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New Natural Product from *Botryosphaeria australis*, an Endophyte from Mangrove *Avicennia marina*

Produk Alami Baru dari Botryosphaeria australis, Jamur Endopit dari Tumbuhan Mangrove Avicennia marina

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ABSTRACT

Chemical investigation of the endophytic fungus *Botryosphaeria australis* isolated from *Avicennia marina* originally from Hainan Province, P.R. China, yielded a new compound botryosphaenin (**1**), from the class of napthoquinone, together with 5 known compounds, botryosterpene (**2**) and 5-hydroxy-2,7-dimethoxynaphthalene-1,4-dione (**3**) and its derivatives, 6-ethyl-5-hydroxy-2,7-dimethoxynaphthalene-1,4-dione (**4**), O-methylaspmenone (**5**), O-methylasparvenone (**6**) and 5-(carboxymethyl)-7-hydroxy-1,4a-dimethyl-6-methylene decahydron aphthalene-1-carboxylic acid (**7**). Their structures were determined on the basis of spectroscopic methods including 1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, HMQC, HMBC, and ROESY) NMR experiments and by mass spectroscopic measurements The new compounds, **1** showed activity against the bacterial pathogens *Staphylococcus aureus*, several *Streptococcus species* and *Bacillus subtilis*, but also against the eukaryotic cell lines THP-1 (human leukemia monocyte) and BALB/3T3 (mouse embryonic fibroblast).

Keywords: Mangrove, *Avicennia marina, Botryosphaeria australis*, antibiotic activities, cytotoxicity

ABSTRAK

Telah dilakukan penelitian kimiawi tentang jamur endofit *Botryosphaeria australis* yang diisolasi dari bakau *Avicennia marina* yang tumbuh di kawasan Provinsi Hainan, Republik Rakyat China. Dari penelitian ini diperoleh senyawa botryosphaenin (**1**), yang merupakan senyawa baru , dari kelas napthoquinone , bersama dengan lima senyawa yang diketahui sebelumnya yaitu, botryosterpene (**2**) dan 5 - hidroksi - 2,7 - dimetoksinaftalen - 1 ,4 - dion (**3**) dan turunannya , 6 - etil - 5 - hidroksi - 2 ,7 - dimetoksinaftalen - 1 ,4 - dion (**4**) , O – metilaspmenon (**5**) , O – metilasparvenon (**6**) dan 5 - (karboksimetil) - 7 - hidroksi - 1 , asam 4a - dimetil - 6 - 1 - metilendekahidronaftalen karboksilat (**7**). Strukturnya kimia dari senyawa-senyawa tersebut di atas ditentukan berdasarkan analisis spektroskopi Resonansi Magnetik Inti (NMR) termasuk 1D (1H , 13C, dan DEPT) dan 2D (COSY, HMQC, HMBC, and ROESY) serta menggunakan spektroskopi massa. Senyawa baru yang diisolasi (**1**) tidak hanya menunjukkan aktivitas yang kuat melawan bakteri patogen *Staphylococcus aureus*, beberapa spesies Streptococcus dan *Bacillus subtilis*, tetapi juga terhadap galur sel eukariotik THP - 1 (human leukemia monocyte) dan BALB/3T3 (mouse embryonic fibroblast) .

Kata Kunci: Mangrove, *Avicennia marina, Botryosphaeria australis*, antibiotic activities, cytotoxicity

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1. Introduction

Microorganisms have long served mankind by desirable quality of numerous enzymes and secondary metabolites they generate. Furthermore, only a relatively small number of microbes are used directly in industrial applications (e.g. cheese, beer and wine production) as well as biological control of

pathogens and have direct usefulness to the human society. This terminology refers to either the microbes themselves or to one or more of their natural products of them.

The term of endophytes mean organisms that reside with plant. They can serve as symbiont either mutuality, commensalics or parasitics to the plant itself. The demand for chemical defense appears to be the basis for endophytic association between plant and their particular endosymbiont (Carroll ,1988; Clay, 1988). They serve as potential sources of novel natural products for exploitation in medicine, agriculture, as well as in industrial sectors (Strobel & Daisy, 2003). The ability to produce a large number of secondary metabolites by endophytes is mainly related to the filamentous actinomycetes, myxobacteria, *Pseudomonas* and cyanobacteria in bacteria, and mainly to the filamentous fungi for the eukaryotes (Donadio et al., 2002). Nowaday, this terminology is not only limited to microorganism with living together with higher plant, but also in-between algae and bacteria or fungi or other microorganisms that reside in them. Several of these compounds show pronounced pharmacological activities and are interesting candidates for new drugs primarily in the area of cancer treatment and antibiotics. A number of natural products from the plant or marine organisms show prominent structural similarities to known metabolites of microbial origin, suggesting that microorganisms are at least involved in their biosynthesis of these metabolites or host-symbiont modification.

