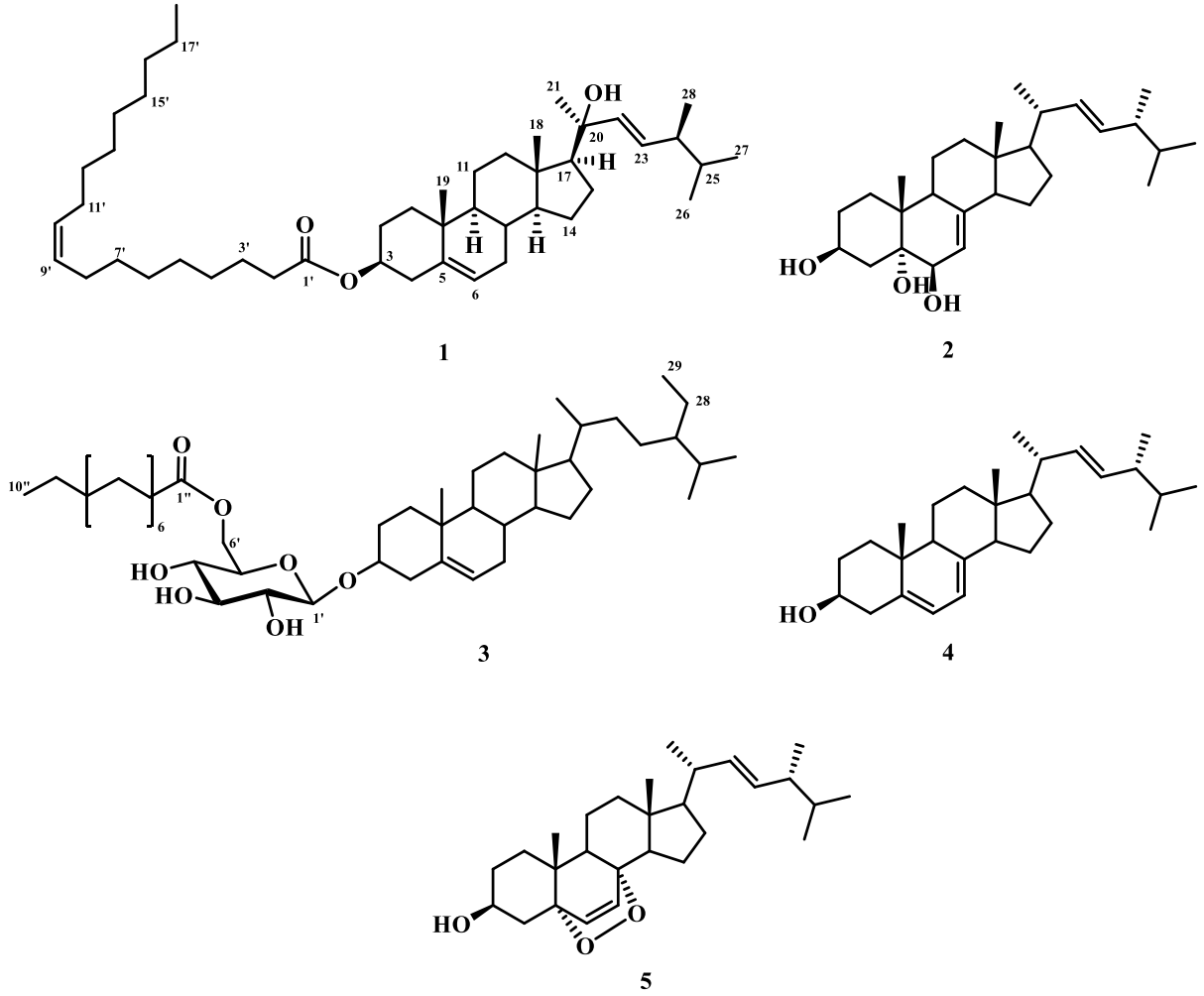

Figure 1. Structures of Compounds **1**-**5**

3. Results and Discussion

3.1. Structure Elucidation

The structure elucidation of fatty acid substituted steroid by NMR is only possible by analysis of ^{13}C, DEPT and 2D NMR experiment due to ^{1}H NMR have predominance peak of methylene groups of fatty acid at δ_H 1.2-1.3 ppm. Furthermore, the fatty acid contents can be identified using mass spectra analysis of their methyl esters product through transesterification reaction [19].

