BIOACTIVE COMPOUNDS FROM MARINE SPONGE DERIVED FUNGUS *Aspergillus unguis* WR8

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ABSTRACT

Three bioactive compounds were isolated from the marine sponge-derived fungus *Aspergillus unguis*. The fungus was isolated from the marine sponge *Haliclona fascigera*. The fungus cultivation on rice medium yielded three bioactive compounds, nidulin, 2-chlorounguinol, and unguinol. All compound was elucidated structurally based on nuclear magnetic resonance spectroscopy data and accurate mass spectrometric data. All compounds had a strong activity with minimal inhibitory concentration values of 0.78 µg - 3.12 µg against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhosa*, *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Vibrio cholera Inaba* and *Methicillin-Resistant Staphylococcus aureus*. All compounds also showed moderate cytotoxic activity against cancer cell lines and normal cells.

Keywords: *Aspergillus unguis*, nidulin, 2-chlorounguinol, unguinol, *Methicillin-Resistant Staphylococcus aureus* (MRSA), Antibacterial Activity, Cytotoxic Activity.

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INTRODUCTION

The oceans are a source of secondary metabolites with unique structures mainly accumulated in invertebrates such as sponges, tunicates, bryozoans, and molluscs¹. The sponge is a food filter that has many small pores on its surface, allowing water to enter and circulate through a series of channels where microorganisms and organic particles can also be filtered as food. Sponges have been known to host large groups of microorganisms, with a percentage of up to 50-60% of the biomass. Microbes' role in the sponge is very diverse, including as a source of nutrients to the mutualistic symbiosis with a sponge^{2,3}.

Based on the results of previous studies, these microbes play an important role in producing bioactive compounds that are useful as a source of medicines^{4,5}. We have investigated several endophytic fungi from marine sponges, and mangrove plants found in West Sumatra, Indonesia. Some of the fungal isolates have been shown to have antibacterial and cytotoxic activity, these include *Aspergillus nomius* NC06, *Cocliobolus geniculatus* WR12, *Diaporthe amygdali* SgKB4, and *Penicillium oxalicum* WR3⁶⁻¹¹.

As part of our research program on bioactive compounds of marine-derived fungi, we investigated the fungus *Aspergillus unguis* WR8 which has been isolated from the marine sponge *Haliclona fascigera*. The sponge, *H. fascigera*, was collected from Mandeh Island, south coast of West Sumatera, Indonesia. Preliminary studies on ethyl acetate extract of *A. unguis* WR8 showed its potential for antibacterial and cytotoxic activities.^{4,5} In the current study, we present details of the isolation, and structure identification of three metabolites, nidulin (1), 2-chlorounguinol (2), and unguinol (3) isolated from ethyl acetate extract of *A. unguis* WR8, as well as, their antibacterial and cytotoxic activities.

EXPERIMENTAL

Solvents for extraction and column chromatography were distilled before use. Fungal growth media *Sabouraud Dextrose Agar* (SDA) (Merck®), Bacteria growth media *Nutrient Agar* (NA) (Merck®), a disk

of chloramphenicol (BD BBL™) dan a *disk* of nystatin (Oxoid®). TLC analysis was performed on silica gel 60 F_{254nm} (Merck®). The lamp of UV_{λ254nm} and UV_{λ365nm} was used as the visualization method on TLC analysis. Open column chromatography was done by using Silica gel 60 (0.040 – 0.005 mm) Merck® (Darmstadt, Germany) and sephadex™LH-20 (GE healthcare®). Melting points were measured using the Innotech® *Melting Point Apparatus*. The IR spectrum and Ultraviolet spectrum were analyzed by the Perkin-Elmer Spectrum One FT-IR instrument and the Pharmaspec 1700 Shimadzu® ultraviolet-visible spectrometer, respectively. 1D and 2D NMR spectra were recorded on the Bruker AVANCE DMX 600 and 300 NMR spectrometers. The mass spectrum (ESI) was measured by the Finnigan LCQ Deca mass spectrometer and the HRESIMS spectrum recorded with the FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer.