The needs for new medicines for relieving human from many deadly diseases are never end. Many new developed medicines become less potent to the resistant to the diseases. A developed resistance bacteria, viruses, and other pathogenic microorganisms makes this problem arise exponentially these few years back.

Genus *Botryosphaeria* is found in many climate environments around the world. Ranging from terrestrial into marine environment, make them able to produce many interesting compounds which have various bioactivities. This genus has been reported as epiphytic fungus on mangrove *Sonneratia apetalla* (Xu et al., 2011), as an endophyte from cacao (*Theobroma cacao* L.) (Rubini et al., 2005) as well as an endosymbiont in sponge *Tetra auratium* (Wiese et al., 2011). The examples of isolated metabolites include Primin(Pongcharoen et al., 2007), 4-hydroxymellein (Venkatasubbaiah et al., 1991), and Lasiodiploin (Yang et al., 2006).

In this present study we focused on the discovery of structurally new and biologically active natural products from endophytic fungus, *Botryosphaeria australis*, was isolated from the leaves of the *Avicennia marina* collected from Hainan Province, P.R. China, and resulted in the isolation and structural elucidation of one new compouns from the class of polyketide.

2. Material and Methods

General. UV spectra were determined on a Dionex instrument with Chromeleon V6.3 as standard software programs. 1D and 2D NMR spectra were obtained at 300 and 500 MHz for ^{1}H and ^{13}C, on a DRX-300 and DRX-500 Bruker in mixture of deuterated CF_3CD_2OD/ D_2O (1:1) solvent with TMS as an internal standard. Mass spectra were recorded using a LCMS HP1100 Agilent Finnigan LCQDecaXP Thermoquest. HRMS (ESI) spectra were obtained with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Column chromatography both vaccum and pressurised were performed with Silica gel 60M (230-3400 mesh ASTM, Macherey-Nagel GmbH & Co.KG, Düren, Germany) and Sephadex column was performed using Sephadex LH-20 (Sigma). TLC was carried out with precoated Silica gel plates (TLC silica gel 60 F-254, Merck KGaA, Darmstadt, Germany). Semi preparative HPLC was carried out with Merck Hitachi L-7100 and L-7400 for pump and UV detector respectively, using C-18 column (Dr. Ing H. Knauler, GmbH, Berlin Germany).

Material. The part of *Avicennia marina* plant was collected from coastal region of Hainan Province P.R. China at the middle of July 2011. The endophytic fungus was isolated from the healthy leaves of related plant by using procedure previously described (Debbab et al. 2009). A strain (strain designation is AMCL7) is kept in of the author´s laboratory (P.P). Sequence data have been submitted to GenBank with accession number JQ974951.1.

Identification of Fungal Cultures. Fungal cultures were identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously (Wang et al., 2006). The BLAST search result showed that the sequence had 100% similarity to the sequence of *Botryosphaeria australis* (FJ037758.1), which is known as well as *Neofusicoccum australe.*

Cell Proliferation Assay. Cytotoxicity against the human leukemic monocyte cell line THP-1 and the embryonic fibroblast cell line BALB/3T3 was measured using a fluorometric Alamar Blue assay. BALB/3T3 THP1 cells were grown in DMEM medium (PAN Biotech GmbH), containing 4.5 g/l glucose and 3.7 g/ l $NaHCO_3$, supplemented with 10% fetal calf serum (v/v), 1% sodium pyruvate and 1% L-glutamine. THP1 cells were cultured in RPMI-1640 medium (PAN

