Active Compound from An Indonesian Endophytic Fungus \textit{Talaromyces veruculosus} BioMCC-f.EP.2165 Inhibiting \textit{Plasmodium falciparum} Dihydroorotate Dehydrogenase as Antimalarial Agent

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\textbf{Abstract.} An Indonesian endophytic fungus \textit{Talaromyces veruculosus} BioMCC-f.EP.2165, was cultured to find compounds that showing inhibitory activity against \textit{Plasmodium falciparum} dihydroorotate dehydrogenase (\textit{PfDHODH}). Preliminary extraction test (PET) was conducted for examination of active compounds properties in fungal culture broth. Active compounds were extracted and purified based on a method derived from PET result. We isolated altenuisn as an active compound from the fungal culture extract. Although, altenuisn has been reported previously as an inhibitor of \textit{PfDHODH} and inhibited the proliferation of \textit{Plasmodium falciparum} 3D7, we isolated this compound from endophytic \textit{Talaromyces} \textit{fungus} for the first time.

\section{1 Introduction}

Malaria remains one of major cause of illness and death in children and adults in countries were become endemic, particularly in Central and Southern Africa, as well as South-East Asia. World Health Organization reported total malaria cases in 2020 was 241 million, with death cases reached 627,000 [1]. Malaria in human is caused by a parasite from the genus \textit{Plasmodium}, and among them, \textit{Plasmodium falciparum} is responsible for most malaria deaths, become the number one killing parasite for human.

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WHO recommended artemisinin-based combined therapy (ACT) as the first-line treatment of uncomplicated *P. falciparum* malaria. However, artemisinin-resistance *P. falciparum* had been reported in Cambodia in 2008. Thus, development of new anti-malarial drug become an urgent task to ensure successful of global effort in controlling malaria [1].

Many targets for antimalarial drug development have been developed. Among them, *P. falciparum* dihydroorotate dehydrogenase (*PfDHODH*), an enzyme that involved in mitochondrial electron transfer chain, was validated as target for antimalarial drug discovery [2]. *PfDHODH* is essential in *de novo* pyrimidine biosynthesis pathway of the parasite since *Plasmodium* parasite does not have pyrimidine salvage pathway and completely depend on *de novo* pyrimidine biosynthesis pathway [3]. It has different specific sites compared to the human ortholog (*HsDHODH*) [4]. Therefore, this enzyme has become an attractive target for antimalarial drug discovery.

Several compounds have been investigated in screening, purification, identification, preclinical and clinical stages as novel selective inhibitors of *PfDHODH* including DSM265 as a significant milestone [5-7]. Most study in the discovery of promising anti-malarial drug based on chemical library screening, e.g., the screening and identification of 3,4-dihydro-2H,6H-pyrimido[1,2-c] [1,3] benzothiazin-6-imine derivatives [8].

Despite the huge study in drug discovery with the chemical library, the other choice is using a variety of unique chemical compounds produced by primary and secondary metabolism of living organisms i.e., natural products [9]. A lot of natural products, over one million, have been discovered until now. About 200,000-250,000 of these natural products are bioactive, more than 22,000 of which are produced by microorganisms [10-12]. Of the microbial bioactive, about 45% are from actinomycetes and about 38% obtained from fungi, suggesting the taxonomic importance of these two groups of microbes for drug development [9].

During our drug discovery campaign against malaria by utilizing microorganisms deposited in Biotech Center-BPPT Culture Collection (BioMCC), we screened more than 17,000 microbial extracts against target *PfDHODH*. In this study, we isolate and identify an active compound from a fungal culture extract of *Talaromyces veruculosus* BioMCC-f.EP.2165 that showed significant inhibitory activity (96.5%) from the first screening result. Altenuisin has been reported previously isolated from soil and litter *Talaromyces*, we isolated this compound from endophytic *Talaromyces* fungus for the first time.

### 2 Materials and Methods

#### 2.1 Microorganism

Fungus (*Talaromyces veruculosus* BioMCC-f.EP.2165) was isolated from a plant sample collected in Bengkulu province of Indonesia. This endophytic fungus was isolated by surface sterilization method [13-14] and was identified based on internal transcribed spacer (ITS) and 28S ribosomal DNA (28S rDNA) sequence.

