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Antiparasitic Compounds from the Panamanian Marine Bacterium *Pseudomonas aeruginosa*

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Fractionation of the ethyl acetate extract of the bacterium *Pseudomonas aeruginosa* led to the isolation of five compounds, *cyclo*–(L-Phe-L-Pro) (**1**), 3-heptyl-3-hydroxy-1,2,3,4-tetrahydroquinoline-2.4-dione (**2**), 2-heptyl-4-hydroxyquinoline (**3**), 2-nonyl-4-hydroxyquinoline (**4**), and 1-phenazinecarboxylic acid (**5**). The structures of compounds **1**–**5** were established by spectroscopic analyses. Compounds **2**–**4** produced inhibition on the growth of *Plasmodium falciparum*, with IC_{50} values of 3.47, 2.57 and 2.79 μg/mL, respectively. Compounds **3**–**4** had activity against *Trypanosoma cruzi*, with IC_{50} values of 3.66 and 3.99 μg/mL. Finally, all compounds were found inactive when tested against *Leishmania donovani* at 10 μg/mL.

Keywords: *Pseudomonas aeruginosa*, *Trypanosoma cruzi*, Malaria, Chagas disease.

Pseudomonas aeruginosa is a bacterial species that possess a large genome, which allows it to adapt to almost all kinds of ecosystems. This broad genome also confers a broad metabolic capacity, allowing to produce a wide variety of secondary metabolites [1]. There are reports of interesting biomedical activities attributed to extracts or compounds isolated from species belonging to the *Pseudomonas* genus, including anticancer, antiparasitic, antibacterial and antifungal, among others [1, 2a].

Interestingly, it has been shown that some factors (such as the addition of compounds, or co-cultures with other microorganisms) modulate the production of certain secondary metabolites in marine *P. aeruginosa* strains. This fact has biotechnological relevance because the controlled variation of this kind of factors could allow the selective production of certain compounds by *P. aeruginosa*, especially those with antibiotic and antimalarial properties [2b]. This work was designed to investigate the potential of a *P. aeruginosa* strain isolated from marine sediments of Panama as a source of antiprotozoal compounds. Herein we report the isolation, identification and anti-parasitic activity of compounds **1-5** produced by *P. aeruginosa* strain GL0334, isolated from the marine sediment collected near Coiba Island, Pacific coast of Panama. This bacterium was identified as *P. aeruginosa* based on the analysis of its partial 16S rRNA gene sequences.

P. aeruginosa strain GL0334 was cultured in 10 L of liquid media and extracted with ethyl acetate to obtain the organic crude extract. The extract was fractionated using C-18 reverse phase solid phase extraction cartridges (SPE) followed by HPLC purification to yield compounds **1-5**. Compounds isolated were identified as *cyclo*–(L-Phe-L-Pro) (**1**), 3-heptyl-3-hydroxy-2,4(1H,3H)-quinolinedione (**2**), 2-nonyl-3-hydroxy-4(1H)-quinolinone (**3**), 2-heptyl-3-hydroxy-4-quinolone (**4**), and 1-phenazinecarboxylic acid (**5**), (Figure 1), by spectroscopic analysis including HRESITOF-MS and NMR, and we found them to be reported previously in the literature [2c-5].

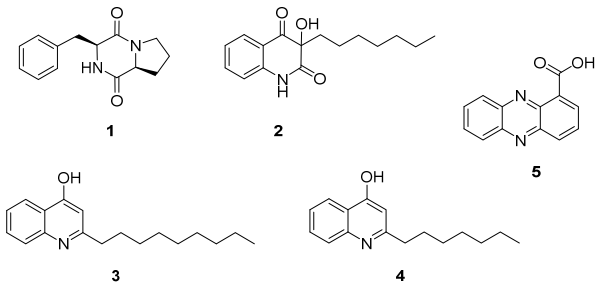

Figure 1: Compounds isolated from *P. aeruginosa* strain GL0034.

Table 1: Biological activity (IC_{50} μg/mL) of compounds isolated from Pseudomonas aeruginosa against *Lesihmania donovani*, *Plasmodium falciparum* and *Trypanosoma cruzi*.

Compounds	*L. donovani*	*P. falciparum*	*T. cruzi*
1	I	I	I
2	I	3.47	I
3	I	2.57	3.66
4	I	2.79	3.99
5	I	I	I
chloroquine		0.03	
nifurtimox			1.6

I = Inactive at 10 μg/mL.