Biotech GmbH) containing 2.0 g/l $NaHCO_3$, supplemented with 10% fetal calf serum (v/v), 1% L–glutamine and 1% penicillin-streptomycin (10000 U/ml penicillin and 10 mg/ml streptomycin; PAN Biotech GmbH). Cells were seeded in 96-well plates at 10^4 cells/well in 190 µl of cell culture medium without penicillin-streptomycin. Test compounds were dissolved in DMSO at a concentration of 10 mg/ml and twofold serial dilutions were prepared in cell culture medium. 10 µl sample of each compound dilution was added to the cells, resulting in final concentration of 0.016 – 32 µg/ml test compound in 0.00016 to 0.32 % DMSO. Cell culture medium and cycloheximide (Applichem) served as negative and positive controls, respectively. Plates were incubated for 48 h at 5% CO_2 and 37°C. Then, 10 µl Alamar Blue/well (Invitrogen) were added and the incubation continued for another 24 h. After 72 h of total incubation time in the presence of test compounds, fluorescence was read using a 96-well fluorometer (Tecan, infinite M200) with excitation at 560 nm and emission at 600 nm. Relative fluorescence units (RFU) were determined by the ratio of fluorescence emitted by treated cells vs. untreated cells and served for determination of the 50% inhibitory concentrations (IC_{50}). Experiments were carried out in duplicates.

Antibacterial activity: The Minimal Inhibitory Concentration (MIC) for each bacterial strain was determined by the broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI and M7-A8 2008). For preparation of the inoculum the direct colony suspension method was used and the final inoculum contained 2-5x10^5 Cfu/ml. The strain panel included antibiotic-susceptible CLSI quality control strains (*Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27799, a standard laboratory strain (*Bacillus subtilis* 168 (Burkholder and Giles 1947), a high-level quinolone-resistant laboratory mutant (*Escherichia coli* WT-3-1 MB2, Peter Heisig, University of Hamburg, Germany) and the following (multi)drug-resistant clinical isolates: *Staphylococcus aureus* Mu50 (Hiramatsu *et al.* 1997), *Staphylococcus aureus* 25697 (AiCuris, Wuppertal, Germany), *Streptococcus agalactiae* 013761 and *Streptococcus pyogenes* 014327 (Hans-Georg Sahl, University of Bonn, Germany), *Enterococcus faecalis* UW 2689 (Wolfgang Witte, Robert Koch Institute, Wernigerode, Germany), *Enterococcus faecium* 6011(Klare et al., 1995) and *Pseudomonas aeruginosa* B 63230 (Henrichfreise *et al.* 2005).

3. Results and Discussion

The ethyl acetate extract of *Botryosphaeria australis* were subjected into column chromatography over LH-20 sephadex column eluted using DCM/MeOH (1:1) continued by 100% MeOH as running eluents thus subjected into semipreparative HPLC to afford a new compounds from polyketide named as botryosphaenin (**1**) and 5 known compounds, botryosterpene (**2**) and 5-hydroxy-2,7-dimethoxyna phthalene-1,4-dione, (**3**) and its derivatives, 6-ethyl-5-hydroxy-2,7-dimethoxy naphthalene-1,4-dione (**4**), O-methylaspmenone (**5**), O-methylasparvenone (**6**) and 5-(carboxymethyl)-7-hydroxy-1,4a-dimethyl-6-methylen edecahydrona phthalene-14-carboxylic acid (**7**).

Compound **1** was obtained as a yellow amorphous powder. The UV spectrum showed absorbance at l_{max} 218, 262, 308 and 430 nm. The molecular formula was determined as $C_{15}H_{16}O_6$ on the basis of prominent signal detected at m/*z* 293.1024 $[M+H]^+$; 315.0833 $[M+Na]^+$; 607.1773 [2M+Na] in the HRESIMS. The ^{13}C NMR data of **1** exhibited fifteen carbon signals corresponding to four methyls whereas three of them were methoxy, three methine groups and eight quaternary C-atoms whereas five of them O-bearing carbons, including two C=O groups at *d* at 180.5 (C-1) and 191.7 (C-4) on the basis on DEPT spectrum (Table 1). Analysis of ^1H NMR spectrum indicated the

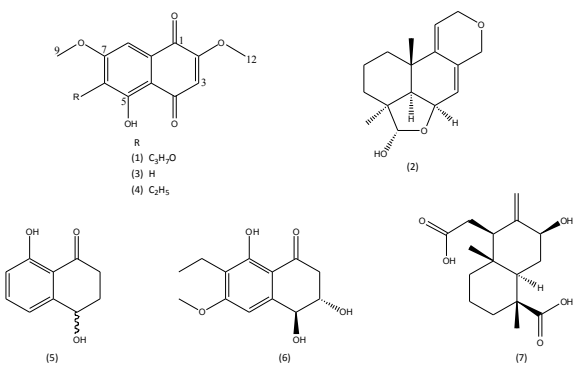

Figure 1. Isolated compounds of *B. australis.*

Table 1. ^{13}C and ^{1}H NMR data of 1. Recorded at 300MHz in CD_3OD; *d* in ppm; *J* in Hz