#### 2.2 Fungal Strain Cultivation and Extract Preparation

One piece of agar culture disk of the pure fungal colony (taken from agar culture or frozen stock) was inoculated into 30 mL of seed culture medium (rice powder 2%, glucose 1%, soybean meal 2%, KH$_2$PO$_4$ 0.1%, MgSO$_4$,7H$_2$O 0.05%, sterilized by autoclave) in a 250 mL Erlenmeyer flask. The seed culture was incubated in a shaker incubator (220 rpm) at 28°C for 3 days. The culture was transferred into 500 mL Erlenmeyer flasks containing 100 mL of main culture medium (malt extract 1%, glucose 3%, yeast extract 2%, KH$_2$PO$_4$ 0.1%,...
glycerol 2%, dextrin 1%, NH₄NO₃ 0.1%, tryptone 0.1% & adjusted at pH 6.5), so the final concentration of seed culture in main culture was 2% (v/v). The main culture was incubated in a shaker incubator (220 rpm) at 28°C for 7 days. A total of 5L main culture broth was transferred into a 15L pan and was subjected for extraction based on Pre-extraction Test result.

2.3 Pre-extraction Test (PET)

A small volume (1-4 mL) of microbial broths was centrifuged (10,000 rpm at 4°C for 5 min) to separate the biomass and supernatant. Subsequently, the biomass was extracted with methanol (1:1). The supernatant was subjected to a stability and extraction test (Figure 1). Stability test was performed by adjusting pH of the supernatant fraction to acidic (pH 2), neutral (pH 7), or basic (pH 9) condition, before heated at 60°C for 1 h. Extraction test was performed by adjusting pH of the supernatant fraction to acidic (pH 2) or weak base (pH 8) condition, followed by extraction with ethyl acetate (1:1) or butanol (1:1), and separated solvent and water layer by centrifugation (10,000 rpm, 5 min, room temperature). All fractions were dried up in a vacuum concentrator and resolved in 100% dimethyl sulfoxide (DMSO) for enzymatic inhibitory activity measurement.

2.4 Isolation and Identification of Active Compounds

Silica gel column chromatography was carried out using Silica gel 60 (0.063–0.200 μm, Merck, Darmstadt, Germany). Supernatant dried extract (1g) was subjected to silica gel column chromatography and was eluted stepwisely using chloroform-methanol mixed solvent (compositions are as described in the text). Gel-filtration chromatography was conducted using a SephadexTM LH-20 (GE Healthcare, Uppala, Sweden). Dried fraction (100 mg) was subjected to LH-20 chromatography and was eluted using methanol. TLC analysis of extract after PET using chloroform-methanol (9:1) and chloroform-MeOH-water (8:2:0.1) as developing solvent. TLC analysis of column chromatography fractions using chloroform-methanol (9:1) and BuOH-acetic acid-water (4:1:2) as developing solvents. Preparative TLC using BuOH-acetic acid-water (4:1:2) as a developing solvent. TLC was performed on a Silica gel 60 F254 TLC glass plate (Merck, Darmstadt, Germany) and visualized under UV 254 nm. Spectra of liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) was obtained using a Xevo G2-XS quadrupole coupled with time-of-flight (QToF) mass spectrometer (Waters, Milford, Massachusetts, USA) connected with Acquity ultra-performance liquid chromatography (UPLC) systems (Waters, Milford, Massachusetts, USA).

2.5 Enzymatic Inhibition Assay

Inhibition assay of PfDHODH were carried out by monitoring the reduction of a readily observable electron acceptor 2,6-dichloroindophenol (DCIP; Sigma-Aldrich, Missouri, USA), as described previously [15-16] with some modifications. Decylubiquinone (dUQ; Cosmo Bio Co., Ltd., Tokyo, Japan) and DCIP were added in 20 ml of reaction buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100) to a final concentration of 12 μM and 120 μM, respectively. Subsequently, the recombinant PfDHODH was added into the mixture to a final concentration of 20 nM. The assay solution was gently mixed and aliquot volumes of 190 μl were transferred into a 96 well microplate that containing 2 μl of tested samples (dissolved in 100% DMSO). After incubation at 25°C for 2 min, the enzymatic reaction was initiated by adding the L-dihydroorotate (L-DHO) to a final concentration of 200 μM. The reduction of DCIP was monitored at 600 nm every 1
min for a total of 20 min, with 5s of shaking between each cycle, using a Spectramax Paradigm multi-mode micro plate reader (Molecular Device, San Jose, California, USA). The percentage of DHODH inhibition was calculated relatively to the positive and negative control, which was simultaneously prepared by replacing the test compound with DMSO and without addition of L-DHO, respectively.