Fungal Material

The marine-derived fungus *A. unguis* WR8 was isolated from marine sponge *H. fascigera*. The marine sponge was collected from Mandeh Island, South Coast of West Sumatera, Indonesia. The fungus was grown in *Sabouraud Dextrose Agar* (SDA) media and incubated at 25° C for 4-7 days.¹²

Identification of Fungal Cultures

The fungal cultures were identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously.¹³ The sequence data has been submitted to GenBank, accession number MN273740. The fungal strain was identified as *Aspergillus unguis*. A voucher strain (strain designation WR8) is kept in the Laboratory of Sumatra Biota/Faculty of Pharmacy, Andalas University, Padang, Indonesia.

Cultivation

The fungus was grown in ten Erlenmeyer flasks (1 L each) containing 100 g of rice and 110 mL of distilled water at room temperature (25°C) for 40 days.¹²

Extraction and Fractionation

The solid-state rice culture was extracted with EtOAc. The crude extract (33.8 g) was dried and partitioned between n-hexane and 90% MeOH. The 90% MeOH soluble material was chromatographed over silica gel using stepwise elution with mixtures of n-hexane, EtOAc, and MeOH, respectively. The fractions of 2, 3, 4 are combined and recrystallized to obtain compound **1**. A combination of fractions 6 and 7 was purified by recrystallization to afford compound **2**. Fractions 8, 9, and 10 were also purified by recrystallization with hexane and EtOAc, yielding compound **3**.

MIC Assay Test for Antibacterial Activity

For the MIC (Minimum Inhibitory Concentration) assay of antibacterial activity, the antibacterial activity of compounds **1**, **2**, and **3** was tested against *S. aureus* and *Methicillin-Resistant Staphylococcus aureus* (MRSA) using the agar diffusion method. DMSO and chloramphenicol (30 µg/disc) were used as a negative and positive control, respectively. The paper disc was placed on the surface of *Nutrient Agar* medium containing 10⁸ of the tested bacteria and incubated at 37° for 24 hours. The lowest concentration contained a clear zone around the paper disc was described as a minimum inhibitory concentration (MIC) value.^{13,14}

MTT Assay Test for Cytotoxic Activity

The isolated compounds were tested against WiDr (colon adenocarcinoma cell line), and Vero (normal cell line) using MTT assay¹⁵. All compounds were tested in the Laboratory of Parasitology, Gadjah Mada University, Yogyakarta, Indonesia.

RESULTS AND DISCUSSION

The sequences were aligned and compared with all the 18S rDNA gene sequences available in the GenBank database using the multisequence advanced BLAST comparison tool. Based on the molecular characteristics of 18S rDNA sequencing, the isolate WR8 was identified as *Aspergillus unguis* Strain F3000054. The phylogenetic trees were constructed using MEGA software version 7.0 (Fig.-1).

Fig.-1: The Phylogenetic Tree using the Neighbor-joining Method of the 18S rRNA Gene Sequence from the Fungi Isolate WR8 derived Marine Sponge *Haliclona fascigera* and Some Strain of *Aspergillus*. The Scale Bar indicates a 0.05 substitution Nucleotide Position

Aspergillus unguis has been reported as a source of bioactive secondary metabolite production. The secondary metabolites isolated from *A. unguis* were inguinal and noridulin. Several other bioactive secondary metabolites that have been successfully isolated, such as 2-chlorounguinol¹⁶, nidulin¹⁶, 3-ethyl-5,7-dihydroxy-3,6-dimethylphthalide¹⁶, emeguisin A-C¹⁷, folipastatin¹⁸, guisinol¹⁹, unguisin A-D²⁰, aspergillusidones A-C²⁰, aspergillusidones D-F²¹, aspergillusphenol A-B²¹, 2,4-chlorounguinol²¹, asperlides²², methyl orellinate²², (+)-montagnetol²², aspersidone²², orsellinate²², pilobolusate²², 3-chlorounguinol²², Asperunguisones A-D²³ and Aspergiside A-C.²⁴

