Short Communication

Gentisyl alcohol and homogentisic acid: *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors isolated from fungi

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Two Indonesian fungi *Aspergillus assiutensis* BioMCC-f.T.7495 and *Penicillium pedernalense* BioMCC-f.T.5350 along with a Japanese fungus *Hypomyces pseudocorticicola* FKI-9008 have been found to produce gentisyl alcohol (1), which inhibits *Plasmodium falciparum* dihydroorotate dehydrogenase (PfDHODH) with an IC₅₀ value of 3.4 μM. Another Indonesian fungus, *Penicillium citrinum* BioMCC-f.T.6730, produced an analog of 1, homogentisic acid (4), which also inhibits PfDHODH with an IC₅₀ value of 47.6 μM.

Key Words: dihydroorotate dehydrogenase; inhibitor; malaria; *Plasmodium falciparum*

Malaria is one of the most prevalent parasitic infectious diseases in the world. The emergence of parasites resistant to existing drugs and the lack of diversity of antimalarial agents necessitates urgent development of drugs with novel modes of action. *Plasmodium falciparum* dihydroorotate dehydrogenase (PfDHODH) is an essential enzyme for parasite survival in the intraerythrocytic and liver stages. PfDHODH inhibitor molecules are potential targets as lead compounds for antimalarial drug

development (McCarthy et al., 2017). We have been undertaking a screening programme of microbial extracts to find new PfDHODH inhibitors. We have reported on inhibitors of PfDHODH isolated from a fungus extract in a previous paper in this journal (Pramisandi et al., 2020). Our continuing study for inhibitors has recently created several bioactive fungal fermentation extracts from Indonesian and Japanese fungi. The inhibition selectivity toward the enzyme orthologs between PfDHODH and human DHODH (HsDHODH) is an essential aspect of this screening.

The inhibition studies of both PfDHODH and HsDHODH were carried out by measuring the reduction of a readily-observable electron acceptor 2,6-dichloroindophenol (DCIP) as described previously (Baldwin et al., 2005; Pramisandi et al., 2020). Each tested sample was dissolved in 10 μl of 50% (v/v) aqueous DMSO. Decylubiquinone (dUQ) and DCIP were added in a 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 18 μM and 120 μM, respectively. The recombinant PfDHODH or HsDHODH was added into the mixture to a final concentration of 20 nM. 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 for 20 minutes using a

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Table 1. IC₅₀ values of isolated compounds.

Compound	IC ₅₀ (μM)			
	<i>Pf</i> DHODH	<i>Hi</i> DHODH	<i>Pf</i> 3D7 cells*	<i>Pf</i> DD2 cells**
1	3.4	>4,500	79	395
2	>9,700	>9,700	>600	>600
3	Unmeasurable	Unmeasurable	73	88
4	48	>190	94	109
HQNO	18	946	0.45	Not tested
Atovaquone	Not tested	Not tested	0.0002	Not tested

*Chloroquine-sensitive strain.

**Chloroquine-resistant strain.

Gentisyl alcohol (**1**) Gentisic acid (**2**) 3-Chlorogentisyl alcohol (**3**) Homogentisic acid (**4**)

HQNO

Fig. 1. Chemical structures of isolated compounds and a known *Pf*DHODH inhibitor.

microplate reader and the IC₅₀ values of the compounds were evaluated in triplicate independent experiments.

An extract of *Aspergillus assiutensis* BioMCC-f.T.7495 isolated from a soil sample collected in Ambon, Indonesia, showed potent *Pf*DHODH inhibition. The strain was cultured for 5 days at 28°C in fifty 500 ml Erlenmeyer flasks containing 100 ml of fermentation medium consisting of 2% glycerol (Univar, Illinois, USA), 1% dextrin (Merck KGaA, Darmstadt, Germany), 3% glucose (Merck KGaA, Darmstadt, Germany), 1% malt extract (HiMedia Laboratories Pvt., Ltd., Mumbai, India), 2% yeast extract (Oxoid Ltd., Hants, UK), 0.1% tryptone (Pronadisa, Madrid, Spain), 0.1% NH₄NO₃ (Sigma-Aldrich, Oakville, Canada), and 0.1% KH₂PO₄ (Univar, Illinois, USA) (pH 6.5 before sterilization). We performed a bioassay-guided fractionation of this culture broth using EtOAc extraction, a Diaion® HP-20 (Nippon Rensui Co., Tokyo, Japan) column chromatography with stepwise elution of a water-acetonitrile mixture (100:0 to 0:100), a preparative HPLC by a Waters Spherisorb® NH₂ column (Waters, Dublin,

