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CHEMICAL CONSTITUENTS OF *Aspergillus carbonarius* ISOLATED FROM MARINE SPONGE *Aaptos suberitoides*

Muhammad Syahrur Ramadhan[1], Riga Riga[2], Euis Holisotan Hakim[1]*

Address(es):
[1] Natural Products Research Group, Organic Chemistry Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40116, Indonesia.
[2] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Jalan Prof. Dr. Hamka, Padang 25131, Indonesia.

*Corresponding author: holisotan@gmail.com

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ABSTRACT

An endophytic fungus identified as *Aspergillus carbonarius* was obtained from from sea sponge *Aaptos suberitioides*. Phytochemical study of fungal *A. carbonarius* led to the isolation of four compounds (**1-4**) of which compound **1** was a new isocoumarin derivative, (*R*)-6-hydroxy-3-(1-hydroxypropan-2-yl)-8-methoxyisocoumarin. The others (compounds **2-4**) were the known compounds. Isolation of these compounds was performed using vacuum liquid dan column chromatography. The structure elucidation and identification of these compounds were conducted with the help of NMR and HR-ESI-MS spectroscopic data. All isolated compounds (**1-4**) were tested for their antibacterial activity against five bacterial strains and displayed inactive activity against all tested microorganisms.

Keywords: *Aaptos suberitoides*, *Aspergillus carbonarius*, endpohytic fungus, isocoumarin

INTRODUCTION

Marine sponge *Aaptos suberitoides* is a species belonging to the Subiritidae family. Previous phytochemical investigation of genus *Aaptos* identified the presence of alkaloids, lipopeptides, and phenolic compounds. The compounds obtained from *Aaptos* have various biological activities including cytotoxic, antibacterial, and antifungal activity **(Rashid *et al.*, 2018; Hamada *et al.*, 2019; Tang *et* al., 2020; Wang *et al.*, 2020)**. In continuation of research on potentially bioactive compounds from marine sponge *Aaptos*, we investigated the endophytic fungi derived from *A. suberitoides*.

Endophytic fungi are microorganisms residing in the internal tissues and cause no apparent harm to the host. The study of endophytic fungi is one sustanaible way to reveal important compounds under the sea because they only requires a small fractions of marine organisms. Fungi can be easily cultivated and have a very short life span allowing for the production of sufficient amounts of organisms and compounds without harming the marine environment. Moreover, there are a huge number of bioactive compounds belonging to various structures have been reported from endophytic fungi **(Bramhachari *et al.*, 2019; Bovio *et al.*, 2019; Riga *et al.*, 2020; Roat and Saraf, 2020; Suryelita *et al.*, 2021; Ignjatovic *et al.*, 2021; Gue *et al.*, 2023)**. An endophytic fungus, *Aspergillus carbonarius*, has been obtained from the internal tissues of sea sponge *A. suberitoides* collected from Kepulauan Seribu, Indonesia. *Aspergillus* is the most ubiquitous genus in the environment and has been long exploited to be a rich source of pharmaceutically potent and diverse compounds such as isocoumarines, alkaloids, steroids, flavonoids, azolones, etc (**Arastehfar *et al,* 2021; Youssef *et al,* 2021**). The endophytic fungus, *A. Carbonarius*, helped its host to survived in the extreme conditions of marine environtments such as elevated hydrostatic pressure, low temperatures, and high concentrations of metals in the deep sea. However, there are no phytochemical studies of *Aspergillus* colonized with sea sponge *Aaptos suberitoides.* Based on them, the aim of the research was to determine the chemical constituents of fungus *A. carbonarius* isolated from *A. suberitoides*.

MATERIALS AND METHODS

General experimental procedure

The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on an Agilent spectrometer (Agilent Technologies, Santa Clara, United States). Chemical shifts are in ppm referring to the deuterated solvent signals at δ_H 2.05 and δ_C 206.3 (Acetone-d_6) for ^{1}H and ^{13}C, respectively. Q-TOF HRMS data were obtained by using HRMS Waters LCT Premier XE (Waters Corporation, Milford, United States). Optical rotations were measured using an Autopol IV polarimeter (Rudolph Research Analytical, United States). Vacuum liquid chromatography and gravitational column chromatography were performed on silica gel Kieselgel 60, 0.04–0.063 mm (Carl Roth, Germany). Thin-layer chromatography was conducted on silica gel plates (Silica gel 60 F_{254}, 20 x 20 cm, Merck, Germany), detected under UV light 254 nm, and sprayed by vanillin-sulfuric acid in ethanol followed by heating for 2 minutes.