Position	$\delta^{13}C$	$\delta^{1}H$ (*J* in Hz)
1	180.5	
2	162.3	
3	110.4	6.18 (1H; s)
4	191.7	
5	162.3	
6	123.6	
7	164.8	
8	103.6	7.29 (1H; s)
9	133.3	
10	110	
11	72.1	5.12 (1H; q; *J* = 6.7; 6.7;
12	19.2	1.56 (3H; d; *J* = 6.7)
13	56.8	3.99 (3H; s)
14	57.3	3.92 (3H; s)
15	56.9	3.23(3H; s)

presence of one methyl which resonating at d^H 1.56 (3H; d; *J*=6.7; H-12) and three methoxy resonating at d^H 3.23 (3H; s; H-15), d^H at 3.92 (3H; s; H-14) and d^Hat 3.99 (3H; s; H-13), three methine groups divided as one O-bearing methane resonating at d^H 5.12 (1H; q; *J*= 6.7; H-11) and two aromatic methine protons resonating at d^H 6.18 (1H; s; H-3) and at d^H 7.29 (1H; s; H-8). Analysis of HMBC spectrum showed correlation between H-3 to keto- groups C-1 and C-4 and C-2 and C-10. The aromatic H-8 correlates to C-1, C-6, C-7, C-9 and C-10. Thus, proton bound to oxygenated carbon C-11 correlates to C-5, C-7, methyl C-12 and methoxy C-15. Proton H-12 correlates to C-6 and C-11 Figure 2).

Cross peaks were observed from methoxy protons to their adjacent carbon C-13, C-14 and C-15. Both olefinic protons H-3 and H-8 correlate to keto- group C-1. In fact, COSY data confirmed direct correlation of H-11 and H-12 and also long range correlations of H-3 and H-14 and H-8 and H-13. Therefore, from the data above, the structure of compound **1** was determined as 5-hydroxy-2,7-dimethoxy-6-(1-methoxyethyl) naphthalene-1,4-dione, named botryosphaenin. The NMR data were slight similar as reported in literature (Poch *et al.* 1992), except the additional aliphatic methoxy (H-15).

Botryosphaenin (= 5-hydroxy-2,7-dimethoxy-6-(1-methoxyethyl)naphthalene-1,4-dione, **2**), yellow amorphous. UV maxima at l_{max} 218; 262; 308; 430 nm. [*á*]D20 +29.5 (*c* 0.02, acetone). EI-MS=277 (loosing CH_3) and 292. HRESIMS *m/z* 293.1024 $[M+H]^+$; 315.0833 $[M+Na]^+$; 607.1773 [2M+Na] (calcd for $C_{15}H_{17}O_6$, 293.1025; $C_{15}H_{16}O_6Na$, 315.0833). ^{1}H and ^{13}C: see table 1.

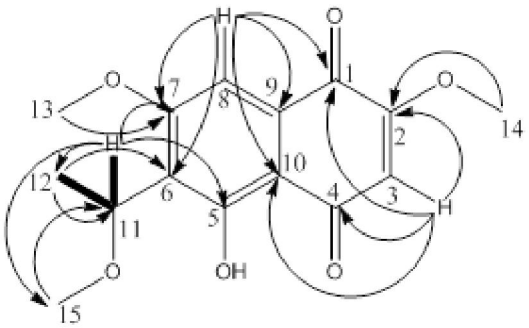

Figure 2. Structure of 1, and selected HMBC (arrows) and HH-COSY correlations (bold lines).

3.1. Antibacterial activity

The new compounds **1** and the known compounds **3**-**7** were evaluated for their antibacterial activity against a diverse spectrum of bacterial species, including antibiotic-susceptible reference strains and multi-resistant clinical isolates (Table 2). Among these, only compound **1** and its close congener **4** displayed antibacterial activity, similar in strength and spectrum. Both compounds inhibited the growth of Gram-positive bacteria of the genera *Staphylococcus*, *Streptococcus* and *Bacillus* with minimal inhibitory concentrations in the range of 2 to 32 µg/ml and affected even high-level (multi)drug-resistant clinical isolates like *S. aureus* Mu50, *S. aureus* 25697, *S. agalactiae* 013761 and *S. pyogenes* 014327. In preceding studies on the basis of diverging strain panels and agar dilution as the predominate method for MIC determination, a lack of antibacterial activity had been observed for compound **2**, **3**, **4**, **6** and **7** (Gerber and Wieclawek 1966; Otomo *et al.* 1983; Yuan *et al.* 2009; Xu *et al.* 2011), while compound **5** had been active against *B. subtilis* at an elevated concentration of 100 µg/ml (Kokubun *et al.* 2003).

