ANTIBACTERIAL COMPOUND FROM MARINE SPONGE DERIVED FUNGUS *Aspergillus sydowii* DC08

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**ABSTRACT**

A bioactive compound was isolated from ethyl acetate extract of the marine sponge-derived fungus *Aspergillus sydowii* DC08. The compound's structural details were determined based on the study of nuclear magnetic resonance spectroscopy and mass spectrometric data. p-Iodonitrotetrazolium chloride (INT), a marker of bacterial growth, was used in the microdilution assay to assess the isolated compound's antibacterial efficacy. The isolated chemical was identified as sterigmatocystin (1) using spectroscopic data. This compound showed moderate activity against some pathogenic bacteria. The Minimum Inhibitory Concentration (MIC) values for this substance were 64, 128, 16, 32, and 32 µg/mL for Methicillin-Resistant *Staphylococcus aureus* (MRSA), Multi-Drug Resistant *Pseudomonas aeruginosa* (MDRPA), *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, respectively.

**Keywords:** *Aspergillus sydowii*, Sterigmatocystin, p-iodonitrotetrazolium chloride, MIC, MRSA, MDRPA.

**INTRODUCTION**

WHO (World Health Organization) has reported that several pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are resistant to antibiotics. They pose a significant threat to human health globally. The high resistance level to these pathogenic bacteria causes the treatment options used in therapy to be limited and require an expensive cost. Therefore, it is necessary to search for new antibacterial compounds. One source of antibacterial compounds comes from marine biota. Marine sponges are known as interesting marine invertebrates due to the content of secondary metabolites they produce. This invertebrate has a wide array of bioactive compounds. It is estimated that over 12,000 compounds are reported, and hundreds of new metabolites are still being discovered every year from marine sponges. Marine Sponges *Dactylospongia* sp are reported repeatedly regarding its secondary metabolites, such as new merosesquiterpenoid compounds, aillimaquinone, smenosponsgine, and dycitioceratine C, which showed antibacterial activity against *Bacillus megaterium* with a Minimum Inhibitory Concentration (MIC) of 32 µg/mL. It is suspected that most of the metabolites found from marine sponges are not produced by themselves but are produced by related microorganisms. The sponge's body contains many microbes, such as bacteria and fungi, accounting for up to 50-60% of the sponge's dry weight the sponge. Fungi and bacteria are potential producers of bioactive metabolites. Our previous study reported that the ethyl acetate extract from fungus *Aspergillus sydowii* DC08 derived from marine sponge *Dactylospongia* sp. had antibacterial activity against *S. aureus*, and *E. coli* with an inhibitory zone of 14.25 mm and 12.31 mm, respectively. This fungus has attracted much attention because of the discovery of several bioactive compounds, such as sesquiterpenes, alkaloids, xanthones cyclopentanoids, and some of these compounds have antibacterial activity against pathogen bacteria. This paper details the bioactive metabolite from the marine sponge-derived fungus *Aspergillus sydowii* DC08 that exhibits antibacterial activity.

**EXPERIMENTAL**

**Sponge Material and Fungus Isolation**

Scuba divers were used to harvest the marine sponge *Dactylospongia* sp. from the Mandeh, South Coast, West Sumatra, Indonesia island. The sponge was immediately put in a sterile plastic bag and kept in an icebox. Dr. Nicole J. De Voogd from the Natural Biodiversity Center in the Netherlands identified the...
sponge. The fungus *Aspergillus sydowii* DC08 was obtained from marine sponge *Dactylospongia* sp. by our previous study.\(^9,16\)

**Cultivation and Extraction of Secondary Metabolite**

Cut the fungus isolate in the Petri dish to 1-2 cm\(^2\), then grow it in a rich medium in a 1000 mL Erlenmeyer flask for 4-6 weeks at room temperature. When the fungus isolate completely coated the rice, the fungi flourished at their fastest rate.\(^16\) A pure fungal isolate cultured for 4-6 weeks was extracted using a three-step process that involved macerating the isolate in ethyl acetate (EtOAc) at a ratio of 1:1 for 24 hours before filtering it.\(^17\) To create the extract of fungal secondary metabolites, the macerate of EtOAc was evaporated using a rotary evaporator.\(^18,19\)

**Isolation of a Secondary Metabolite**

The EtOAc extract was defatted with a liquid-liquid separation between n-hexane and aqueous 90% MeOH. The MeOH extract (12.1 g) was subjected to liquid chromatography (LC) on silica gel 60 with step gradient polarity eluent using n-hexane, EtOAc, and MeOH solvent. From this process, we afford nine fractions (F1-F9). These fractions were screened for their antibacterial activity with the agar diffusion method. Fraction 6 (F6) showed antibacterial activity with a diameter zone of 19.32 mm against *E. coli*, 17.12 mm against *S.aureus*, 8.10 mm against MRSA, 6.14 mm against MDR-PA, and 17.92 mm against *P. aeruginosa*. The bioactive fraction (F6) is then subject to purification by recrystallization method using n-hexane and EtOAc solvent to afford compound 1 (28 mg).

