

**Anti-Mycobacterial Activity of Polycarpine and Polycarpaurine A from an Indonesia Marine Ascidian *Polycarpa* sp.**Wilmar Maarisit^{1*}, Sonny D. Untu¹, Yessi K. Lengkey¹, Torry C.C Lomban¹, Jabes W. Kanter¹, Hiroyuki Yamazaki², Michio Namikoshi², Fitje Losung³, Veibe Warouw³, and Remy E.P. Mangindaan³¹Faculty of Mathematics and Natural Sciences, Universitas Kristen Indonesia Tomohon, North Sulawesi, 95362 Indonesia.²Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan³Faculty of Fisheries and Marine Science, Sam Ratulangi University, Kampus Bahu, Manado, North Sulawesi, 95115 Indonesia.

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ABSTRACT

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Marine ascidians are an abundant source of new bioactive metabolites. The current investigation aimed at characterizing anti-mycobacterial compounds from Indonesian marine ascidian *Polycarpa* sp. After extracting ethanol, Ascidian *Polycarpa* sp. was segregated with EtOAc and H₂O. Ethyl acetate extract was purified using preparative HPLC. Identification and characterization of compounds using spectroscopic analysis. Anti-mycobacterial activity testing using the disc diffusion method on *Mycobacterium smegmatis*. This study showed that the ethyl acetate extract of ascidian *Polycarpa* sp produced two known compounds polycarpine (1) and polycarpaurine A (2). Compounds 1 and 2 exhibited activity against *Mycobacterium smegmatis* at 50 µg/disc, with 12- and 10-mm inhibition zones, respectively. This study suggests that polycarpine (1) and polycarpaurine A (2) have potential compounds for anti-mycobacterium.

Keywords: *Anti-mycobacterium*, polycarpine, polycarpaurine A, alkaloid, *Polycarpa* sp.

Introduction

Mycobacterium tuberculosis is the primary cause of Tuberculosis (TB). There are alarming numbers of TB cases reported by WHO in 2020. Between 2019 and 2021, there were an anticipated 1.6 million fatalities from TB globally, including 1.4 million of these deaths predicted to occur in HIV-negative people and 187,000 in HIV-positive people.¹ On the other hand, drug-resistant TB (DR-TB) cases have become a severe problem in treating TB. In particular, 450,000 additional cases of rifampicin-resistant TB (RR-TB) are predicted to emerge between 2020 and 2021¹. Therefore, efforts to look for new drugs to combat drug-resistant *M. tuberculosis* and the quest for novel bioactive substances as anti-tuberculosis originating from nature are urgently needed.

Marine organisms, notably ascidians, play a significant part as a provider of secondary bioactive substances with the prospect of being used in medication development. Nitrogenous chemicals are plenty in marine ascidians²⁻⁴: included in this group are the β-carboline alkaloids,⁵ siladenoserinols,⁶ purine alkaloids,⁷ polyaurines,⁸ quinolone, pyridocridine alkaloids, tyrosine, phenylalanine, and indole alkaloids.⁹

These compounds showed various biological activities, for example, anti-HIV, anti-cancer, anti-tumor, anti-malarial, and anti-trypanosomal, anti-diabetic, phosphatase inhibitors, acetylcholine signaling inhibitors,^{7,9} anti-inflammatory,¹⁰ and anti-tuberculosis.¹¹

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As a continuation of the study for biologically active metabolites from Indonesian marine organisms water,¹²⁻¹⁴ it was observed that the EtOH extracts of marine ascidian *Polycarpa* sp showed inhibitory efficacy towards *M. smegmatis* at 50 µg/disc with the inhibition zone 10 mm. Using bioassay-guided purification, two identified compounds were isolated polycarpine (1),¹⁵ and polycarpaurine A (2).¹⁶ We disclosed isolation, structure determination and anti-mycobacterial activity of these compounds.

Materials and Methods*Animal material*

The animal material was collected in Manado water in North Sulawesi, Indonesia (1°29'08"N 125°14'28"E), in November 2022. Faculty of mathematics and natural sciences, Universitas Kristen Indonesia Tomohon kept a voucher specimen (236) and identified as *Polycarpa* sp by Dr. Fitje Losung (Sam Ratulangi University).

Extraction and Isolation

Immediately upon collection, the ascidian (200 gr, wet mass) was sliced into little pieces and immersed in ethanol (1.0 L) on a watercraft. To yield 480 mg, the EtOH residue was disintegrated in MeOH: H₂O and extracted with EtOAc. Utilising preparative HPLC pegasil ODS, compounds 1 (16.8 mg) and 2 (6.0 mg) were separated from EtOAc extract (480 mg) eluted with MeOH: H₂O (40:60, v/v)].

