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Azmi Azhari
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia

Desi Harneti
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia

Asri Peni Wulandari
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia

Yeni Mulyani
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia

Sari Purbaya
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia

See next page for additional authors
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Azmi Azhari, Desi Harneti, Asri Peni Wulandari, Yeni Mulyani, Sari Purbaya, Aprilia Permata Sari, Galih Bayu Pratama, Unang Supratman, and Yoshihito Shiono

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Diketopiperazine *Cyclo-(S-Pro-R-Leu)* Produced by *Periconia pseudobyssoides* K5 Isolated from *Toona sureni* (Meliaceae) and its Heme Polymerization Inhibition Activity

Azmi Azhari[1,2], Desi Harneti[1], Asri Peni Wulandari[3], Yeni Mulyani[1], Sari Purbaya[1], Aprilia Permata Sari[1], Galih Bayu Pratama[1], Unang Supratman[1,4*], and Yoshihito Shiono[5]

1. Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia
2. Department of Chemistry Education, Faculty of Tarbiyah and Teacher Training, Institut Agama Islam Negeri Syekh Nurjati Cirebon, Cirebon 45132, Indonesia
3. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia
4. Central Laboratory of Universitas Padjadjaran, Jatinangor 45363, Indonesia
5. Department of Food, Life, and Environmental Science, Faculty of Agriculture, Yamagata University, Yamagata 997-8555, Japan.

[*]*E-mail: unang.supratman@unpad.ac.id*

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Abstract

Fungi endophytes are living microorganisms colonizing inside the internal tissue of a plant and provide ecological benefits for their host. Endophytes provide various metabolites for plant adaptation toward biotic and abiotic stresses and have tremendous pharmacological activities. *Toona sureni* (*T. sureni*) (Meliaceae) belongs to the *Toona* genera and is reported to have antimalarial activity. To determine the compounds produced by the endophytic fungus from this plant, we isolated a compound from *Periconia pseudobyssoides (P. pseudobyssoides)* K5 endophytes from the stem bark of *T. sureni*. Diketopiperazine cyclo-(*S*-Pro-*R*-Leu), a non-ribosomal peptide, was isolated from brown rice fermented at 28 ± 2 °C for 30 days. The structure was determined by spectroscopic methods including fourier-transform infrared spectroscopy, mass spectrometry, and 1D and 2D nuclear magnetic resonance techniques. This compound was evaluated for heme polymerization inhibition activity (HPIA) with an IC_{50} value of 9.89 ± 0.24 mmol/L compared with positive control chloroquine phosphate with an IC_{50} value of 3.08 ± 0.58 mmol/L. This compound has been categorized as having low activity three times lower than positive control chloroquine phosphate. This information provides new leads about the compound diketopiperazine cyclo-*(S-Pro-R-Leu)* produced by *P. pseudobyssoides* K5 endophytes having low activity in inhibiting heme polymerization. In the future, to explore the potency of this compound as antimalarial agent, the other antimalarial test such as lactate dehydrogenase assay might be useful.

Keywords: Diketopiperazine, Periconia pseudobyssoides, Toona sureni, Heme polymerization inhibition activity

Introduction

Fungi endophytes are living microorganisms colonizing inside the internal tissue of a plant and provide ecological benefits for their host [1, 2]. They provide improved nutrient intake, pathogen protection, and significant adaptation toward biotic and abiotic stresses [3–5]. Endophytic fungi can produce various secondary metabolites, such as alkaloids, phenols, polyketides, steroids, and peptides [6, 7]. Moreover, these compounds have diverse pharmacological activities for antioxidants, such as antibacterial, antifungal, antidiabetic, and antimalarial activities [6–9]. Endophytic fungi have become a tremendous source for natural product exploration over the last decades [10, 11].