Compound **1** (36.6 mg) was isolated as colorless crystals, m.p 118.5-121.8 °C. Its molecular formula was determined as C₂₀H₁₇Cl₃O₅ and the MS isotope pattern of the ions at *m/z* 441.2; [M+2+H]⁺ 443.3; [M+3+H]⁺ 444.9 and [M+4+H]⁺ 445.8 (the ratio 10:6.5:2.5:1) explained the presence of three chlorine atoms. UV analysis showed λ_{max} 203.80 and 319.60 nm. The IR spectrum showed absorption bands at 3596 cm⁻¹ for hydroxyl, 2929-2857 cm⁻¹ for C-H aromatic, 1710.69 cm⁻¹ for C=O, 1568-1402 cm⁻¹ for C=C, 1205 cm⁻¹ for alcohol or phenol carbonyl and 1030 cm⁻¹ for ether carbonyl functional groups. The ¹H and ¹³C NMR data of **1** (Table 1) exhibited five methyl groups at δ_H 5.37 (d, 1H); 3.73 (s, 3H); 2.40 (s, 3H); 2.26 (s, 3H); 1.89 (d, 3H) and 1.77 (d, 3H). Chemical shift at δ_C = 10.7; 14.5; 17.9; 19.2 ppm were characteristic for 4 methyl groups (CH₃), and δ_C 60.8 ppm indicating the presence of methoxy group. Whereas, chemical shifts of δ_C at 114.4; 121.2; 124.3; 128.0; 129.0; 136.0; 140.2; 142.0 and 152.5 ppm were indicative for the quaternary C atom. The comparison of ¹H and ¹³C NMR for compound **1** with literature data^{16,20} and concluded that compound **1** was nidulin.

Compound **2** (545.2 mg) was also a colorless crystal, m.p 220.9-222.6 °C. Its molecular formula was C₁₉H₁₇ClO₅ at *m/z* [M]⁺ 358.9 and [M+2+H]⁺ 361.0 (the ratio abundance 3:1), indicating the presence of a chlorine atom. The IR spectrum showed absorption bands at 3460, 2597, 1704, 1578-1431, 1231, and 1095 cm⁻¹ for hydroxyl, C-H aromatic, C=O, C=C, alcohol or phenol carbonyl, and ether carbonyl functional groups, respectively. The UV spectrum exhibited absorption bands at 314 and 206.80 nm, indicating the presence of a conjugated double bond. The ¹H and ¹³C NMR data of compound **2** were presented in Table-1. Compound **2** has 4 methyl groups at δ_H 2.38 (3H, s); 2.05 (3H, s); 1.99 (3H, m); 1.81 (3H, d). The two methyl groups at δ_H 2.38 and 2.05 had peak singlet multiplicity which was methyl function connected to aromatic ring. The proton signals at δ_H 6.60 and 6.44 showed aromatic protons, while δ_H 9.65 and 11.42 as hydroxyl protons. In chemical shifts at δ_C 9.7; 14.2; 17.9; and 18.6 showed four methyl groups (CH₃), while δ_C 105.5; 111.4; 125.4 indicated the presence of C-H. The comparison of ¹H and ¹³C NMR for compound **2** with literature data¹⁰ revealed that compound **2** was as 2-chlorounguinol.

