Full Paper

Microbial inhibitors active against Plasmodium falciparum dihydroorotate dehydrogenase derived from an Indonesian soil fungus, Talaromyces pinophilus BioMCC-f.T.3979

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An Indonesian soil fungus, Talaromyces pinophilus BioMCC-f.T.3979 was cultured to find novel scaffolds of Plasmodium falciparum dihydroorotate dehydrogenase (PfDHODH) inhibitors. We obtained altenusin (1), which inhibits PfDHODH, with an IC_{50} value of 5.9 µM, along with other metabolites: mitorubrinol (2) and mitorubrinic acid (3). Compounds 1 and 2 inhibited PfDHODH but displayed no activity against the human orthologue. They also inhibited P. falciparum 3D7 cell growth in vitro. Compound 3 showed little PfDHODH inhibitory activity or cell growth inhibitory activity.

Key Words: dihydroorotate dehydrogenase; malaria; Plasmodium falciparum; Talaromyces pinophilus

Introduction

Malaria remains the most life-threatening parasitic disease worldwide. Recently, the World Health Organization estimated 219 million cases and 435,000 deaths from malaria. Although immeasurably large amounts of funding have been invested globally in malaria control and elimination efforts by governments of malaria-endemic countries and international partners, the disease remains a significant global challenge and malaria eradication remains a long way off (WHO, 2018).

Concerted and innovative efforts have been made to eliminate malaria, including the development of vaccines, transmission control, and the use of preventive and therapeutic drug combinations. Pharmacological interventions to tackle the disease, aimed at various life stages of the parasite, including the asexual phase in the human host and the sexual phase in vector female Anopheles spp. mosquitoes (Flannery et al., 2013), have generally been unsuccessful. The efficacy of all existing antimalarial drugs has been hampered by the rapid appearance of resistant parasites (Singh et al., 2017). Consequently, there has been a continuing and urgent need for the development of new and innovative antimalarial agents. Seeking compounds which inhibit the metabolic enzymes essential for the survival of the Plasmodium parasite may prove to be a more successful approach.

Pyrimidines are essential precursor molecules for the synthesis of DNA, RNA, and other metabolites, such as phospholipids and glycoproteins. They are usually supplied in two ways; (1) de novo pyrimidine biosynthesis, (2) via a salvage pathway. Plasmodium parasites lack a pyrimidine salvage pathway and completely depend on de novo pyrimidine biosynthesis pathway (Sherman, 1979).

Dihydroorotate dehydrogenase (DHODH)
catalyzed the fourth and the rate-limiting step in pyrimidine de novo biosynthesis pathway by oxidizing dihydorotate into orotate and ubiquinone into ubiquinol. The reduced ubiquinol enters the mitochondrial electron transport chain and the reducing equivalents are finally transferred to molecular oxygen via complex III (quinol:cytochrome c reductase), cytochrome c and complex IV (cytochrome c oxidase). The proton pumping activity of complexes III and IV generates the electrochemical gradient which is used for ATP production by complex V (ATP synthase). Hence, DHODH is an important enzyme that contributes not only to the biosynthesis of pyrimidines, but also to cellular bioenergetics. Due to the essentiality of \( P. falciparum \) DHODH \((PfDHODH)\) for parasite survival at intraerythrocytic and liver stages, \(PfDHODH\) inhibitors are strong candidates for the treatment and prophylaxis of \( Plasmodium \) infection.

Most organisms, including human and malaria parasites, possess a DHODH enzyme. Human DHODH (\(HsDHODH\)) inhibitors are used as therapeutics for human autoimmune disease. Therefore, the inhibition specificity towards \(PfDHODH\) is an essential aspect to guide the search for \(PfDHODH\) inhibitors and possible development into antimalarial drug candidates. Importantly, X-ray structures of \(HsDHODH\) and \(PfDHODH\) have highlighted an extensive variation in the structure of ubiquinone binding site (Singh et al., 2017), thereby providing the structural basis for the identification of species-specific inhibitors.