Periconia is a member of the Periconiaceae fungal family (Ascomycetes) and can live in some habitats, such as plant symbionts, as endophytes. Several bioactive compounds from *Periconia* have antibacterial, antivirus, anticancer, and anti-inflammatory activities. In the last 50 years, this genus has produced terpenoids, polyketides, macrolides, aromatic compounds, and carbohydrate derivatives [12–19]. To widen its research interest, we

isolated *Periconia* species, *Periconia pseudobyssoides (P. pseudobyssoides)* K5 from the stem bark of *Toona sureni (T. sureni)*, traditional plant medicine for diarrhea, diabetes, and malaria [20–22]. *T. sureni* and some reported endophytes belonging to the Meliaceae family have antimalarial activity [23–26]. Malaria is a disease that causes deaths worldwide, especially in tropical and subtropical regions. The number of malaria-associated deaths was 435,000 in 2017 with 219 million cases [27]. The cause of this problem is the resistance of several antimalarial drugs: semisynthetic derivatives (artesunate, artemether, and dihydroartemisinin), chloroquine, mefloquine, quinine, proguanil, atovaquone, and sulfadoxine-pyrimethamine since 2003 [28]. Hence, malaria is still a world concern. Heme polymerization inhibition activity (HPIA) is an *in vitro* method that mimics free hemes resulting from the degradation of hemeoglobin, which is toxic to *Plasmodium* sp. The free heme is converted to hemozoin by *Plasmodium* sp. so that it is not toxic. If the production of hemozoin could be inhibited, the plasmodium dies because of this mechanism. The antimalarial drug chloroquine disulfate uses the same principles. This method can be used with the advantage for its rapidity and low cost [29].

To date, there has been no research on the endophytes of *T. sureni* for natural product exploration. To determine the compounds produced from endophytes from *T. sureni*, especially *P. pseudobyssoides* K5, we isolated the compound and tested it for HPIA. We report here the isolation of a non-ribosomal peptide, diketopiperazine cyclo-(*S*-Pro-*R*-Leu) from *P. pseudobyssoides* K5, with structure elucidation and HPIA.

Materials and Methods

General. Melting points were measured with a Fisher–Johns melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded in a KBr plate using a Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin Elmer, Shelton, USA). The high-resolution time-of-flight mass spectrometry (HR-TOFMS) was determined on a Waters Xevo Q-TOF direct probe/MS system, utilizing the electrospray ionization mode and microchannel plate detector (Milford, MA, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECX500R/S1 spectrometer (JEOL, Tokyo, Japan) using 500 MHz for ^{1}H and 125 MHz for ^{13}C using tetramethylsilane as an internal standard. Column chromatography was conducted on silica gel 60 (70–230 and 230–400 meshes, Merck, Darmstadt, Germany). Thin-layer chromatography plates were precoated with silica gel GF_{254} (0.25 mm, Merck, Darmstadt, Germany), and spot detection was performed by spraying with 10% H_2SO_4 in ethanol, followed by heating. The solvent used includes *n*-hexane, ethyl acetate, methanol (technical grade), and chloroform (p.a). The fermentation took 30 days, at a temperature of 28 ± 2 ℃. Each sample was tested three times for the HPIA test.

Collection of fungal and plant material. The plant material was collected from Kuningan District, West Java, Indonesia (6°56'30.5"S 108°26'19.5"E). The plant was determined as a *Toona sureni* (Blume) Merr by a herbarium staff of the Plant Taxonomy Laboratory, Department of Biology, Universitas Padjadjaran Indonesia, with identification no. 30/HB/09/2019. The fungi were isolated from the internal tissue of the stem bark through a surface sterilization [30]. This endophytic fungus was identified through a molecular analysis of the internal transcribed spacer [31,32] by a staff member of the Division of Biological Activity, Central Laboratory, Universitas Padjadjaran Indonesia, and is known as *P. pseudobyssoides* (97% similarity; NCBI Sequence ID: MW444851.1) K5 strain.

Fermentation and compound isolation. *P. pseudo byssoides* K5 were fermented on sterilized unpolished brown rice (total 10 kg; 35 g/flask × 285 flask) at 28 ± 2 ℃ for 30 days. The rice culture was extracted with ethyl acetate and evaporated under a vacuum, yielding a 270 g extract. The extract was dissolved in 500 mL distilled H_2O and partitioned with *n*-hexane and ethyl acetate. The total *n*-hexane extract (230 g) was separated on vacuum liquid chromatography with a stepwise gradient elution *n*-hexane/ethyl acetate/methanol (100:0–0:100–100:0) to provide 13 fractions (Fr. A to H). Fr. E (875 mg) was chromatographed on silica gel column chromatography using a stepwise gradient of *n*-hexane:ethyl acetate to provide 11 fractions (Fr. E1 to E11). Fr. E4 (86 mg) was subjected to a silica gel column chromatography with $CHCl_3$/methanol (50:1) as an eluent to afford compound **1** (5.3 mg, 2.3 % of the total extractum, i.e., 230.0 g).