Biological material

Sea sponge, *Aaptos suberitodes*, was collected from Taman Nasional Kepulauan Seribu, Indonesia (5°46'0.05"S, 106°35'2.34"E) at 7-10 m depth. The sample was stored at -20^{0}C with sea salt water until being further processed in laboratory. The sponge was sterilized by immersed in 70% ethanol for 10 s. After surface sterilization, the sample was cut and inoculated on sterilized malt extract agar media (15 g/L bacto agar, 15 g/L malt extract in distilled water, at pH 7.4–7.8) with chloramphenicol 0.2 g/L and incubated at 28 °C. After 5 days incubation, the fungi were transferred into the other sterilized MEA to obtain a single strain of endophytic fungus labelled with voucher specimen S1-2. Voucher strain was deposited at Natural Product Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung.

Identification of endophytic fungus

The strain voucher specimen S1-2 was identified based on the sequence homology of molecular rDNA ITS. The fungus was grown in 250 mL of Potato Dextrose Broth (PDB) and incubated for 72 hours. The DNA extraction of the fungus was conducted by using nucleon PHYTOpure reagent. The PCR amplification of ITS region was amplified using Primer ITS4 (5`-TCC TCC GCT TAT TGA TAT GC–3`) dan Primer ITS5 (5`-GGA AGT AAA AGT CGT AAC AAG G–3`) **(Raja *et al.*, 2017; Riga *et al.*, 2019)**. The purification of PCR product carried out with PEG precipitation method and then re-purified with ethanol purification method. The pure PCR product was sequenced with automated DNA sequencer. Sequence analyses were carried out using ChromasPro program (version 1.7.5) (Technelysium Pty Ltd., South Brisbane, Australia) and blasted at National Center for Biotechnology Information (NCBI). The molecular analysis of ITS region (GenBank accession number: MH854855) exhibited that S1-2 sequence were well similar to comprising strain of Aspergillus carbonarius IMI016136 with 99% sequence identity.

Sequence ITS region of fungus *A. carbonarius*

5'- TATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCT
GGAAAAAAAGGTTGGAGTTGTCGGCAGGCGCCGGCCAATCCTACAGA
GCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCC

GCTGCCTTTCGGGCCCGTCCCCCCAGACAGGGGGACGGCGACCCAAC
ACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC
CCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATT
CACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTC
ATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTG
AAAACAATCGACTCAGACTTCACGATTTCAGACAGTGTTCGTGTTGGT
GTCTCCGGCGGGCGCGGGCCCGAGGGGCAGAGATGCCCCCCCGGCGG
CCGACAAGCGGCGGGCCCGCCGAAGCAACAGGTACAATAGACACGG
GTGGGAGGTTGGGCCCAAAGGACCCGCACTCGGTAATGATCCTTCCG
CAGGTTCACCTACGGAAACCTTGTTAC-3'.

Fermentation, extraction, and isolation

The mature cultures were cut into small squares and transferred into autoclaved 1 L Erlenmeyer flasks (10 flasks) with solid rice medium (containing 100 g of rice in 110 mL of water) incubated at 28 °C for 28 days **(Kjer *et al*., 2010 ; Suryelita *et al*., 2023)**. The fungus cultures were extracted with ethyl acetate and concentrated via reduced pressure to yield 11.24 g crude extract. The crude extract was fractionated by vacuum liquid chromatography using gradient elution with *n*-hexane-EtOAc (100:0-0:100) and followed by acetone-methanol to obtain 4 fractions (A-D). Based on the weight of fraction, three potential fractions (A, B, and C) were further fractionated. Fraction B (1238 mg) was subjected by gravitational column chromatography and eluted with a stepwise gradient of *n*-hexane-EtOAc (8:2-7:3) to obtain 18 fractions (B1-B18). The potential subfraction B.12 (30.1 mg) was further purified over silica gel column chromatography, eluted with chloroform-acetone (19.5:0.5-8:2) to yield **1** (17 mg). A 628 mg of Fraction C was eluted by chloroform:acetone (19.5:0.5-8:2) over silica gel column chromatography to give two pure compounds **2** (5.5 mg) and **3** (5.1 mg). A 1258 mg of fraction A was fractionated using silica gel column chromatography eluted with *n*-hexane:EtOAc (9.5:0.5-8:2) to give 16 fractions (A1-A16). Sub-fractions A.10–A.13 (22.4 mg) were further separated with column chromatography over silica gel to yield compound **4** (6 mg).