To date, numerous \(PfDHODH\) inhibitors with various chemical scaffolds have been explored by high-throughput screening, structure-based virtual screening, and their chemical modifications (Azeredo et al., 2017; Baldwin et al., 2005; Pavada et al., 2016; Thillainayagam et al., 2018). Currently, triazolopyrimidine-based \(PfDHODH\) inhibitor (DSM265) has shown potent antimalarial activity in Phase IIa clinical trials (McCarthy et al., 2017), and the enzyme proved to be a validated target for antimalarial chemotherapy (Gujjar et al., 2009). This compound is a potent and selective inhibitor of \(PfDHODH\), exhibiting excellent \textit{in vitro} and \textit{in vivo} antimalarial activity against \(P. falciparum\), including strains that are resistant to multiple first-line antimalarial drugs. Thus, \(PfDHODH\) represents a promising target for the development of new antimalarial drugs (Llanos-Cuentas et al., 2018). As for a natural inhibitor, coptisine, an alkaloid derived from plants, was recently reported to inhibit \(PfDHODH\), with an IC\textsubscript{50} value of 1.83 \(\mu\)M (Lang et al., 2018). To the best of our knowledge, no report has yet been published of work focusing on the search for \(PfDHODH\) inhibitors derived from microorganisms.

In this study, we screened fungal and actinomycetes culture extracts to discover microbial metabolites targeting \(PfDHODH\). A fungal solid culture extract of \(Talaromyces pinophilus\) BioMCC-f.T.3979 showed an inhibitory activity against the \(PfDHODH\). We isolated several inhibitors using a bioassay-guided fractionation of an extract of the fungal culture. We elucidated the inhibition of both \(PfDHODH\) and \(HsDHODH\), the \textit{in vitro} efficacy of isolated compounds against \(P. falciparum\) cell viability, and the cytotoxicity against the human fibroblast MRC-5 cells of the obtained inhibitors.

**Materials and Methods**

**Microorganism.** Fungus strain BioMCC-f.T.3979 was isolated from a soil sample collected in Manokwari, Papua Island, Indonesia. The strain BioMCC-f.T.3979 was identified as the genus \(Talaromyces\) from its morphological characteristics. In order to determine the most closely related \(Talaromyces\) species, the DNA sequence (500 bp) of the ITS region of BioMCC-f.T.3979 was compared with the sequences in the GenBank database. From the morphological characteristics and the BLAST analysis, the strain BioMCC-f.T.3979 was identified as \(Talaromyces pinophilus\), with 98.8% similarity (Fig. 1).

**Cultivation of fungal strain.** One loop of the pure colony was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of seed medium consisting of 2% glucose, 0.2% yeast extract, 0.05% MgSO\(_4\)•7H\(_2\)O, 0.5% hi-polypeptone, 0.1% KH\(_2\)PO\(_4\), and 0.1% agar (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 3 culture bags (200 rpm) at 27°C for 13 days.

**Isolation and identification of active compounds.** Silica-gel column chromatography was carried out using Silica gel 60 (0.063–0.200 \(\mu\)m, Merck, Darmstadt, Germany). The gel-filtration chromatography was conducted using a...
Sephadex™ LH-20 (GE Healthcare, Uppsala, Sweden) and the ODS open column chromatography was performed over the YMC*Gel® ODS-A (YMC Co., Ltd., Kyoto, Japan). The optical rotation data were measured using Jasco P-2200 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The HR-ESI-MS spectra were measured on a JEOL JMS-AX505HA (JEOL Ltd., Tokyo, Japan). The $^{1}$H and $^{13}$C NMR spectra were recorded using a JEOL JNM ECP500 (JEOL Ltd., Tokyo, Japan).

**Purification of HsDHODH and P/DHODH.** HsDHODH was purified essentially as previously reported (Miyazaki et al., 2018). Codon optimized P/DHODH gene equivalent to code from amino acid residues 158 to 569 was constructed and optimized for expression in *Escherichia coli* by GenScript, Japan. The gene was amplified by PCR and cloned into pETSUMO expression vector to originate pETSUMO/P/DHODH. After confirmation of the P/DHODH sequence (Fasmac, Kanagawa, Japan), 25 pg of pETSUMO/P/DHODH was mixed with 200 µl of BL21Star™(DE3) chemically competent cell (Invitrogen, Massachusetts, USA) and transformed by a heat shock method, according to the manufacturer’s protocol. Transformed cells were cultured in 500 µl of S.O.C medium at 37°C for 1 h and 200 µl was spread into LB agar plates supplemented with 50 µg/ml of kanamycin and incubated overnight at 37°C.