**Antibacterial Assay**

The EtOAc extract of fungus was tested with the agar diffusion method against some pathogenic microbial, such as *S. aureus* ATCC 25923, MRSA (clinical isolate), *P. aeruginosa* ATCC 27853, MDR-PA (clinical isolate), and *E. coli* ATCC 2592. We obtained those bacteria from M. Djamil Hospital, West Sumatera, Indonesia. The Petri dish was filled with Natrium Agar (Merck®), which contained 0.5 McFarland of the bacterial solution. Positive control was a chloramphenicol (Oxoid®) disc at a concentration of 30 μg/ml. A 100 μl dose of DMSO was used to inoculate the paper disc as a negative control. There were three repetitions of each treatment. For fungi, the plates were incubated overnight at 25°C–27°C, and for bacteria, at 37°C. The antibacterial assay was conducted based on the method described previously. The clear zone on the disc was measured as an indicator of antibacterial activity.\(^20-24\)

**Microdilution Tests**

The pathogenic bacteria (MRSA, MDR-PA, *E. coli*, *S. aureus*, and *P. aeruginosa*) were inoculated on Nutrient Agar (NA) media, then incubated for 24 hours at 37°C. The concentration of the bacterial suspension was measured with a densitometer to obtain a concentration of 0.5 McFarland, equivalent to a cell density of 1.5x10\(^8\) CFU/mL. All the test wells on the plates received 100 μL of Mueller Hinton Broth medium. Each test well (apart from the blank well) had added 50 μL of the appropriate standardized inoculum. In the first well (columns 1 to 5) of each line (lines A to H) was added 50 μL of sample solutions in DMSO solvent (serial dilution from 128 μg/mL to 2 μg/mL). For the negative control (column 7, lines A to E), 50 μL of inoculum and 50 μL of DMSO solvent were added to the well. For the positive control, chloramphenicol (Oxoid®) disc at a concentration of 30 μg/mL. A 100 μL dose of NaCl solution (0.9%) was supplied to the blank wells (column 11, lines A to E).\(^25-27\) The plates were incubated for 22 hours at 37°C. Following incubation, each well received ten μL of the INT solution (0.6 mg/mL) before the plates underwent a second 2-hour incubation. The lowest concentration was thought to be where there was no color development to calculate the MIC. Optical measurements were made after the incubation by watching the development or not of red color arising from the reduction of INT (colorless) to formazan (violet). The measurements were made using a microplate photometer set to 570 nm.\(^28\) The schematic portrayal of the procedure is introduced (Fig.-1). The test was carried out in 5 repetitions.

**RESULTS AND DISCUSSION**

Compound 1 was obtained as pale-yellow crystals. It has a melting point of 246°C. The UV spectrum of compound 1 showed λmax at 246.20 and 320.80 nm. Its molecular formula was determined as C\(_{18}\)H\(_{12}\)O\(_7\) by the ESI-MS at m/z 325.0734 [M\(^+\) + H\(^+\)]. The FT-IR spectrum displayed bands at 3222.81; 2923.17; 2846.98;
1623.13; 1581.66; 1458.21; and 1125.48 cm\(^{-1}\) indicating the existence of the O-H group, C-H group, methoxy group, olefinic group, aromatic group, and C-O (carbonyl group) respectively.\(^9\)

Replicates

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Fig.-1: 96-Well Plates used to Represent Serial Dilution, with PC (Positive Control), NC (Negative Control), and BL (Blank)
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Precursor ion of 1 was fragmented to produce product ion at m/z 310.0489 by separating fragment CH\(_3\); then it was turned into product ion m/z 268.2648 by separating fragment of C\(_2\)H\(_2\)O. This compound's fragmentation patterns were similar to those described in (Fig.-2). The fragmentation of this compound's pattern matched that of sterigmatocystin previously reported in the reference.\(^10\)