Antimicrobial assay

Agar dilution assay was conducted to evaluate antimicrobial activities of isolated compounds. The protocols of antimicrobial assay referred to the previous procedure **(Klančnik *et al*., 2010; Riga *et al*., 2021)**. The isolated compounds were measured against three gram-positive bacteria: *S. aureus*, *E. faecalis*, and *S. saprophyticus* and two gram-negative bacteria: *P. mirabilis* and *S. enterica*. Bacterial suspensions (500 µL) were blended with 35 mL solid MHA at 55 ^{0}C. Each compound (50 μL) was delivered into the 6 mm hole. After 20 minutes, the plates were sealed with parafilm and then incubated at 37 ^{0}C for all bacterial cultures. After 24 h incubation, antimicrobial activities were measured in mm and compared to the positive control chloramphenicol (1 mg/mL) and negative control methanol.

RESULTS AND DISCUSSION

Structure elucidation

The endophytic fungus with voucher specimen S1-2 was isolated from the internal tissue of sea sponge *A. suberitodes*. The strain S1-2 was molecularly identified based on analyses of the internal transcribed spacer (ITS) region of the rDNA and reported as *A. carbonarius* (99% sequence identity). Four compounds (**1**-**4**) (Figure 1) were yielded from EtOAc extract of fungal *A. carbonarius* using various chromatography techniques, including vacuum liquid and column chromatography.

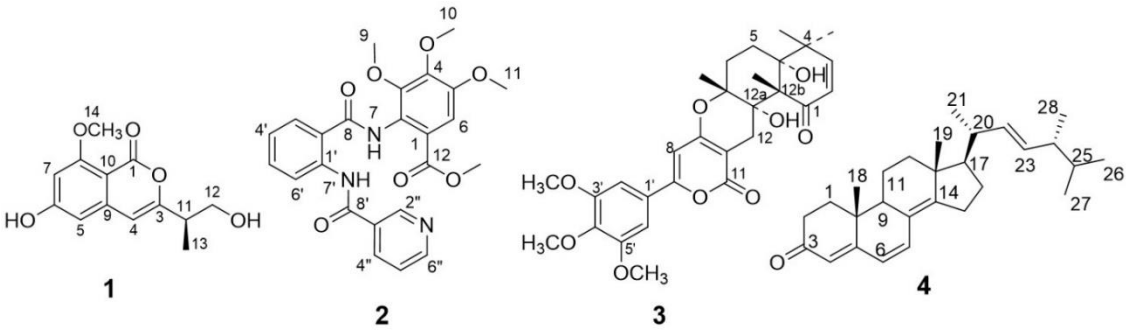

Figure 1 The structures of isolated compounds

(*R*)-6-Hydroxy-3-(1-hydroxypropan-2-yl)-8-methoxyisocoumarin (**1**) was isolated as a brown solid with $[\alpha]_D^{20}$: +41.60 (c 0.25, MeOH). The molecular formula $C_{13}H_{14}O_5$ was assigned based on the positive HR-ESIMS ions (*m/z* 251.0912 $[M+H]^+$) suggested seven degrees of unsaturation. Proton signals in the ^{1}H-NMR spectrum of **1** displayed a pair of meta aromatic proton [δ_H 6.51 (1H, *d*, J = 2.3 Hz); 6.44 (1H, *d*, J = 2.3 Hz)], indicating a tetrasubstituted aromatic ring; a vinyl methine proton at δ_H 6.48 (1H, *s*); a methine proton (δ_H 4.17, 1H, *sext*); a methoxy proton at δ_H 3.89 (3H, *s*); an oxygenated methylene proton at δ_H 2.60 (2H, *dd*, J = 7.7, 3.95); and a methyl group (δ_H 1.23, 3H, *d*, J = 6.2 Hz). The ^{13}C-NMR and HSQC data (Table 1) of **1** displayed the presence of 13 carbons. Nine of them were sp^2 hybridized carbons including a carbonyl ester carbon (δ_C 167.1), six carbons for a benzene ring (δ_C 167.9, 164.2, 140.8, 106.5, 100.9, 100.6), two carbons for an alkene (δ_C 156.7, 101.8). The other carbons were four sp^3 hybridized carbons where two of them (δ_C 56.3, 43.9) were directly connected with oxygen. The planar structure of **1** was mainly established by HSQC and HMBC spectra.