A single colony of BL21Star™ (DE3) pETSUMO/P/DHODH was inoculated into 10 ml of LB medium containing 50 µg/ml of kanamycin and incubated at 37°C for 4 h with shaking at 160 rpm. Eight ml of pre-culture was inoculated into 500 ml of TB medium supplemented with 50 µg/ml of kanamycin, and incubated at 37°C rotating at 250 rpm. The OD$_{600}$ was checked every hour, and 0.2 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added when the OD$_{600}$ reached 0.4–0.6. After induction of expression by IPTG, the temperature was decreased to 20°C, and the culture was continued for additional 16 h.

All of the following purification processes were conducted at 4°C. The *E. coli* was harvested by centrifuging for 10 min at 5,000 x g. The pellet was suspended in 20 ml of lysis buffer [50 mM HEPES-KOH pH 7.6, 500 mM NaCl, 5 mM imidazole, 20% (v/v) glycerol and 0.25 mM of phenylmethanesulfonyl fluoride (PMSF)] and cells were broken by a single passage into French Press (Ohtake, Tokyo, Japan) at 180 MPa (lysat). Unbroken cells and debris were removed by centrifugation at 25,000 x g for 20 min to obtain the extract. Polidocanol (Anatomiche, Ohio, USA) was added into the extract to a final concentration of 0.05% (w/v), and it was stirred for 30 min and further centrifuged at 40,000 x g for 90 min. The supernatant was mixed with 3 ml of pre-equilibrated Cobalt resin Talon® Superflow metal affinity resin (Clontech, California, USA) and rotated for 1 h at 10 rpm. After centrifugation at 1,500 x g for 15 min, the supernatant was collected, resin was suspended in a minimal volume of supernatant and packed into a gravity-flow column. The column was consecutively washed with 60 ml of Buffer A [50 mM HEPES-KOH pH 7.5, 300 mM NaCl, 10% (v/v) glycerol and 0.2 mM orotate], 60 ml of Buffer A containing 0.05% (w/v) Polidocanol, 60 ml of buffer A containing 5 mM imidazole and 0.05% (w/v) Polidocanol and finally, bound His6-SUMO-P/DHODH were eluted by 30 ml of buffer A containing 300 mM imidazole and 0.05% (w/v) Polidocanol, with 1 ml per fraction collected. The protein elution from each fraction was monitored at 280 nm using Nanodrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). The active fractions were pooled and concentrated using Ultracel 50 kDa MWCO at 3,500 x g until the volume reached below 0.5 ml. Buffer A was added to the final 15 ml and concentrated once more to reduce the imidazole concentration.

To remove the His$_{6}$-SUMO tag, SUMO protease was added in proportion of 1:20 (w:w) with P/DHODH, and it was diluted with the cleavage buffer (50 mM Tris HCl pH 8.0, 300 mM NaCl, 0.05% (w/v) Polidocanol and 0.2 mM orotate) and incubated for 14.5 h. Purification of His6-SUMO tag-free native P/DHODH was achieved by passing the cleavage mixture into 4 ml of Talon® Superflow metal affinity resin and collecting the flowthrough. Finally, purified P/DHODH was concentrated using Ultracel 50 kDa MWCO at 3,500 x g. Ice-cold glycerol was added to the final 50% (v/v) and the enzyme was stored at –30°C until use.

**Enzymatic inhibition assay.** Inhibition studies of both P/DHODH and HsDHODH were carried out by measuring the reduction of a readily-observable electron acceptor 2,6-dichloroindophenol (DCIP; Sigma-Aldrich, Missouri, USA), as described previously (Baldwin et al., 2005) with some modifications. Each tested sample was placed into a 96-well microplate and dissolved in 10 µl of 50% (v/v) aqueous DMSO. 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 18 µM and 120 µM, respectively. Subsequently, the recombinant P/DHODH or HsDHODH was added into the mixture to a final concentration of 20 nM. The assay solution was gently mixed and aliquot volumes of 182 µl were transferred into a 96-well microplate. After incubation at 25°C for 2 min, the enzymatic reaction was initiated by adding the l-dihydropyruvate (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 5 s of shaking between each cycle, using a microplate reader SH-9000 Lab (Corona Electric, Ibaraki, Japan). The percentage of DHODH inhibition was calculated relatively to the positive control which was simultaneously prepared without the addition of the test compound. The IC$_{50}$ values of the isolated compounds were evaluated in three independent experiments.