Compounds **1**–**5** were tested for activity against parasites causative of three tropical diseases: *Leishmania donovani*, *Trypanosoma cruzi* and *Plasmodium falciparum*. Compounds **2**–**4** displayed activity against *P. falciparum*, while compounds **3** and **4** were also active against *T. cruzi* (Table 1). Active compounds were less active than reference drugs (chloroquine, IC_{50} = 80–100 nM for *P. falciparum*, and nifurtimox, IC_{50} = 0.15–13.4 μM for *T. cruzi*) for these strains and clone assessed with the same methodology. Regarding toxicity, compounds **3-5** have been reported with moderate to weak cytotoxicity against cancerous (KB, MCF-7, NCI-H187) and non-cancerous (Vero) cells [6]. Although there are not previous reports about the activity of compounds **1-5** against the strains of parasites evaluated in this study, compounds **2-5** were previously tested against the K1 multidrug-resistant strain of *Plasmodium falciparum*

[7]. However, that strain has different antimalarial drug-susceptibility profile and geographical origin than the strain W2 used in this study. Finally, it is important to point out that corresponding tautomers for compounds **3** and **4** showed strong *in vitro* activity against chloroquine sensitive (D6, Sierra Leone) and resistant (W2, IndoChina) strains of *Plasmodium falciparum* by measuring plasmodial LDH activity [2b]. This is relevant because it confirm that this type of compounds maintains the antimalarial activity independently of their tautomeric form.

Experimental

Biological material collection and identification: Sediment sample were collected in April 2010 in sterile plastic bags near Coiba Island, in the Golf of Chiriquí, Pacific of Panama, by SCUBA diving. The sediment sample was suspended in sterile seawater, filtered and processed using a standard methodology to obtain the bacterial isolates employing a seawater-based nutrient media. Agar plates were taken to the laboratory and observed for bacterial isolation, at room temperature over the period of one month. Strain GL0334 was further isolated from the collection plate and successively re-plated until a pure strain was obtained. Taxonomy of the strain GL0334 was carried out by sequencing of the 1409 bp of 16S rRNA gene. The gene sequence was submitted to NCBI-BLAST tool and showed 99.7 % similarity to 16S rRNA gene sequence of a strain identified as *Pseudomonas aeruginosa.* Sequences are available in GenBank with the accession number MK040572.

Fermentation and extraction: The bacterium *Pseudomonas aeruginosa* was inoculated in ten Erlenmeyer flasks (1 L), containing 500 mL of seawater-based liquid medium (agar, starch of potatoes, yeast extract and peptone). Erlenmeyer flasks were placed in an orbital shaker at 172 rpm at room temperature for 10 days. After this period, the culture broth was extracted with ethyl acetate (200 mL x 3). The organic extract was washed with distilled water (200 mL x 3) in order to remove traces of culture media from the organic extract. The extract was then dried under reduced pressure to obtain 456 mg of crude extract.

Isolation of compounds: Extract (202.6 mg) was fractionated using C-18 solid phase extraction (SPE) cartridges eluted with a stepwise gradient of 20 % (43.8 mg), 40 % (33.8 mg), 50% (7.0 mg), 60 % (10.6 mg), 70 % (30.5 mg), 80 % (6.4 mg), 90 % (15.3 mg), and 100 % (9.5 mg) of methanol in water to yield 8 fractions (F1-F8). All the 8 fractions were subjected to antiparasitic assays. Fractions C displayed 100% of inhibition of *P. falciparum* and fraction D showed 100% of inhibition of *P. falciparum* and 78.0% of inhibition of *T. cruzi*. All the fractions were analyzed by means of NMR to observe their complexity and it was detected that fraction B and E contained a major component. Fraction B eluted with 40% of methanol, was purified by reverse phase HPLC (Synergi Hydro-RP 250 x 10 mm column, isocratic elution of 85% Methanol: 15% distilled water, UV detector at 254 nm, flow of 1.0 mL/min) to afford 1.3 mg of compound **1**. Fraction C eluted with 50% of methanol, was purified by reverse phase HPLC (Synergi Hydro-RP 250 x 10 mm column, isocratic elution of 75% methanol: 25% distilled water, UV detector at 254 nm, flow of 1.0 mL/min) to afford 0.9 mg compound **2**. Fraction D eluted with 60% of methanol, was purified by reverse phase HPLC (Synergi Hydro-RP 250 x 10 mm column, isocratic elution of 75% methanol: 25% distilled water, UV detector at 254 nm, flow of 1.0 mL/min) to afford 1.2 mg of compound **3**, and 1.0 mg of compound **4**. Finally, Fraction E eluted with 30% of methanol, was purified by reverse phase HPLC (Synergi Hydro-RP 250 x 10 mm column, isocratic elution of 50% methanol: 50% distilled water, UV detector at 254 nm, flow of 1.0 mL/min) to afford 1.4 mg of compound **5**.

In vitro antiparasitic assays: For *L. donovani* (amastigotes) and *P. falciparum* (chloroquine-resistant strain, Indochina W2), a DNA cross linking agent is used to determine the amount of parasites in culture. Positive controls were amphotericin B for *L. donovani* and Chloroquine for *P. falciparum* [8]. For *T. cruzi*, a colorimetric method was used to determine the inhibition of parasite growth as detected by reduction of β-galactosidase (β-Gal) as a reporter gene, expressed by the Tulahuen clone C4 of *T. cruzi*. Nifurtimox was used as a positive control [8].

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