Compound **1** was obtained as a pale-yellow oil. The molecular formula was determined as $C_{46}H_{78}O_3$ on a basis of the HR-TOFMS ion peak at *m/z* 679.6030 $[M+H]^+$ (calcd. for $C_{46}H_{79}O_3^+$, *m/z* 679.6029) from positive-ion-high-resolution measurements, indicating eight degrees of unsaturation originating from three pairs of C sp^2, C ester from fatty acid moiety, and the remaining tetracyclic ergostane-type steroid. The IR spectra showed absorption peaks which implies the existence of hydroxyl (3427 cm^{-1}), aliphatic (2926 cm^{-1} and 2854 cm^{-1}), carbonyl (1738 cm^{-1}), alkene (1456 cm^{-1}), and *gem*-dimethyl groups (1374 cm^{-1}). The ^{1}H NMR spectrum displayed proton resonance related to three tertiary methyls (δ_H 0.53, 0.99 and 0.99, each 3H), three secondary methyl (δ_H 0.79, 0.80 and 0.89, each 3H), and huge signal at δ_H 1.22-1.26 suggested the presence of fatty acid moiety. Three olefinic methine group resonating at δ_H 5.31 (1H, m, H-6), δ_H 5.18 (1H, m, H-23), and two doublets at δ_H 5.14 (d, *J* = 17.0, H-22) and δ_H 5.18 (d, *J* = 17.0, H-23) were typical for olefinic proton of ergosterol

derivatives. The ^{13}C NMR spectrum of **1** together with the DEPT-135 spectrum revealed the presence of six methyl groups, three olefinic methine, one olefinic quaternary carbon, which confirmed the C_{28}-ergostane-type steroid skeleton substituted with fatty acid in ester linkage. The spectral data of steroid core structure of **1** was similar to leucisterol isolated from *Leucas urticifolia* [21] except the presence of fatty acid attached at C-3 (δ 73.7) in ester form. The mass spectrum of the transesterification products of **1** allowed the identification of methyl oleate $[M+H]^+$ *m/z* 297. 2779 (calcd. for $C_{19}H_{37}O_2^+$, *m/z* 297.2794) and the steroid core moiety $[M+H]^+$ *m/z* 415.3569 (calcd. for $C_{28}H_{47}O_2^+$, *m/z* 415.3576). The IR spectra of steroid core moiety of **1** showed absorption peaks which implies the existence of hydroxyl (3371 cm^{-1}), aliphatic (2965 cm^{-1} and 2865 cm^{-1}), olefinic (1625 cm^{-1}), alkene (1456 cm^{-1}), and *gem*-dimethyl groups (1370 cm^{-1}). The IR spectrum support the loss of carbonyl group of **1** replaced with signal of olefinic carbon with medium band absorption at 1625 cm^{-1}. The ^{13}C NMR spectrum provided additional supporting evidence, with typical signals at δc 173.3 (C-1'), 34.5 (C-2'), 24.8 (C-3'), 27.2 (C-8' and C-11'), 130.0 (C-9') and 130.2 (C-10') for the oleate moiety with (*Z*)-configuration [22]. The characteristic for the allylic C-8' and C-11' were typical for *cis*-olefins than *trans*-olefins [23]. The position of fatty acid was determined by HMBC correlation (Figure 2) which clearly showed the correlation peak between 3α-H (δ_H 5.24) and C-1' (δc 173.3). The steroid core was determined by a detail analysis of the COSY and HMBC data (Figure 2), and the configurations of the sterol ring system was deduced from the NOESY correlations (Figure 3). In addition, the coupling constant of C-22 at δ_H 5.14 (d, *J* = 17.0 Hz) was consistent with a *trans*-configuration between C-22 and C-23. No NOESY correlation between H-19/H-3 and the existence of cross peak correlation between H-3/H-9 confirmed the position of 3*β*-oleic substituent. Originated from brassicasterol, no correlation between H-21 and H-28 confirmed the different position of H-21*α* and H-28*β*. From all of data mentioned above, the structure of **1** was determined as ergost-5,22*E*-dien-3*β*-oleate-20-ol.

The known compounds cerevisterol (**2**) [24], atroside (**3**) [25], ergosterol (**4**) [26], and ergosterol peroxide (**5**) [27] were confirmed by comparison and biogenetic analysis of these compounds with previously reported data.