**P. falciparum culture and parasite lactate dehydrogenase (pLDH) assay.** *P. falciparum* strain 3D7 was maintained in human type O-positive red blood cells (RBCs) in RPMI-1640 (Gibco, Thermo Fisher Scientific, Massachusetts, USA), supplemented with 25 mM sodium hydrogen carbonate, 10 µg/ml hypoxanthine, 40 µg/ml gentamicin sulfate, and 0.5% (w/v) Albumax II (Gibco, Thermo Fisher Scientific, Massachusetts, USA) under 5% O$_{2}$ and 5% CO$_{2}$ at 37°C. Human RBCs were received from the Japanese Red Cross Society. The experiments were performed under the guidelines of the ethical committee of The Uni-
dilutions were made in a final volume of 100 μl/well in RPMI media. Plates were then incubated with a standard gas mixture of 5% O₂, 5% CO₂, 90% N₂, for 72 h at 37 °C. Plates were then spun for 5 min at 1,300 RPM to lyse the pellet. Samples were frozen at –30 °C to lyse the erythrocytes. Pre-thawing plates were then supplemented with 100 μl of buffer containing 100 mM Tris-HCl pH 8.0, 50 mM sodium-l-lactate, 0.25% (v/v) Triton X-100, 0.7 mM 3-acetylpipridine adenine dinucleotide (APAD; Sigma-Aldrich, Missouri, USA), 0.2 mg/ml nitroblue tetrazolium (Sigma-Aldrich, Missouri, USA), and 1 unit/ml diaphorase. After 30 min incubation, absorbance was measured by SpectraMax® Paradigm® (Molecular Devices, CA, USA) at a wavelength of 650 nm. Inhibition values were measured in three independent experiments. The IC₅₀ values were calculated by least squares curve fitting of the dose inhibition curves using GraphPad Prism (GraphPad Software Inc., San Diego, USA).

**Evaluation of cytotoxicity against MRC-5 cells.** Human fibroblast cells, MRC-5, were plated on 96-well flat bottom plates at a density of 1.5 × 10⁴ cells/well with 100 μl of MEM medium (Life Technologies, Grand Islands, NY, USA) containing 10% fetal bovine serum (Hana-nesco Bio, Tokyo, Japan) and 1% penicillin-streptomycin (Life Technologies) and incubated at 37°C with 5% CO₂ for 48 h. Approximately 100 μl of MEM medium, containing 5 μl of each compound dissolved in 500 μl of DMSO, was added to each well. After 48 h cultivation at 37°C with 5% CO₂, cell density and morphological changes were observed under a microscope. After observation, 10 μl of WST-8 solution (Dojindo, Kumamoto, Japan) was added to the cells and the plate was incubated at 37°C with 5% CO₂ for 2 h. The absorbance was measured at 450 nm by spectrophotometer (SH-9000 Lab., Corona Electric, Ibaraki, Japan). The growth of MRC-5 cells was measured in three independent experiments.

| Compound          | IC₅₀ (µM) | Selectivity to PyDHODH | IC₅₀ (µM) |
|-------------------|----------|------------------------|----------|
|                   | PyDHODH  | HsDHODH                | Py3D7    |
|                   |          |                        | MRC-5    |
| Altenusin         | 5.9      | >430                   | >71.8    | 60.2  | 24.8 |
| Mitoxantrone      | 41.7     | >313                   | >7.5     | 17.6  | 51.2 |
| Mitoxantrin acid  | 160      | >303                   | >1.9     | 432   | 206  |
| HQNO              | 17.7     | 946                    | 53.4     | 0.45  | Not tested |
| Atovaquone        | Not tested | Not tested            | Not tested | 0.0002 | Not tested |