3.2. Cytotoxicity

Compounds **1**-**7** were further tested for their cytotoxic activity against THP-1 human leukemic monocyte cells and BALB/3T3 mouse embryonic fibroblast cells (Table 3). The IC_{50} values of compound **1** for both cell lines were in the range of 0.5-2 µg/ml, indicating that compound **1** lacks specificity for prokaryotes. Again, compound **4** displayed a similar

Table 2. Minimal inhibitory concentration [µg/ml] of compound **1-7** MIC values were determined by the broth microdilution method in Mueller-Hinton broth according to the rec ommendations of the Clinical Laboratory Standards Institute

Tested organism	Resistance phenotype[a]	Compound						
Gram-positive		**1**	**2**	**3**	**4**	**5**	**6**	**7**
Staphylococcus aureus ATCC 29213	susceptible	8	>64	>64	16	>64	>64	(32)[b]
Staphylococcus aureus Mu50	CAZ^{Rc}, CLA^{R}, CIP^{R}, CLI^{R}, DOX^{R}, ERY^{R}, MET^{R}, MXF^{R}, KAN^{R}, TEL^{R}, RIF^{R}, (MRSA[d], VISA[e],)	16	>64	>64	8	>64	>64	>64
Staphylococcus aureus 25697	AMX^{R}, CHL^{R}, CIP^{R}, CLI^{R}, ERY^{R}, FOS^{R}, GEN^{R}, KAN^{R}, NIT^{R}, TET^{R} (MRSA)	8	>64	-64	8(4)	>64	>64	64
Streptococcus pneumoniae ATCC 49619	susceptible	2	>64	32	2	64	64	32
Streptococcus agalactiae 013761	DOX[f]	32	>64	>64	32	>64	>64	>64
Streptococcus pyogenes 014327	DOX[i]	16	>64	32	8	>64	>64	64
Enterococcus faecalis UW 2689	CLA^{R}, ERY^{R}, MXF^{R}, TEL^{R}, (VRE[g])	>64	>64	>64	>64	>64	>64	>64
Enterococcus faecium 6011	ERY^{R}, CLA^{R}, TEL^{R} (VRE)	>64	>64	>64	>64	>64	>64	>64
Bacillus subtilis 168	susceptible	32	>64	64	4(2)	>64	>64	-64
Gram-negative								
Pseudomonas aeruginosa B 63230	CAZ^{R}, CIP^{R}, CPM^{R}, GEN^{R}, IMI^{R}, MER^{R}, PIP/TAZ^{R}	>64	>64	>64	>64	>64	>64	>64
Escherichia coli ATCC 25922	susceptible	>64	>64	>64	>64	>64	>64	>64
Escherichia coli WT-3-1 MB2	CIP^{R}	>64	>64	>64	>64	>64	>64	>64
Klebsiella pneumoniae ATCC 27799	DOX^{R}, KAN^{R}	>64	>64	>64	>64	>64	>64	>64

Table 3. IC_{50} values [µg/ml] of compound **1-7** in THP-1 human leukemic monocyte and BALB/3T3 mouse embryonic fibroblast cells. IC_{50} values were determined using a fluorometric Alamar Blue assay

Tested cell line	Compound						
	1	2	3	4	5	6	7
THP-1	0.5	3	7	3	>32	>32	15
BALB/3T3	2	>32	>32	10	>32	>32	>32

result. Compound **2** and **3** inhibited only THP-1, but not the generally more robust BALB cells and compounds **5**-**7** demonstrated no cytotoxicity against the cell lines tested.

4. Conclusion

The new compounds (**1**) shows strong antibacterial activity toward several bacteria including multi drugs resistant clinical isolate *S. aureus* Mu50. However, it was also shows strong activity against cell lines tested, indicating its lack specificity to prokaryotes and eukaryotes cells.

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