Anti-Mycobacterial assay

The anti-mycobacterial test was performed by utilizing *disc* diffusion method.^{17,18} The strain NBRC 3207 of *Mycobacterium smegmatis* was obtained from a stock culture laboratory and maintained at 80 °C in 20% glycerol. These bacteria were grown with Middlebrook 7H9 broth medium, which comprised 0.5% polysorbate 80 (BD), 0.5% glycerol, and 10% Middlebrook OADC (BD), at 37 °C for two days and was adapted to 1.0 106 CFU/mL. The inoculum 1 mL was applied on Middlebrook 7H9 medium 100 mL at 40 °C, with 1.5% agar in a plate. Compounds 1 and 2 (50 µg/disc) were adsorbed on a 6 mm paper disc manufactured by Advantec in Tokyo, Japan. After the MeOH had evaporated, the paper disc was put in a dish and maintained for two

days at 37 °C. As a positive control, streptomycin sulphate (2 µg/disc) was employed, and MeOH served as a negative control.

Results and Discussion

The marine ascidians (200 gr wet weight) were collected from Manado, North Sulawesi, Indonesia, and extracted with ethanol. Upon partitioning the ethanol residue with MeOH: H₂O (200 mL, 9:1, v/v) and EtOAc (200 mL), resulting in 480 mg, compounds 1 and 2 were formed by HPLC deploying an ODS column. By comparing the spectroscopy data for 1 and 2 with those of the previously published values for polycarpine and polycarpaurine A, two identified substances could be detected by their structures.^{15,16,19}

Compound 1 was isolated as an orange solid. The molecular ion peak at *m/z* 469 [M+H]⁺ was observed in the LRFABMS spectrum and was compatible with the following molecular formula: C₂₂H₂₄N₆O₂S₂. The ¹H-NMR spectrum showed four signals in MeOH-d₄, an O-Methyl signal at δ 3.90 (6H, s), an N-Methyl signal at δ 3.23 (6H, s) and bands for a para-disubstituted benzene ring at δ 7.49 (*d*, 8.0 Hz), and 7.03 (*d*, 8.0 Hz) (Table 1). Compound 1 was identified as Polycarpine, originated from the marine ascidian *Polycarpa clavate*¹⁵ and *Polycarpa aurata*.^{16,19}

Compound 2 was isolated as an orange solid. The LRFABMS spectrum was visible at the molecular ion peak at *m/z* 437 [M+H]⁺ and was consistent with the molecular formula: C₂₂H₂₄N₆O₂S. The ¹H-NMR spectrum showed four signals in MeOH-d₄, an O-Methyl signal at δ 3.85 (6H, s), an N-Methyl signal at δ 3.30 (s) and bands for a para-disubstituted benzene ring at δ 7.97 (2H, *d*, 8.0 Hz), and 6.98 (*d*, 8.0 Hz) (Table 1). One sulfur atom was remained after the molecular formula of compound 2 was reduced by the combined value of two phenyl imidazole units. Therefore, the structure of 2 was determined as a dimeric monosulfide alkaloid. Compound 2 was identified as Polycarpaurine A and was isolated from ascidian *Polycarpa aurata*.¹⁶ Polycarpine (1) and Polycarpaurine A (2) was gained as a bis-TFA salt, because the separation process was conducted by HPLC applying a solvent mixture composed of 0.1% trifluoroacetic acid (TFA).

Ascidian Polycarpa sp is reported to contain various secondary metabolites such as Polycarpamines A-E, polycarpine, N,N-didesmethylgrossularine, polycarpaurines A, B, C, polycarpathamines A and B, Polycarpine dihydrochloride,²⁰ polycarpathiamine A and B,²¹ and polyaurine B.⁸

Anti-Mycobacterial activity

Marine ascidian crude extract *Polycarpa* sp. demonstrated efficacy against *M. smegmatis* at 50 µg/disc, with an inhibition value of 10 mm. Meanwhile, the isolated compounds polycarpine (1) and polycarpaurine A (2) was active against *M. smegmatis* at a dosage of 50 µg/disc with inhibition levels of 10 and 12 mm respectively (Table 2). The positive control used in this test produced an inhibition of 25 mm at a concentration of 2 µg/disc. Polycarpine (1) has a higher inhibition level in comparison to Polycarpaurine A (2). This indicates that the two sulfur groups in polycarpine (1) are essential for antimycobacterial activity.

Studies on phenotarget have reported that extracts from *Polycarpa aurata* actively bond to *Mycobacterium tuberculosis* Rv 1466 protein. Based on the results of the compounds present in the active extract using ¹H-NMR and mass spectroscopy it was found to contain a polycarpine compound with a mass weight of 468 Da. So far, in vitro antimycobacterium activity has not been reported²². This study provides in vitro information on the antimycobacterium activity of polycarpine (1).