Ireland), a preparative TLC with Silica gel 60 F₂₅₄ (Merck KGaA, Darmstadt, Germany), and finally a Sephadex™ LH-20 (GE Healthcare, Uppsala, Sweden) column chromatography by MeOH solvent to give a purified **1** (14 mg). The TLC analysis of **1** on Silica gel 60 F₂₅₄ plates (Merck KGaA, Darmstadt, Germany) with CHCl₃-MeOH (90:10) afforded a single spot visualized by spraying FeCl₃-bipyridyl EtOH solution (Emmerie-Engel's reagent) at R_f 0.27. The red color reaction with the reagent suggested the compound had a reducing property. HR-EI-MS analysis of **1** indicated the molecular formula C₇H₈O₃, with observed [M⁺] *m/z* 140.0468, compared with a calculated value of 140.0473. The ¹H NMR spectrum (500 MHz, acetone-*d*₆) showing signals at δ 4.58 (2H, s), 6.53 (1H, dd, *J* = 8.5 Hz, 2.9 Hz), 6.60 (1H, d, *J* = 8.5 Hz), and 6.76 (1H, br d, *J* = 3.0 Hz) confirmed the compound **1** is 2,5-dihydroxybenzyl alcohol (gentisyl alcohol) (Fig. 1, Ishikawa et al., 1986).

Another fungal strain *Penicillium pedernalense* BioMCC-f.T.5350, isolated from a soil sample collected in Pangandaran, Indonesia, showed inhibitory activity against *Pf*DHODH. The strain was cultured in 50 Erlenmeyer flasks (500 ml), each containing 100 ml of the same fermentation medium used for *A. assiutensis* BioMCC-f.T.7495 as described above, and incubated in a rotary shaker (220 rpm) for 5 days at 28°C. We centrifuged 5 L of the culture broth and extracted the biomass with MeOH. The concentrated extract was separated by silica gel column chromatography with stepwise elution of hexane-EtOAc-MeOH mixture. The active fractions (hexane-EtOAc (50:50) and EtOAc (100)) were combined and purified by Sephadex™ LH-20 gel filtration with MeOH elution. We isolated **1** (8.7 mg), as well as the related compound **2** (1.0 mg), from the culture mixture. The negative HR-ESI-MS analysis of **2** indicated a [M-H]⁻ signal at *m/z* 153.01831, indicating the formula C₇H₅O₄ compared with the calculated value of 153.01878. The ¹H NMR spectrum (400 MHz, DMSO-*d*₆) showing signals at δ 6.76 (1H, d, *J* = 9.0 Hz), 6.94 (1H, dd, *J* = 9.0 Hz, 3.0 Hz), 7.13 (1H, d, *J* = 3.0 Hz) and 9.12 (1H, br) and the ¹³C NMR spectrum (100 MHz, DMSO-*d*₆) showed signals at δ 112.6, 114.5, 117.8, 123.7, 149.4, 154.1, and 171.8. The MS and NMR data suggested the compound **2** was identified as 2,5-dihydroxybenzoic acid (gentisic acid). The identity was confirmed by comparison of the compound **2** with a commercial reagent of gentisic acid (Tokyo Chemical Industry, Tokyo, Japan) by TLC analysis on Silica gel 60

F₂₅₄ plates (Merck KGaA, Darmstadt, Germany) with a solvent CHCl₃-MeOH-H₂O (70:30:5), showing an R_f value of 0.20 colored by the Emmerie-Engel's reagent.

Similarly, a solid culture extract of *Hypomyces pseudocorticicola* FKI-9008, isolated from a mushroom of *Trametes versicolor* collected in Yamanashi, Japan, expressed *Pf*DHODH inhibition. We cultured the strain in a 500 ml Erlenmeyer flask containing 100 ml of seed medium consisting of 2% glucose (Wako, Tokyo, Japan), 0.2% yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 0.05% MgSO₄·7H₂O (Kanto Chemical Co., Inc., Tokyo, Japan), 0.5% hi-polypeptone (Nihon Seiyaku, Tokyo, Japan), 0.1% KH₂PO₄ (Kanto Chemical Co.), and 0.1% agar (Becton, Dickinson and Company, Le Pont de Claix, France) (pH 6.0 before sterilization). The seed culture was incubated in a rotary shaker (200 rpm) at 27°C for 3 days. Subsequently, 25 ml of seed culture was inoculated into each of four culture bags (containing 500 g of water-sodden rice with 1% (w/w) of aqueous seaweed solution). The cultures were incubated in a static solid-state at 25°C for 13 days. About 2 kg of the fungal rice culture was extracted with EtOAc. The concentrated extract was separated by silica gel column chromatography with stepwise elution of a hexane-EtOAc-MeOH mixture. We purified the active material by SephadexTM LH-20 gel filtration with EtOH elution, followed by ODS (YMC Co., Ltd., Kyoto, Japan) column chromatography with acetonitrile elution. From this culture broth we also isolated 12.3 mg of **1** and another compound **3** (15.3 mg) which showed a positive reaction to Emmerie-Engel's reagent. The structure of **3** was elucidated by HR-EI-MS and NMR experiments. HR-EI-MS analysis of **3** indicated the molecular formula C₇H₇O₃Cl, with observed [M⁺] *m/z* 174.0084, compared with the calculated value of 174.0083. The ¹H NMR spectrum (500 MHz, methanol-*d*₄) showed signals at δ 4.61 (2H, s), 6.66 (1H, d, *J* = 2.90 Hz), and 6.73 (1H, d, *J* = 2.85 Hz), and the ¹³C NMR spectrum (125 MHz, methanol-*d*₄) showed signals at δ 61.1, 114.4, 115.3, 121.8, 132.0, 144.2, and 151.7. According to the spectral data, **3** was compared to published data and identified as 3-chlorogentisyl alcohol (Kwong et al., 2006).