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **1** in acetone-d_6

Position	δ_H (*mult*., J_{Hz})	δ_C (C-type)	HMBC
1	-	167.1 (C)	-
3	-	156.7 (C)	C11, C12
4	6.48 (*s*)	101.8 (CH)	C3, C4, C9, C10
5	6.51 (*d*, 2.3)	106.5 (CH)	C5, C7
6	-	164.2 (C)	-
7	6.44 (*d*, 2.3)	100.9 (CH)	C6, C8
8	-	167.9 (C)	-
9	-	100.6 (C)	-
10	-	140.8 (C)	-
11	4,17 (*sext*, 6.15)	65.4 (CH)	-
12	2.60 (*dd*, 7.7, 3.95)	43.9 (CH_2)	C3, C11, C13
13	1.23 (*d*, 6.2)	23.6 (CH_3)	C11, C12
14	3.89 (*s*)	56.3 (CH_3)	C8

HMBC spectrum of **1** displayed ^{1}H-^{13}C correlations between H-12/C-3 and C-13 suggested the attachment of the C-12 side chain to C-3. Another HMBC correlation from H-14 to C-8 exhibited the methoxy group was linked to C-8 of an aromatic ring (Figure 2). The configuration of C-11 in **1** was identified by comparison of optical rotation of **1** with the NM-3 compound **(Tsuchida *et al*., 2003)**. From the spectroscopic data analysis, structure **1** was elucidated as a new isocoumarin derivative and named (*R*)-6-hydroxy-3-(1-hydroxypropan-2-yl)-8-methoxyisocoumarin.

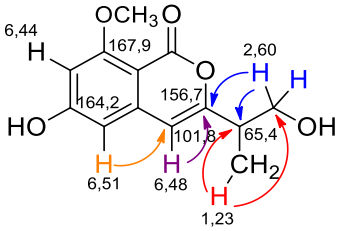

Figure 2 Selected HMBC correlations of compound **1**

The structures of three known compounds (Figure 1), methyl 3,4,5-trimethoxy-2-(2-(nicotinamido)benzamido)benzoate (**2**) **(Wang *et al*., 2011)**, territrem B (**3**) **(Peng *et al*., 1992)**, ergosta-4,6,8(14),22-tetraen-3-one (**4**) **(Qiao *et al*., 2010)** were recognized by the examination NMR, MS, and optical rotation data, as well as comparison of spectroscopic data published before.

Antimicrobial activity

Compounds (**1**-**4**) were tested for their antibacterial activities against three gram-positive bacteria (*S. aureus*, *E. faecalis*, and *S. saprophyticus*) and two gram-negative bacteria (*P. mirabilis* and *S. enterica*) using agar dilution method. All compounds displayed no inhibition zone against all tested bacteria.In addition, Compound **2** was also reported no antibacterial activities against *E. aerogenes*, *P.*

aeruginosa, *S. aureus* and *C. albicans* but exhibited moderate to pronounced cytotoxicity against the murine cancer cell line L5178Y, with an IC_{50} value of 0.2 µm **(Wang *et al*, 2011; Zhou *et al*, 2011)**. Territrem B **(3)** shown strong anticholinesterase ability with IC_{50} value of 0.071 mM **(Bunbamrung *et al*, 2020)**. Compound **4**, ergosta-4,6,8(14),22-tetraen-3-one, previously reported moderate cytotoxicity (IC_{50} = 6.24 µg/mL) against HCT-8 tumor cells **(Gallo *et al*, 2009**).

CONCLUSION

In conclusion, (R)-6-hydroxy-3-(1-hydroxypropan-2-yl)-8-methoxyisocoumarin, a new isocoumarin derivative, together with three known compounds were isolated from *Aspergillus carbonarius*, an endophytic fungus from marine sponge *Aaptos suberitoides*. Antibacterial activities of isolated compounds were determined against tested microorganisms.

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