Compound **3** (1056 mg) was obtained as a colorless, m.p. 193.1-193.7 °C. Its molecular formula was assigned as C₁₉H₁₈O₅ and showed fragment ion peaks at m/z 327.4. UV analysis of **3** exhibited absorption bands at 207.8 nm. Compound **3** exhibited IR absorption at 3492 and 3313, 2984, 1694, 1581 and 1428, 1290, and 1067 cm⁻¹, suggesting the presence of hydroxyl, C-H aromatic, C=O, C=C, alcohol or phenol carbonyl, and ether carbonyl functional groups. The ¹H and ¹³C NMR data of compound **3** were shown in Table-1. The two methyl groups at δ_H 2.33 and 2.05 had peak singlet multiplicity which was methyl aromatic. The proton signals at δ_H 6.55 and 6.43 showed aromatic protons, while δ_H 9.59 and 10.57 as hydroxyl protons. In chemical shifts at δ_C 9.9; 14.4; 18.0; and 21.2 showed four methyl groups (CH₃), while δ_C 105.2; 111.2; 116.5; and 125.7 indicated the presence of C-H. The ¹H and ¹³C NMR of **3** were compared with literature data¹⁰, which indicated that compound **3** was unguinol.

Table-1: ¹H and ¹³C NMR Data of Compounds 1, 2, and 3 in DMSO-d₆

Position	Compound 1		Compound 2		Compound 3	
	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)
1	140.2		141.4		145.1	
2	121.2		119.7		116.5	6.55, d (2.2)
3			161.3		164.3	
4			105.5	6.60, s	105.2	6.32, dd (2.3)
4a			158.0		163.0	
5a			140.7		140.9	
6	136.0		135.8		135.8	
7			111.4	6.44, s	111.2	6.43, s
8	152.5		153.1		152.9	
9	124.3		115.3		115.2	
9a	142.0		143.7		143.8	
11					162.2	
11a	114.4		113.8		112.2	
1-Me	19.2	2.40, s	18.6	2.38, s	21.2	2.33, s
9-Me	10.7	2.26, s	9.7	2.05, s	9.9	2.05, s
8-OMe	60.8	3.73, s				
1'	129.0		132.2		132.8	
2'	128.0	5.37, dq (6.8,1.6)	125.4	5.50, qd	125.7	5.50, qd (6.8,1.6)
3'	14.5	1.77, dd (6.9)	14.2	1.81, dd (6.8,1.3)	14.4	1.80, dd (6.8,1.4)
4'	17.9	1.89, s(1.4)	17.9	1.99, m	18.0	1.99, t
8-OH				9.64, s		9.59, s
3-OH				11.41, s		10.57, s

^aChemical shifts are extracted from HSQC and HMBC spectra. bnot observed.

The NMR data of 2-chlorounguinol was almost the same as that of unguinol. 2-chlorounguinol was a derivative of unguinol, in which one hydrogen atom of unguinol at position C-2 was replaced with chlorine in the structure of 2-chlorounguinol. The presence of two aromatic protons appears at δ_H 6.60 and 6.44 ppm as broad singlets, indicating that 2-chlorounguinol was chlorinated in the C-2 position of unguinol¹⁶. Comparing the ¹H NMR spectrum of compounds 1-3, the methyl group attached to aromatic ring at position 1-CH₃ resonating at δ_H 2.33 ppm for unguinol (**3**), while it appeared at δ_H 2.38 and 2.40 ppm for 2-chlorounguinol (**2**) and nidulin (**1**). The chemical shift of the respective proton shifted downfield in compounds 1 and 2 due to the presence of chlorine atom in the aromatic ring at position C-2.

The isolated compounds (**1**, **2**, and **3**) were evaluated for antibacterial (against *S. aureus*, *E. coli*, *S. typhosa*, *B. subtilis*, *E. faecalis*, *M. luteus*, *V. cholerae* Inaba, and MRSA) and cytotoxic (against WiDR and Vero cell lines) activities (Table-4). Compound **1** was more active against seven tested bacteria with MIC values of 0.78 µg, except for *P. aeruginosa* and *S. typhosa* bacteria. Compound **2** displayed activity with MIC values of 0.78 µg for *E. faecalis*, *B. subtilis*, *V. cholerae*, *M. luteus*, and MRSA bacteria. Compound **3** also displayed MIC values of 0.78 µg against *B. subtilis*, *V. cholerae*, and *M. luteus* bacteria.