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Fig.-2: The Fragmentation Pattern of the Precursor ion of Compound 1 and the ESI-MS Spectrum
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Compound 1's NMR spectrum can be seen using \(^1\)H NMR, \(^{13}\)C NMR, and DEPT. The \(^1\)H NMR spectrum displayed 10 protons. Methoxy protons as singlet signals were detected with \(\delta H\) 3.90 ppm for OCH\(_3\)-12. Four signals of aromatic protons consisted of two doublet signals with a \(\delta H\) 6.75 ppm (d, 1H, H-4) and 6.82 ppm (d, 1H, H-6), and one triplet signal at 7.50 (t, 1H, H-5) and one singlet signal at 6.42 ppm (s, 1H, H-11). One hydroxy (OH) group was identified at \(\delta H\) 13.22 ppm as a single signal. Four signals of dihidrofuranofuran proton were \(\delta H\) 6.81 ppm (d, 1H, H-14), \(\delta H\) 4.80 ppm (dt, 1H, H-15), \(\delta H\) 5.43 ppm (t, 1H, H-16) and \(\delta H\) 6.49 ppm (t, 1H, H-17).\(^{13}\)C NMR data of this compound exhibited one carbonyl group
at 181.5 ppm (C-1), nine quaternary carbons at 181.5 ppm (C-1), 109.1 ppm (C-2), 162.4 ppm (C-3), 155.0 ppm (C-7), 154.1 ppm (C-8), 106.6 ppm (C-9), 164.7 ppm (C-10), 163.4 ppm (C-12), 106.0 ppm (C-13) eight methine units (CH) 111.3 ppm (C-4), 135.8 ppm (C-5), 106.0 ppm (C-6), 90.6 ppm (C-11), 113.3 ppm (C-14), 145.5 ppm (C-15), 102.6 ppm (C-16), 48.20 ppm (C-17), one methoxys (O-CH$_3$) 163.4 ppm (C-12), and one hydroxyl group 162.4 ppm (C-3). After comparing the spectra to the available literature, it was determined that chemical one was identical to sterigmatocystin (Fig.-3 and Table-1).

![Chemical Structure of Compound 1](image)

### Table-1: NMR $^1$H and $^{13}$C Comparing the Data From Compound 1 to Those From the Literature

| No | $^{13}$C NMR Data in CDCl$_3$ (δ in ppm) | $^1$H NMR Data in CDCl$_3$ (δ in ppm, J in Hz) |
|----|----------------------------------------|-----------------------------------------------|
| 1  | 181.5                                 | 181.6                                         |
| 2  | 109.1                                 | 109.1                                         |
| 3-OH | 162.4                             | 162.5, 13.22, s, 1H                          |
| 4  | 111.3                                 | 111.4, 6.75, d, 1H (J = 8)                    |
| 5  | 135.8                                 | 135.9, 7.50, t, 1H (J = 8.5)                  |
| 6  | 106.0                                 | 106.1, 6.82, d, 1H (J = 7.5)                  |
| 7  | 155.0                                 | 155.1                                         |
| 8  | 154.1                                 | 154.2                                         |
| 9  | 106.6                                 | 106.7                                         |
| 10 | 164.7                                 | 164.7                                         |
| 11 | 90.6                                  | 90.7, 6.42, s, 1H                             |
| 12-OMe | 163.4                                 | 163.5                                         |
| 13 | 106.0                                 | 106.1                                         |
| 14 | 113.3                                 | 113.4, 6.81, d,1H                             |
| 15 | 145.5                                 | 145.5, 4.80, dt, 1H (J = 1,5; 2)              |
| 16 | 102.6                                 | 102.7, 5.43, t, 1H (J = 3)                    |
| 17 | 48.20                                 | 48.2, 6.49, t,1H (J = 2)                      |
| 18-Me | 56.9                                  | 57.0, 3.90, s, 3H                             |

The HSQC spectrum reveals a correlation between the methoxy proton at δH 3.90 ppm and carbon at δC 163.4 ppm. Additionally, the signals from the four protons seem to be related to aromatic carbon, and the proton of the hydroxy (OH) group appears to be related to carbon at a concentration of δC 162.4 ppm. The dihydrofuranofuran ring correlates with the signals of three protons. Proton and carbon are correlated in the HMBC spectra (Fig.-4).