Diketopiperazine cyclo-(*S*-Pro-*R*-Leu) (1), white powder, m.p. 163–165 ℃, IR (KBr) ν_{max} (cm^{-1}) 3251 (N–H stretch), 2949 (C–H sp^3 stretch), and 1717 (C=O stretch). ^{1}H-NMR ($CDCl_3$, 500 MHz); see Table 1; ^{13}C-NMR ($CDCl_3$, 125 MHz); see Table 1; HR-TOFMS (positive ion mode) *m/z* 211.1457 $[M+H]^+$ (calcd. for $C_{11}H_{18}N_2O_2^+$, *m/z* 211.1447).

HPIA test. An HPIA test was conducted following the previously reported method with some modified sample dosages and without Enzyme-Linked Immunosorbent Assay (ELISA) reader validation method [33]. A 100 µL sample was prepared with a series of 5.0, 2.5, 1.25, 0.625, and 0.3125 mg/mL concentrations diluted in 10% dimethyl sulfoxide solution. 50 µL of the sample was added to an Eppendorf tube containing 100 µL of 1 mM haematin dissolved in 0.2 M NaOH. Chloroquine diphosphate was prepared as a positive control, and 10% was also prepared as a negative control. All the samples and controls were added with 50 µL of glacial acetic acid

Table 1. Nuclear Magnetic Resonance (NMR) Data of Compound 1 (Left) Compared with the Literature Results of Diketopiperazine Cyclo-(*S*-Pro-*R*-Leu) (Right) [34]

No.	^{13}C (ppm)*	HMQC δH (∑H, mult., *J* in Hz,)*	^{13}C (ppm)**	HMQC δH (∑H, mult., *J* in Hz)**
1	59.1 (d)	4.12 (1H, t, 8.8)	59.1 (d)	4.12 (1H, m)
2	28.3 (t)	2.36 (1H, m) 2.13 (1H, m)	28.2 (t)	2.33 (1H, m) 2.13 (1H, m)
3	22.9 (t)	1.90 (1H, m) 2.03 (1H, m)	22.8 (t)	1.94 (1H, m) 2.02 (1H, m)
4	45.7 (t)	3.57 (2H, m)	45.6 (t)	3.60 (2H, m)
5	53.5 d)	4.02 (1H, dd, 2.8, 9.8)	53.4 (d)	4.01 (1H, m)
6	38.7 (t)	1.52 (1H, m) 2.08 (1H, m)	38.7 (d)	1.52 (1H, m) 2.01 (1H, m)
7	24.9 (d)	1.72 (1H, m)	24.8 (d)	1.76 (1H, m)
8	23.5 (q)	1.00 (3H, d, 6.6)	22.8 (q)	1.00 (3H, d, 6.6)
9	21.3 (q)	0.95 (3H, d, 6.6)	21.2 (q)	0.94 (3H, d, 6.6)
10	166.3 (s)		167.1 (s)	
11	170.2 (s)		171.4 (s)	
		5.72 (s)		5.72 (s)

* ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$)
** ^{1}H-NMR (300 MHz, $CDCl_3$), ^{13}C-NMR (75 MHz, $CDCl_3$)