Polycarpine and polycarpaurine A compounds have been reported to be able to inhibit Tobacco Mosaic Virus. Polycarpine with two sulfur groups is known to have higher activity compared to polycarpaurine A which only has one sulfur.²³

The mechanism of inhibition of alkaloid compounds towards antibacterial activity is very dependent on the unique chemical structure.²⁴ Alkaloids prevent bacterial growth in a number of ways, including by hindering the production of nucleic acids and proteins, altering the permeability of bacterial cell membranes, damaging bacterial cell membranes and cell walls, impairing bacterial metabolism,²⁵ and impairing the efflux pump.²⁴ Alkaloid compounds

can also inhibit bacterial functional protease activity and affect DNA topoisomerase and respiration. These substances may interact with the intestinal flora and have a protective impact on the intestinal mucosa. An enzyme called DNA topoisomerase controls the nucleus's DNA superhelix. It can affect the topological state of DNA by catalyzing the breakage and binding of DNA strands.²⁴ Pharmacological activity studies of the compound Polycarpine (1) have been reported, some of which have cytotoxic activity against the human colon tumor cell line HCT-116 at a concentration of 0.9 µg/ml,¹⁵ cytotoxic against Chinese hamster cell colonies with EC₅₀ 3.8 µM¹⁶ and inhibited inosine monophosphate dehydrogenase IMPDH.¹⁹ Meanwhile, the compound polycarpaurine A (2) was reported to have cytotoxic against Chinese hamster cell colonies with an EC₅₀ value of 6.8 mM.¹⁶ This means that the study invitro of the antimycobacterium activity of the compounds polycarpine (1) and polycarpaurine A (2) is the first time reported. These compounds have potential in the development of medicinal raw materials, especially as anti-tuberculosis.

Conclusion

In the present study, two known alkaloid compounds polycarpine (1) and polycarpaurine A (2) were isolated from Indonesia marine ascidian *Polycarpa* sp. Following a thorough investigation of the spectroscopic data, their structure was established. With inhibition zones of 12 and 10 mm, respectively, the isolated chemicals polycarpine (1) and polycarpaurine A (2) exhibited towards *M. smegmatis* at 50 µg/disc. The chemicals polycarpine (1) and polycarpaurine A (2) hold considerable promise for the development of anti-tuberculosis medications.



Figure 1: Indonesia marine ascidian *Polycarpa* sp.

Table 1: ¹H-NMR (100 MHz) of metabolites 1 and 2 (in CD₃OD)

| Position | Compound 1 | Compound 2 |
|----------|----------------------------------|----------------------------------|
| | ¹ H-NMR (Mult./Hz) | ¹ H-NMR (Mult./Hz) |
| 1 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 (11) | 7.49, (<i>d</i> , 8.0) | 7.97, (<i>d</i> , 8.0) |
| 8 (10) | 7.03, (<i>d</i> , 8.0) | 6.98, (<i>d</i> , 8.0) |
| 9 | | |
| 12 | 3.90, s | 3.85, s |
| 13 | 3.23, s | 3.30, s |

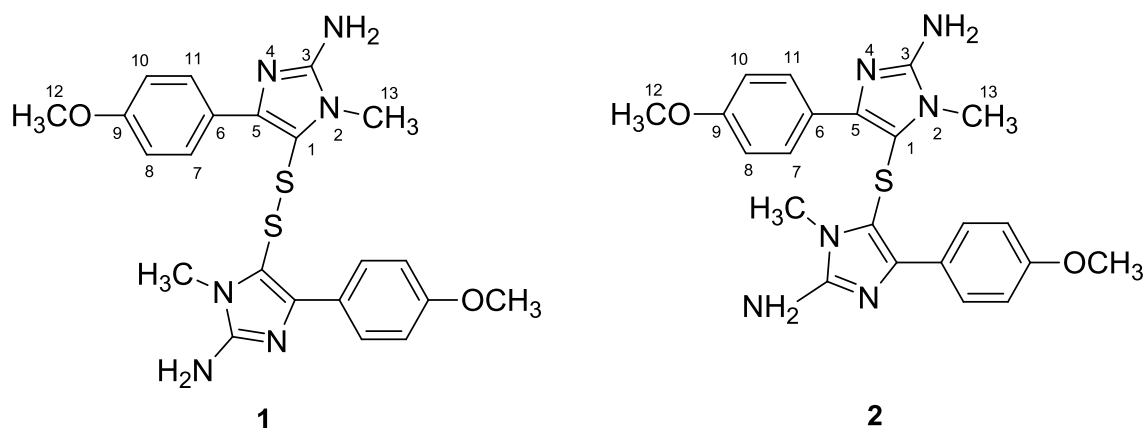


Figure 2: Structure of polycarpine (1) and polycarpaurine A (2) isolated from Indonesian marine ascidian *Polycarpa* sp.

Table 2: Anti-mycobacterial activity of compounds 1 and 2 against *Mycobacterium smegmatis*

| Compound | <i>Mycobacterium smegmatis</i> 50 µg/disc |
|-----------------------------|--|
| Polycarpine (1) | 12 |
| Polycarpaurine A (2) | 10 |
| Streptomycin sulfate (2 µg) | 25 |
| Ethanol (negative control) | - |

Diameter of inhibition zone (mm)
Diameter of paper disc : 6 mm

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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