Fig. 1. Scheme of preliminary extraction test (PET) (a) and activity assay result of samples from PET (b).
3 Results and Discussion

Pre-extraction test (PET) was performed to predict the chemical properties of the active compound in the active microbial extract, such as polarity, pH, and stability, so that active compound can be efficiently extracted from the extract. PET result against small volume of microbial fermentation broth showed that both supernatant and biomass of the culture broth inhibited PfDHODH enzyme activity at relatively similar level (54%~67%). Inhibitory activity of the supernatant remained after being heated at 60°C for 1h under pH 2, 7, and 9, suggested that the active compound may be stable under such conditions. When supernatant layer of the culture broth was extracted using ethyl acetate and butanol, butanol layer showed higher activity than ethyl acetate one at both pH 2 and 8 (Figure 1). From these results, targeted active compounds were accumulated in both supernatant and biomass fractions, relatively stable in a wide range pH, and can be extracted from supernatant fraction by butanol efficiently.

Based on the PET result, the supernatant and biomass of the remaining fermentation broth were extracted with butanol (1:1) and methanol (1:1), respectively, and then dried up in a centrifugal concentrator. To further examine suitable active compounds isolation method, the extracts were dissolved in methanol and subjected to silica TLC analysis. As shown in Figure 2, components in the butanol extracts could be separated better than methanolic ones. From this result, the supernatant was extracted separately from the biomass.

The supernatant was then subjected for fractionation using silica gel column chromatography by employing chloroform-methanol as mix solvent as eluent. Fractions with chloroform-methanol (CM) ratio of 8:2, 7:3 and 5:5 showed medium to high PfDHODH inhibitory activity compared to other fractions (Figure 3). Silica TLC analysis of these 3 active fractions showed fraction CM=8:2 & CM=5:5 have less spots (Figure 3). We then changed the development solvent for TLC analysis of fraction CM=8:2 to a more polar and acidic solvent. As shown in Figure 3 two bands were observed from the fraction, indicating that fraction CM=8:2 contained fewer components compared to that of fraction CM=7:3. Since fraction CM=5:5 was less active than the other 2 fractions, we focused on fraction CM=8:2 for further purification.
Fig. 3. Purification of target compound in supernatant with silica column chromatography and it’s TLC analysis. (a) \(Pf\)DHODH inhibitory activity of fractions from silica column fractionation. (b) Silica TLC of active fractions from silica column with chloroform-MeOH (9:1) under UV 254 nm, A: fraction CM=8:2, B: fraction CM=7:3, C: fraction CM=5:5. (c) Silica TLC of fraction CM=8:2 with BuOH-acetic acid-water (4:1:2) under UV 254 nm.

To isolate the two bands that appeared in the previous step, fraction CM=8:2 subjected into preparative TLC. Each band that appeared (Figure 4) was collected by scratching the silica surface and extracted using developing solvent. The inhibitory activity assay result of these fractions against \(Pf\)DHODH revealed that the band with highest retention factor (band 1) showed highest activity compared to other fractions (Figure 4). HPLC analysis result of this active fraction showed that the fraction composed from a major peak at retention time of 22 min and two minor peaks appeared near the major peak (Figure 4). This indicated that the target active compound was not purified yet.
Fig. 3. Purification of target compound in supernatant with silica column chromatography and its TLC analysis. (a) PfDHODH inhibitory activity of fractions from silica column fractionation. (b) Silica TLC of active fractions from silica column with chloroform-MeOH (9:1) under UV 254 nm, A: fraction CM=8:2, B: fraction CM=7:3, C: fraction CM=5:5. (c) Silica TLC of fraction CM=8:2 with BuOH-acetic acid-water (4:1:2) under UV 254 nm.

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Fig. 4. Purification of target compound in fraction CM=8:2 with preparative silica TLC. (a) Preparative TLC of fraction CM=8:2 visualized under UV 254 nm, (b) PfDHODH inhibitory activity of 3 bands appeared on preparative TLC, (c) HPLC profiling of band 1 of fraction CM=8:2 with gradient eluent system of 5-10-100% MeOH.

Fraction CM=8:2 was then subjected to LH-20 gel-filtration column chromatography and eluted by methanol. Each fraction was collected and subjected to PfDHODH inhibitory activity assay. The result revealed that fractions 31 to 42 showed medium to high inhibitory
activity (Figure 5). Silica TLC analysis result of these active fractions showed that there were two components in each of these fractions (Figure 5). Fractions with the highest inhibitory activity (fraction 38-39 and 40-41) were mixed and subjected to HPLC profiling analysis. As shown in Figure 6, both mixed fractions showed identical chromatogram profiles. This indicated that these fractions contained the same active compounds.