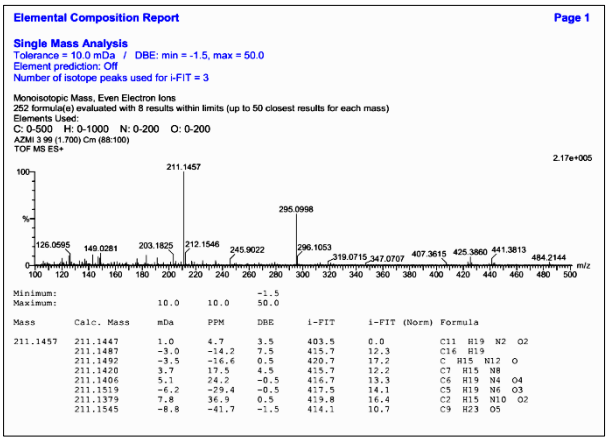
(pH 2.6) to start the polymerization reaction and incubated at 37 ℃ for 24 h. Then, all the samples were centrifuged at 8000 rpm for 10 min, and then the precipitate was separated from the supernatant. The residue from each sample was washed using DMSO and centrifuged at 8000 rpm for 10 min with four repetitions. The washed precipitate was then diluted with 200 µL of 0.1 M NaOH. Each 100 µL of the solution obtained was added into 96-well microplates, and the absorbance was recorded by an ELISA reader at λ = 405 nm. Values for the HPIA were presented as IC_{50} or a concentration that could reduce heme polymerization by 50% compared to the negative control. A standard haematin curve was constructed by making a series of concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.9063 mM). Each concentration was added into 96-well microplates reaching 100 µL, and the absorbance was recorded by an ELISA reader at λ = 405 nm. The percentage of inhibition was calculated using Equation (1), and IC_{50} was determined using the linear equation from each sample.

$$\% \text{ Inhibition} = \frac{(\text{Control Absorbance} - \text{Sample Absorbance})}{\text{Control Absorbance}} \text{ x } 100\% \quad (1)$$

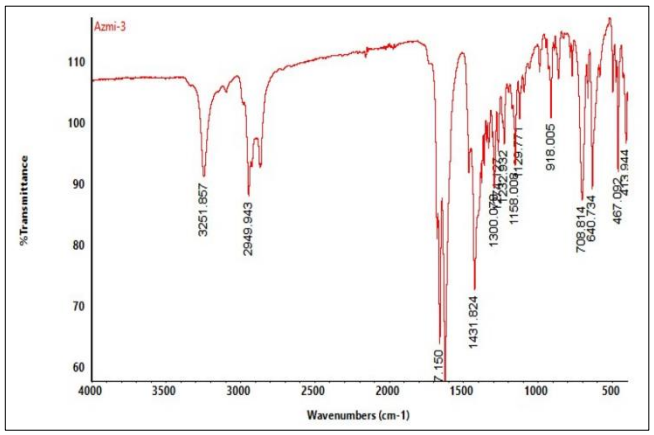
Results and Discussion

Compound **1** was obtained as a white powder. The molecular formula was determined as $C_{11}H_{18}N_2O_2$ from the HR-TOFMS (Figure 1a) ion peak at *m/z* 211.1457 $[M+H]^+$ (calcd. for $C_{11}H_{18}N_2O_2^+$, *m/z* 211.1447) from positive-ion-high-resolution measurements, indicating four degrees of unsaturation originating from two C=O and two remaining bicyclic rings. The IR spectra (Figure 1b) showed absorption peaks, which implies the existence of N–H (3251 cm^{-1}), C–H sp^3 (2949 cm^{-1}), and carbonyl C=O (1717 cm^{-1}). The ^{1}H NMR spectrum (Figure 1c) showed proton resonances related to three peaks of signal CH sp^3 resonating at δ_H 4.12 ppm (1H, t, *J* = 8.8 Hz), 4.02 ppm (1H, dd, *J* = 2.8, 9.8 Hz), and 1.72 ppm (1H, m). Several CH_2 sp^3 were resonating at δ_H 3.57 ppm (2H, m), 2.36 ppm (1H, m), 2.13 ppm (1H, m), 2.08 ppm (1H, m), 2.03 ppm (1H, m), 1.90 ppm (1H, m), and 1.52 ppm (1H, m). There are also two methyl peaks at δ_H 0.95 ppm (3H, d, *J* = 6.6 Hz) and δ_H 1.00 ppm (3H, d, *J* = 6.6 Hz) and also one proton for NH at δ_H 5.72 ppm (1H, s), with a total of 18 protons.