### Minimum Inhibitory Concentration (MIC) Determination

The broth microdilution assay is more sensitive than agar diffusion methods and thus best suited for determining antibacterial activity quickly. This method saves time and resources while screening various bacteria and plant extract combinations. Tetrazolium has been used to detect bacterial growth in the broth microdilution assay. Reduction causes an easily discernible color change at a cell density relevant for MIC testing. Reduction occurs due to its role in respiration as an artificial terminal electron acceptor. Simultaneously, the spectrophotometer reading is an additional tool for confirming the MICs value results. The results of cell growth or death are shown in Fig.-5 for visual readings and Table-2 for...
spectrophotometric readings. The color intensity could be visually perceived and in the absorbance format obtained.

Fig.-4: The Correlation HMBC (→) of Compound 1

Fig.-5: The MICs Representative of Compound 1 Against Pathogens Bacteria: (a) *S. aureus* ATCC 25923, (b) MRSA (clinical isolate), (c) *P. aeruginosa* ATCC 27853, (d) MDR-PA (clinical isolate), (e) *E. coli* ATCC 2592

Table-2: The Absorbance Data of Compound 1

| Strains                           | Serial Concentration (µg/mL) | Average Absorbance Value (n=5) |
|-----------------------------------|------------------------------|-------------------------------|
|                                   | 128 | 64 | 32 | 16 | 8 | 4 | 2 | 1 |
| *S. aureus* ATCC 25923            | 0.076 | 0.088 | 0.412 | 0.956 | 1.033 | 1.043 | 1.121 | 1.098 |
| *P. aeruginosa* ATCC 27853        | 0.067 | 0.089 | 0.389 | 0.754 | 1.011 | 1.105 | 1.067 | 1.065 |
| *E. coli* (ATCC 2592)             | 0.045 | 0.014 | 0.023 | 0.326 | 0.712 | 1.204 | 1.076 | 1.087 |
| MRSA (clinical isolate),          | 0.098 | 0.720 | 0.946 | 1.034 | 1.132 | 1.165 | 1.089 | 1.090 |
| MDRPA (clinical isolate),         | 0.108 | 0.650 | 0.765 | 0.720 | 0.820 | 1.120 | 1.111 | 1.087 |

n: Replicate
Compound 1 showed moderate inhibitory activity against MRSA, MDRPA, and E. coli with a MIC value of 64 µg/mL, 128 µg/mL, and 16 µg/mL, respectively. Moreover, compound 1 also exhibited moderate activity against S. aureus and P. aeruginosa, with a MIC value of 32 µg/mL. The MIC value of the positive control (chloramphenicol) was obtained at the concentration of 5 µg/mL (Table-3).

Table-3: The MICs Data of Compound 1

| Strains                  | Minimum Inhibitory Concentration (MIC, µg/mL) | Compound 1 | Positive Control |
|-------------------------|-----------------------------------------------|------------|-----------------|
| MRSA (Clinical isolate) | 64                                            | 5          |                 |
| MDRPA (Clinical isolate)| 128                                           | 5          |                 |
| P. aeruginosa ATCC 27853| 32                                            | 5          |                 |
| S. aureus ATCC 25923    | 32                                            | 5          |                 |
| E. coli ATCC 2592       | 16                                            | 5          |                 |

Several Aspergillus species and some Bipolaris species produce sterigmatocystin. Aspergillus Versicolor is the primary producer of sterigmatocystin. Sterigmatocystin was isolated from a mycelial mass of A. Versicolor in 1957.\(^{36}\) This compound is commonly found in pistachio nuts, pecans, wheat, and green coffee beans and has been reported in processed foods.\(^{34}\) Several studies reported that sterigmatocystin is cytotoxic to humans depending on time and dose in various cell lines such as bronchial epithelial BEAS-2B cells, esophageal epithelial Het-1A cells, gastric epithelial GES-1 cells, and hepatic HepG2 cells.\(^{38}\) Recently, three new oxisterigmatocystin’s J, K, and L, and aspergillicin A, were isolated from Aspergillus nomius NC06. The isolated compounds displayed significantly cytotoxic activity against HT 29 colon cancer cells.\(^{39}\)

CONCLUSION

From an EtOAc extract of the marine-derived fungus Aspergillus sydowii DC08, sterigmatocystin was successfully extracted. This compound showed moderate activity. The Minimum Inhibitory Concentration (MIC) values for this substance were 64, 128, 16, 32, and 32 µg/mL for Methicillin-Resistant Staphylococcus aureus (MRSA), Multi-Drug Resistant Pseudomonas aeruginosa (MDRPA), Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa, respectively.

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