**Table 1. IC₅₀ values of isolated compounds.**

We screened 11,160 microbial broth extracts and got 114 active samples. We did activity-confirmation tests on the re-cultured hit extracts, including the dose-dependent bioactivity and the selectivity over the HsDHODH. Since an extract of a fungal strain *T. pinophilus* BioMCC-f.T.3979 was excellent in dose-dependency and enzyme selectivity, we selected the extract as a purification candidate. The 1.5 kg of rice culture of *T. pinophilus* BioMCC-f.T.3979 was extracted three times with 2 L of EtOAc. The concentrated extract (13.1 g) was partitioned between hexane-acetonitrile (MeCN)-H₂O (2:1:1). The lower (MeCN-H₂O) layer was washed three times with 100 ml of hexane. After the MeCN was removed in vacuo, the remaining aqueous mixture was extracted three times with an equal volume of EtOAc. The 5.27 g of dried extract was separated using silica-gel column chromatography employing chloroform-MeOH stepwise elution (1:0; 9:1, 8:2, 7:3, 5:5, 0:1 of chloroform-MeOH). The major active fractions, chloroform-MeOH 9:1 to 8:2, were combined and concentrated to give 2.03 g of brown oily material. The active fractions were further purified by a Sephadex™ LH-20 (500 × 550 mm) with MeOH elution. The major active fraction (277 mg) was refined with Sephadex™ LH-20 (230 × 810 mm) eluted with MeOH again. The purified active fraction was evaporated to dryness and dispersed in 200 μl of MeOH. After centrifugation, the clear solution was separated and concentrated under nitrogen to give 68 mg of 1. The insoluble part was dried to give 30.2 mg of 2. The minor active fraction from gel-filtration was evaporated to give 5.2 mg of 3.

The chemical structures of 1–3 were elucidated by MS and NMR analyses as follows;

High-resolution MS of 1, [M+H]+ m/z 291.0961 indicated the molecular formula to be C₁₅H₁₅O₆. The ¹H NMR spectrum of 1, (500 MHz, MeOH-d₄), δ 1.91 (3H, s), 3.79 (3H, s), 6.12 (1H, d, J = 2.3), 6.39 (1H, d, J = 2.8), 6.48 (1H, s), 6.56 (1H, s), was compared with published data, and 1 was identified as altenusin (Phaopongthai et al., 2013).

High-resolution MS of 2, [M+H]+ m/z 399.1267 indicated the molecular formula to be C₁₅H₁₄O₈. The optical rotation was measured to show [α]D₂₅ = –466 (c = 0.3, di­oxane). The ¹H NMR spectrum of 2 (500 MHz, DMSO-d₆), δ 1.54 (3H, s), 2.45 (3H, s), 4.19 (2H, m), 5.61 (1H, d, J = 1.2), 6.15 (1H, d, J = 2.3), 6.24 (1H, d, J = 1.8), 6.38 (1H, d, J = 15.5), 6.63 (1H, d, J = 16), 8.31 (1H, s), 10.30 (1H, s), 10.32 (1H, s), and the ¹³C NMR spectrum (125 MHz, DMSO-d₆), δ 22.0, 22.6, 60.5, 85.1, 100.6, 104.7, 106.6, 109.3, 111.1, 114.2, 118.8, 139.9, 142.4,

**Results and Discussion**

High-resolution MS of 2 (500 MHz, DMSO-d₆), δ 1.54 (3H, s), 2.45 (3H, s), 4.19 (2H, m), 5.61 (1H, d, J = 1.2), 6.15 (1H, d, J = 2.3), 6.24 (1H, d, J = 1.8), 6.38 (1H, d, J = 15.5), 6.63 (1H, d, J = 16), 8.31 (1H, s), 10.30 (1H, s), 10.32 (1H, s), and the ¹³C NMR spectrum (125 MHz, DMSO-d₆), δ 22.0, 22.6, 60.5, 85.1, 100.6, 104.7, 106.6, 109.3, 111.1, 114.2, 118.8, 139.9, 142.4,
143.3, 155.1, 155.2, 162.4, 162.7, 168.1, 191.2, 192.3 were compared with published data, and 3 was identified as (–)-mitorurbinol (Büchi et al., 1965; Zhu and Porco, 2006).