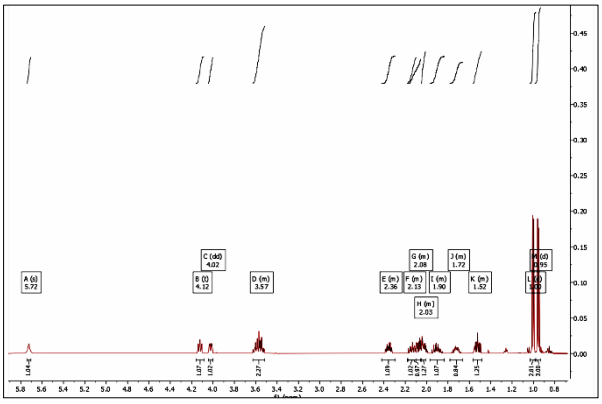
The ^{13}C NMR spectrum of 1, together with the Distortionless Enhancement by Polarization Transfer-135° spectrum (Figure 1d), revealed the presence of two methyl chemical shifts at 21.3 and 23.5 ppm; four methylene resonating at 22.9, 28.3, 38.7, and 45.7 ppm; three methine sp^3 at 24.9, 53.5, and 59.1 ppm; and two quaternary carbon at 166.3 and 170.2 ppm (carbonyl group), with a total of 11 carbons. The heteronuclear multiple quantum coherence (HMQC) experiment was conducted to obtain the correlation between directly bonded ^{1}H and ^{13}C (Figure 1e).



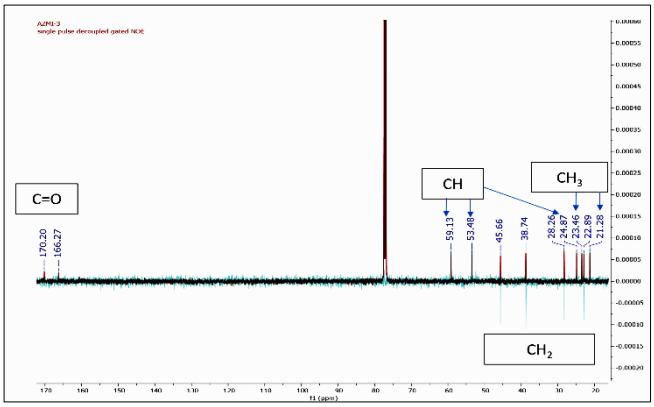
(a)



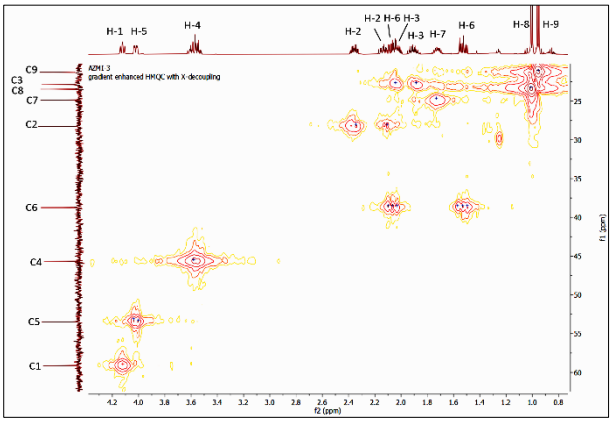
(b)



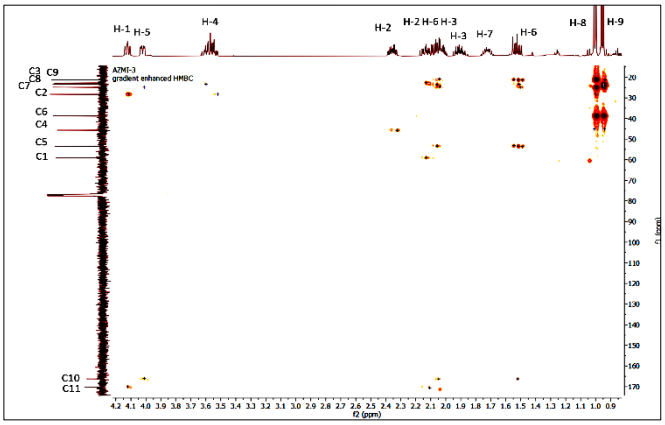
(c)



(d)