The mixed fractions were subjected to LC-MS analysis. In positive mode, the MS electropherogram of the major peak (r.t. 22.044 of the mixture of 40 & 41) showed m/z value of 291.1284. This result was closely like that of our previous finding with predicted molecular weight 290.271 and molecular formula as C_{15}H_{14}O_{6} (altenusin/Figure 7) [15]. To confirm that the isolated active compound was altenusin, both fraction sample and altenusin standard compound were analysed by TLC and HPLC. Both analysis results showed the same pattern for both samples, suggesting that the isolated active compound was confirmed to as altenusin (Figure 8). Active compound inhibited PfDHODH with IC_{50} 5.9 µM.

Altenusin has been investigated to inhibit PfDHODH and P. falciparum 3D7 cells growth [15]. Altenusin also known as inhibitor of several enzymes, including calmodulin-activated myosin light chain kinase [17], neutral sphingomyelinase [18], HIV-1 integrase [19] and trypanothione reductase of the parasitic protozoan Trypanosoma cruzi [20]. It has other activity including antifungal activity against Aspergillus fumigatus, Aspergillus niger, and
Various genera of fungi have been reported to produce altenusin, including *Talaromyces* [15, 19], *Penicillium* [17, 18, 21, 22], and *Alternaria* [20, 23].

In this study, we isolated altenusin as *Pf*DHODH inhibitors from an endophytic fungus *Talaromyces veruculosus* rotatory shaken cultured. We previously isolated altenusin from a soil fungus *Talaromyces pinophilus* static solid-state cultured [15]. Guan also isolated altenusin from a leaf litter fungus *Talaromyces flavus* rotatory shaken cultured [19], suggesting that altenusin may become a common secondary metabolite produced by fungus from *Talaromyces* genera. Cota isolated altenusin from an endophytic fungus *Alternaria* sp. rotatory shaken cultured [20].
Altenusin, which effectively inhibits PfDHODH, was successfully isolated from the culture broth of the endophytic fungus Talaromyces veruculosus BioMCC-f.EP.2165. This isolation process involved fractionation using silica gel column chromatography followed by LH-20 gel-filtration chromatography. This work highlights the significance of carefully selecting microorganisms that provide hits, such as altenusin, a commonly acquired PfDHODH inhibitor from the Talaromyces genus. This method of dereplication is crucial in drug discovery campaigns that rely on microbial isolates as a source.

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Figure 8. Confirmation of active compound compared with altenusin. a, b & c: HPLC chromatogram of active compound (a), altenusin (b), mixed of altenusin and active compound (c), 1000 ppm, eluent 5-10-100% MeCN. d: Silica TLC developed with chloroform-MeOH-water (6:4:1), visualized under UV 254 nm.
Besides altenusin, other bioactives have been reported to be isolated from *Talaromyces* culture. Phellamurin and rubratoxin B with insecticidal activity (LC$_{50}$ value of 674.87 µg/mL) against *Locusta migratoria manilensis* isolated from *Talaromyces purpureogenus* BS5 [24]. Acetylcholinesterase inhibitory activity of asterrer derivative (asterrer acid, methyl asterrer and ethyl asterrer with IC$_{50}$ values of 66.7, 23.3, and 20.1 µM, respectively) produced by *Talaromyces aurantiacus* FL15, an endophytic fungus from *Huperzia serrata* [25]. Thioester-containing benzoate derivatives with α-glucosidase inhibitory activity (eurothiocin C, eurothiocin F and eurothiocin G with IC$_{50}$ value of 5.4, 33.6 and 72.1 µM, respectively) isolated from the deep-sea-derived fungus *Talaromyces indigoticus* FS688 [26]. Sambutoxin derivatives with α-glucosidase inhibitory activity (IC$_{50}$ value was in the range of 12.6 to 57.3 µM) isolated from mangrove endophytic fungus *Talaromyces* sp. CY-3 [27].

4 Conclusion

Altenusin, which effectively inhibits PfDHODH, was successfully isolated from the culture broth of the endophytic fungus *Talaromyces veruculosus* BioMCC-f.EP.2165. This isolation process involved fractionation using silica gel column chromatography followed by LH-20 gel-filtration chromatography. This work highlights the significance of carefully selecting microorganisms that provide hits, such as altenusin, a commonly acquired PfDHODH inhibitor from the *Talaromyces* genus. This method of dereplication is crucial in drug discovery campaigns that rely on microbial isolates as a source.

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