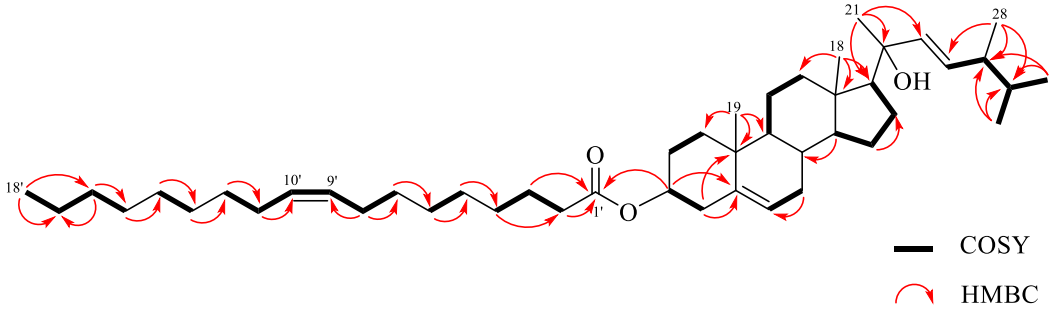

Figure 2. Selected HMBC and ^1H-^1H COSY correlations for **1**

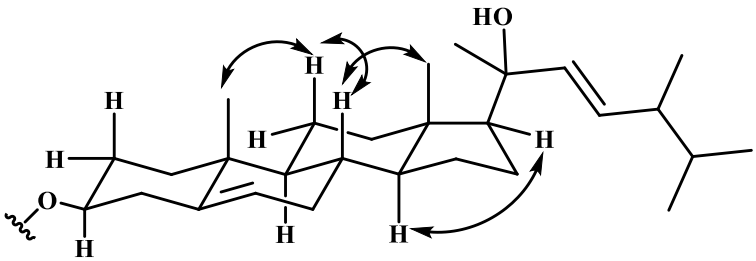

Figure 3. Selected ^1H-^1H NOESY correlations of **1**

Table 1. NMR data of **1** in $CDCl_3$ (δ in ppm, 500 MHz for ^1H and 125 MHz for ^{13}C)

Position	^{13}C NMR δc	^1H NMR δ_H (Int., mult., *J*=Hz)
1	30.8	1.82 (2H, m)
2	31.5	1.66 (1H, m)
		1.52 (1H, m)
3	73.7	5.24 (1H, m)
4	39.5	1.89 (1H, m)
		1.49 (1H, m)
5	144.2	-
6	127.9	5.31 (1H, m)
7	31.4	1.64 (1H, m)
		1.54 (1H, m)
8	43.4	2.07 (1H, m)
9	40.3	1.99 (1H, m)
10	38.9	-
11	22.5	1.27 (2H, m)
12	39.2	2.03 (1H, m)
		1.28 (1H, m)
13	43.8	-
14	54.7	1.89 (1H, m)
15	28.0	1.71 (2H, m)
16	31.8	1.24 (2H, m)
17	55.8	1.26 (1H, m)
18	12.2	0.53 (3H, s)
19	17.9	0.99 (3H, s)
20	75.1	-
21	21.1	0.99 (3H, s)
22	135.4	5.14 (1H, d, 17.0)
23	132.1	5.18 (1H, d, 17.0)
24	42.8	1.82 (1H, m)
25	33.0	1.43 (1H, m)
26	19.6	0.79 (3H, d, 7.2)
27	19.9	0.80 (3H, d, 7.2)
28	17.5	0.89 (3H, d, 6.9)
1'	173.3	-
2'	34.5	2.33 (2H, t, 7.5)
3'	34.0	2.28 (2H, m)
4'	24.8	1.58 (2H, m)
5'-7'	29.1 - 29.7	1.22-1.27 (6H, m)
8'	27.2	1.98 (2H, m)
9'	130.0	5.32 (1H, m)
10'	130.2	5.35 (1H, m)
11'	27.2	1.98 (2H, m)
12'-15'	29.1 - 29.7	1.22-1.27 (8H, m)
16'	31.9	1.22 (2H, m)
17'	22.6	1.27 (2H, m)
18'	14.8	0.86 (3H, t, 7.0)

All compounds (**1**-**5**) were tested for their antibacterial activities against two strains of bacteria, including *S. aureus* ATCC 6538 and *E. coli* ATCC 11229. The result has shown no antibacterial activities between these two bacteria. However, the crude extract has antibacterial activities (eliminate more than 95%) against *S. aureus* ATCC 6538 and *E. coli* ATCC 11229 with 0.156% and 0.313% concentration (w/v) respectively. No antibacterial activity from all isolated compounds (**1**-**5**) compared to the extract might related to synergistic effect of other constituents.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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