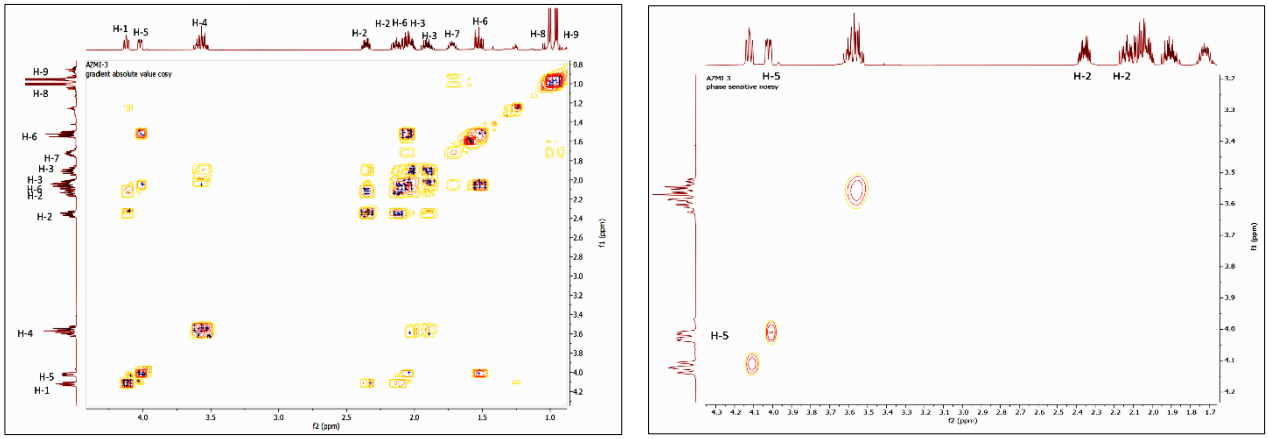


(e)



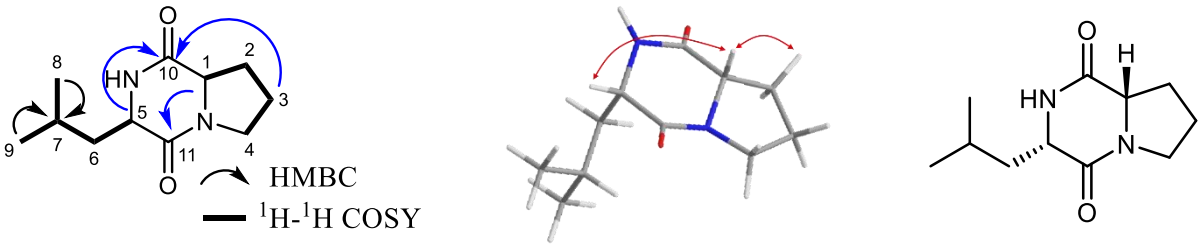
(f)

Figure 1. (a) The High-Resolution Time-of-Flight Mass Spectrometry (HR-TOFMS) Spectra of Compound 1, (b) The Infrared (IR) Spectra of Compound 1, (c) The ^{1}H-Nuclear Magnetic Resonance (NMR) Spectra of Compound 1 (500 MHz, $CDCl_3$), (d) The ^{13}C-NMR (125 MHz, $CDCl_3$) and Distortionless Enhancement by Polarization Transfer 135° Spectra of Compound 1, (e) The Heteronuclear Multiple Quantum Coherence (HMQC) Spectra of Compound 1, (f) The Heteronuclear Multiple Bond Correlation (HMBC) Spectra of Compound 1 (Continued)



(g) (h)

Figure 1. (g) The ^{1}H-^{1}H Correlation Spectroscopy (COSY) Spectra of Compound 1, (h) The Nuclear Overhauser Effect Spectroscopy (NOESY) Spectra of Compound 1



(a) (b) (c)

Figure 2. (a) Selected HMQC Heteronuclear Multiple Bond Correlation (HMBC) and ^{1}H-^{1}H Correlation Spectroscopy (COSY) Correlations of Compound 1, (b) Selected ^{1}H-^{1}H Nuclear Overhauser Effect Spectroscopy (NOESY) Correlations of Compound 1, (c) Chemeical Structure of Compound 1 as a Diketopiperazine Cyclo-(*S*-Pro-*R*-Leu)