High-resolution MS of 3, [M+H]⁺ m/z 413.1003 indicated the molecular formula to be C₃₀H₂₈O₇. The optical rotation was measured to show [α]d₂⁵ = −384 (c = 0.1, ethanol). The ¹H NMR spectrum of 3 (500 MHz, acetone-d₆), δ 1.67 (3H, s), 2.60 (3H, s), 5.75 (1H, s), 6.23 (1H, d, J = 2.3), 6.36 (1H, d, J = 1.7), 6.54 (1H, d, J = 15.4), 7.03 (1H, s), 7.33 (1H, d, J = 15.5), 8.16 (1H, s), 10.75 (1H, s), and the ¹³C NMR spectrum (125 MHz, acetone-d₆), δ 22.5, 24.0, 86.6, 101.6, 104.8, 110.2, 112.6, 115.6, 116.7, 124.9, 134.8, 142.6, 144.8, 153.9, 155.0, 163.9, 166.2, 166.4, 170.6, 192.3, 192.7, were compared with published data, and 3 was identified as (–)-mitorurbinic acid (Marsini et al., 2006; Natsume et al., 1985; Zhu and Porco, 2006).

We evaluated 1–3 for activity against the PfDHODH and the recombinant human orthologue (HsDHODH) to investigate their inhibition selectivity (Table 1). As a result, 1–3 exhibited no significant inhibitory activity against HsDHODH at the concentrations tested. The results suggest that metabolites 1–3 are specific inhibitors for PfDHODH compared with HsDHODH.

Since it was difficult to obtain any commercially available PfDHODH inhibitor, we used 2-heptyl-4-hydroxyquinoline N-oxide (HQNO, Fig. 1) as a positive control. HQNO is a known mitochondrial NDH-II inhibitor (Mogi et al., 2009), and it exhibited moderate inhibition against PfDHODH, with an IC₅₀ value of 17.7 μM.

We investigated the growth inhibition against P. falciparum cells for the three compounds obtained (Table 1). Compounds 1, 2, and 3 suppressed parasite growth with IC₅₀ values of 60.2 μM, 17.6 μM, and 432 μM, respectively. Compound 1 exhibited a larger IC₅₀ value against P. falciparum cells growth than the value against PfDHODH. There is a possibility that most of 1 could not permeate to inhibit the enzyme in vitro. Compound 2 exhibited more potent in vitro activity than 1. These results indicate that the fungal metabolites could become interesting lead compounds for development into new antimalarials. Furthermore, we evaluated the cytotoxicity of the compounds against human fibroblast MRC-5 cells. Compounds 1 and 2 demonstrated cytotoxicity, with IC₅₀ values of 24.8 μM and 51.2 μM, respectively. It has previously been reported that 1 is cytotoxic through inhibition of protein kinases (Aly et al., 2008). Compound 2 exhibited moderate cytotoxicity against MRC-5 cells. Compound 3 displayed no significant cytotoxic effect against MRC-5 cells. Although the isolated PfDHODH inhibitors are cytotoxic, it may be possible to structurally modify them to synthesize derivatives with minimal or absent cytotoxic properties.

Altenusin (1) has been isolated from various genera of fungi, including Talaromyces (Singh et al., 2003), Penicillium (Chua et al., 2017; Komai et al., 2006; Nakanishi et al., 1995; Uchida et al., 1999), and Alternaria (Aly et al., 2008; Cota et al., 2008). Compound 1 exhibits inhibition of the aggregation of tau protein into paired helical filaments in vitro in Alzheimer’s disease (Chua et al., 2017). It shows antifungal activity against Aspergillus fumigatus, Aspergillus niger, and Candida albicans (Komi et al., 2006). It is known to inhibit several enzymes, including calmodulin-activated myosin light chain kinase (Nakanishi et al., 1995), neutral sphingomyelinase (Uchida et al., 1999), and HIV-1 integrase (Singh et al., 2003). Interestingly, it also inhibits trypanothione reductase of the parasitic protozoan Trypanosoma cruzi, with a similar order as PfDHODH inhibition (Cota et al., 2008).

Mitorurbinol (2) and mitorurbinic acid (3) have been recognized as virulence factors of Penicillium marneffei, causing respiratory, skin, and systemic mycosis (Suzuki et al., 1999; Woo et al., 2012). Compound 3, produced by Penicillium funiculosum, induces the formation of chlamydospore-like cells in fungi (Natsume et al., 1985). It also inhibits trypsin, with an IC₅₀ value of 41 μM (Lešvá et al., 2000).

In this study, we discovered several PfDHODH inhibitors from a T. pinophilus culture. This demonstrates that fungal and actinomycetes metabolites may be a good source of lead compounds to develop specific PfDHODH inhibitors for development into much-needed antimalarial drugs.

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