All these three compounds significantly showed strong activity against the tested bacteria. Among them, nidulin (**1**) was more sensitive against all pathogenic bacteria as it contains three chlorine atoms at positions

C-2, C-4, and C-7. The presence of chlorine atoms in the structure of nidulin favored it as chlorine-releasing agents (CRAs), which contribute to its higher antibacterial activity compared to the other two compounds. CRAs are active oxidizing substances that could interfere with the activity of bacterial cell proteins. The penetration of CRAs in the outer layer of cells was strongly influenced by the acidity of the environment and concentration of CRAs²⁵.

Fig.-2: Structures of Compounds 1-3

Table-2: Biological Activities for Compounds 1, 2 and 3

Compound	Antibacterial Activity (MIC, µg/disc)									Cytotoxicity (IC ₅₀ , µg/mL)	
	EC	PA	SA	EF	BC	MRSA	ST	VC	ML	WiDR	Vero
1	0.78	1.56	0.78	0.78	0.78	0.78	1.56	0.78	0.78	28.65	25.74
2	1.56	1.56	1.56	0.78	0.78	0.78	1.56	0.78	0.78	39.14	28.14
3	1.56	3.12	3.12	3.12	0.78	3.12	3.12	0.78	0.78	27.15	28.34
Doxorubicin										9.53	369.15

^a EC= *Escherichia coli* ATCC 25922, PA = *Pseudomonas aeruginosa* ATCC 27853, SA= *Staphylococcus aureus* ATCC 25923, EF= *Enterococcus faecalis* ATCC 29252, BC= *Bacillus subtilis* ATCC 6633, MRSA= *Methicillin-resistant S. aureus*, ST= *Salmonella typhosa* NTCC 786, VC= *Vibrio cholerae* Inaba, and ML= *Micrococcus luteus* ATCC 10240.

Therefore, nidulin with 3 chlorine atoms has the highest antibacterial activity compared to 2-chlorounguinol and unguinol which did not contain chlorine. In the literature study, the strong antibacterial activity of nidulin was also shown against MRSA with a MIC value of 4 µg/mL, against *E. coli* AB3027 with MIC of 2.8 µg/mL and larvicidal activity with LC₅₀ of 2.8 µg/mL²⁶. In contrast to Ryuji (2016) study, 2-chlorounguinol and unguinol showed weak antimicrobial activity against *B. subtilis*, *S. aureus*, and *M. luteus*, and did not inhibit the growth of *E. coli* and *P. aeruginosa*²⁷.

Compound 1–3 exhibited moderate cytotoxicity toward WiDR cancer cells with IC₅₀ values of 28.65; 39.13 and 27.57 µg/mL, respectively. When tested against Vero normal cells, all compounds had smaller IC₅₀ values. This result suggested that compounds 1–3 were toxic against WiDR cancer cells but not selective towards Vero cells. Cytotoxic activity of the compounds had also been tested toward HuCCA-1 (Human Lung Cholangia Carcinoma), HepG2 (Human hepatocellular liver carcinoma), A549 (Human Lung Carcinoma), and MOLT-3 (Acute Lymphoblastic Leukemia) cancer cells.²¹

CONCLUSION

In this study, nidulin (1), 2-chlorounguinol (2), and unguinol (3) were obtained from the EtOAc extract of the marine sponge derived fungus, *A. unguis* WR8. All isolated compounds had a strong antibacterial activity with MIC values ranging from 0.78 to - 3.3.12 µg against the tested pathogenic bacteria. The cytotoxicity test with MTT assay showed that the compounds had moderate cytotoxicity toward WiDR and Vero cells.

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