The heteronuclear multiple bond correlation (HMBC) spectra (Figure 1f) analysis showed the correlation between δ_H 4.12 ppm (H-1) and δ_C 170.2 ppm (C-11), δ_H 4.02 ppm (H-5) and δ_C 166.3 ppm (C-10), and δ_H 2.03 ppm (H-3) and δ_C 166.3 ppm (C-10), confirming the position of the carbonyl functions in the core structure. The isobutyl side chain was confirmed by the HMBC correlation between δ_H 1.00 ppm (H-8) and δ_H 0.95 ppm (H-9) with δ_C 24.9 ppm (C-7) and also by the ^{1}H-^{1}H Correlation Spectroscopy (^{1}H-^{1}H-COSY) spectra correlation (Figure 1g) between δ_H 1.72 ppm (H-7) with δ_H 1.00 ppm (H-8) and δ_H 0.95 ppm (H-9). The HMBC and ^{1}H-^{1}H COSY correlations of compound **1** are shown in Figure 2a. The nuclear overhauser effect spectroscopy (NOESY) (Figure 1h) analysis established the relative configuration of the chiral carbons in C-5 and C-1, as shown in Figure 2b, with the correlation between H-1 (δ_H 4.12 ppm), H$_\beta$-2 (δ_H 2.36 ppm), and H-5 (δ_H 4.02 ppm).

Based on the elucidation structure analysis together with IR spectra, and HR-TOFMS data analysis, with the chemical formula $C_{11}H_{18}N_2O_2$ and the bicyclic ring system, compound **1** was identified as diketopiperazine cyclo-(*S*-Pro-*R*-Leu), as shown in Figure 2c. This result was supported by the comparison between literature results, as shown in Table 1, where this compound has previously been isolated from cyanobacteria in a *Calyx cf. podatypa* sponge (Caribbean islands) [34]. These cyanobacteria are also known as blue green algae. The diketopiperazine cyclo-(*S*-Pro-*R*-Leu) from *Calyx cf. podatypa* sponge were detected in the ectosome and endosome material crude extract observed with analytical high-performance liquid chromatography analyses [34].

The HPIA result of compound 1 shows that HPIA IC_{50} 9.89 ± 0.24 mmol/L. Chloroquine phosphate was used as the positive control and had HPIA IC_{50} 3.08 ± 0.58 mmol/L. The results indicate that compound 1 was less active in inhibiting the *β*-haematin crystal formation than the positive control, chloroquine diphosphate. According to Baelmans [35], the HPIA IC_{50} of a compound has a potent HPIA if it has a lower value than chloroquine (as

a positive control). Therefore, the categorization of compound 1 has lower activity three times than control positive. According to Ignatuschnko *et al.*, [36] the weak HPIA not always conclude low antiplasmodial activity. The compounds tested by Ignatuschnko *et al.*, [36] which is have low HPIA, but have a good antiplasmodial activity because of possible different mechanism of action. *Plasmodium* cell death can be caused by the inhibits of lactate dehydrogenase enzyme in glycolysis causing ATP production problem [37].

In other research, compound 1 has been tested for antimicrobial against *B. subtilis*, *S. aureus*, and *E. coli*, but it had no activity (minimum inhibitory concentration > 100 μg/mL). The antifouling activity shows a significantly reduced larval settlement of the barnacle *Balanus reticulatus* with LC_{50} values of 3.5 μg/cm^2 [38].

Conclusion

Diketopiperazine cyclo-(S-*Pro*-R-*Leu*) was isolated from *P. pseudobyssoides* K5, endophytes of *T. sureni*. The HPIA assay showed an IC_{50} value of 9.89 ± 0.24 mmol/L. Compound **1** has been categorized as having no activity because it is less active than the positive control (chloroquine sulfate). The results provide new bases about compound **1** produced by endophytes *P. pseudobyssoides* K5, which has low activity in the HPIA test. However, ELISA validation methods need to be performed to further validate the assay in the future, to explore the potency of this compound as antimalarial agent, the other antimalarial test such as lactate dehydrogenase assay might be performed.

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Conflict of Interest

The authors declare no conflict of interest.

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