Another fungal solid culture extract of *P. citrinum* BioMCC-f.T.6730, isolated from a soil sample collected in Tual, Indonesia, also exhibited *Pf*DHODH inhibition. We cultured the strain using the same procedures as with *H. pseudocorticicola* FKI-9008 described above. We extracted 2 kg of fungal rice culture with 50% of EtOH. The concentrated filtrate was subjected to ODS column chromatography with a stepwise elution using a water-MeOH mixture. The active fraction (50% of MeOH) was purified by SephadexTM LH-20 gel filtration with 50% of MeOH elution to give 4.0 mg of **4**, which showed a positive reaction to Emmerie-Engel's reagent. HR-EI-MS analysis of **4** indicated the molecular formula C₈H₈O₄ with observed [M⁺] *m/z* 168.0428, compared with the calculated value of 168.0422. The ¹H NMR spectrum (500 MHz, acetone-*d*₆) showed signals at δ 3.54 (2H, s), 6.56 (1H, dd, *J* = 2.85 Hz, 8.60 Hz), 6.67 (1H, d, *J* = 8.00 Hz), and 6.68 (1H, d, *J* = 2.85 Hz), and the ¹³C NMR spectrum (125 MHz, acetone-*d*₆) showed signals at δ 36.8, 115.0, 116.7, 118.4, 123.5, 149.2, 151.0, and 173.7. According to the

spectral data, **4** was identified as 2,5-dihydroxyphenylacetic acid (homogentisic acid) (Cabras et al., 1999).

We studied the inhibition activity of gentisyl alcohol (**1**), gentisic acid (**2**), and homogentisic acid (**4**) with both *Pf*DHODH and *Hs*DHODH (Table 1). As the chlorinated compound **3** had a potent reduction property to the assay reagent DCIP prior to the initiation of enzymatic reaction, we could not evaluate the DHODH inhibitory activity of **3**.

Compound **1** inhibited *Pf*DHODH with an IC₅₀ value of 3.4 μM, whereas it showed no inhibition at 4,500 μM against *Hs*DHODH. This result indicates that **1** is a more specific and potent inhibitor of *Pf*DHODH than the positive control inhibitor, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO). Compound **2** showed no activity against both *Pf*DHODH and *Hs*DHODH at 9,700 μM. Compound **4** exhibited inhibition activity against *Pf*DHODH with an IC₅₀ value of 47.6 μM, while **4** showed no *Hs*DHODH inhibition at 190 μM. The compounds **1–4** have a common structure of 2-substituted hydroquinone. The large difference in the enzyme inhibitory activity between the compounds **1**, **2**, and **4** is considered to be due to the difference in electron donating property of the substituent to the benzene ring.

Furthermore, we investigated the anti-plasmodial activity of the isolated metabolites using two different strains. The two strains *P. falciparum* 3D7 (*Pf*3D7) and *P. falciparum* Dd2 (*Pf*Dd2) are commonly used laboratory strains, the former is chloroquine-sensitive whereas the latter is chloroquine-resistant. We used atovaquone as a positive control for *Pf*3D7 cell growth inhibitor (Table 1). Compounds **1**, **3**, and **4** suppressed *Pf*3D7 cell growth with IC₅₀ values of 79, 73, and 94 μM, respectively. Compound **3** exhibited a similar order of antiplasmodial activity against *Pf*Dd2 cells with an IC₅₀ value of 88 μM. While **1** and **4** suppressed *Pf*Dd2 cell growth with IC₅₀ values of 395 and 109 μM, respectively, **2** exhibited no suppression against either *P. falciparum* strain at 600 μM. Interestingly, **1**, which was frequently isolated from several genera of fungi with antioxidant activity (Alfaro et al., 2003; Gupta et al., 2016; Nenkep et al., 2010; Sakamura et al., 1971), has also been reported to demonstrate activity against *Leishmania donovani*, with an IC₅₀ value of 8.5 μM (Malak et al., 2014).

Compounds **1** and **4** inhibited *Pf*DHODH but not *Hs*DHODH. Their specific inhibition of the plasmodium parasite enzyme indicates that they are worth studying as lead compounds for antimalarial drug candidates. The authors believe that further research, including the screening lead compounds and structural optimization for the *Pf*DHODH inhibitors, could evolve from microbial compounds.

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