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Isolation of Pandangolide 1 from Cladosporium oxysporum, An Endophyte of the Terrestrial Plant Alyxia reinwardtii

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Cover Page Footnote

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Isolation of Pandangolide 1 from *Cladosporium oxysporum*, An Endophyte of the Terrestrial Plant *Alyxia reinwardtii*

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Abstract

Pandangolide 1 was isolated from the ethyl acetate extract of *Cladosporium oxysporum* cultures. The fungus was originally obtained from *Alyxia reinwardtii*. The structure of pandangolide 1 was elucidated on the basis of nuclear magnetic resonance (NMR) spectroscopy and accurate mass spectrometric data. This is the first report of the isolation of pandangolide 1 from endophytic *C. oxysporum* derived from a terrestrial host plant.

Abstrak

Isolasi Pandangolide 1 dari *Cladosporium oxysporum*, suatu Endofit dari Tanaman Terestrial *Alyxia reindwartii*. Pandangolide 1 berhasil diisolasi dari ekstrak etil asetat jamur endofit *C. oxysporum* yang hidup dalam tanaman inang *A. reinwardtii.* Struktur pandangolide 1 dijelaskan berdasarkan data spektroskopi masa dan NMRnya. Pandangolide 1 pertama kali dilaporkan diisolasi dari cendawan endofit *C. oxysporum* yang hidup dalam tanaman inang di darat.

Keywords: alyxia reinwardtii, cladosporium oxysporum, pandangolide 1

1. Introduction

Endophytic fungi are those that grow intra- or intercellularly within the tissues of higher plants without causing overt symptoms of disease [1]. Endophytic fungi are known to produce various bioactive metabolites such as agrochemicals, antibiotics, immunosuppressants, anti-parasitics, antioxidants, and compounds with anticancer, antiviral, insecticidal, and antidiabetic activity [2-4].

We previously reported the isolation of a new compound, lecythomycin, and known compounds (2*R*)-3-(2-hydroxypropyl)-benzene-1,2-diol, kojic acid, 7-*O*-acetyl-kojic acid, *p*-hydroxybenzoic acid, emodine, 7-chloroemodine and ergosterol-5,8-peroxide from the fungus *Lecythophora* sp. growing as an endophyte in the Indonesian medicinal plant *Alyxia reinwardtii* [5,6]. As a continuation of our studies on endophytic fungi, we now describe the chemical analysis of another fungal species (fungus SUB-ALE-A), identified as *Cladosporium oxysporum*, which we have isolated from the same plant.

Endophytic *Cladosporium* spp. have been reported from both terrestrial and marine (sponges, algae, and mangroves) hosts. Metabolites reported from this fungal genus include pan-dangolides 2-4, cladospolide B, *iso*-cladospolide B, Sumiki's acid, acetyl Sumiki's acid [7], herbarin A-B, citreoviridin A, herbaric acid [8], sporiolides A-B [9], pandangolide 1, pandangolide 1a, *iso*-cladospolide B [10], brefeldin A [11], phenylacetic acid, *p*-hydroxyphenylethyl alcohol, L-ß-phenyllactic acid [12], huperzine A [13], aconitine [14], polyketides 1-3, cladospolides A-B, cladospolide A II, *iso*-cladospolide B, *seco*-patulolide C, pandangolide 1-3, and pandangolide 1a [15].

To the best of our knowledge, no report has yet appeared on metabolites of endophytic *C. oxysporum*. In this work, we report the isolation and structural elucidation of a metabolite from the fungus *C. oxysporum* growing as an endophyte in *A. reinwardtii*.

2. Methods

General experimental procedures. Electrospray ionization mass spectrometry **(**ESI-MS) was recorded on a UPLC Dionex Ultimate 3000 coupled to a QTOF Bruker Maxis Impact HD. NMR spectra were recorded on a VNMRS400 Varian 400MHz instrument. TLC was carried out on silica gel 60 F_{254} TLC plates (Merck) and reversed phase C-18 TLC plates (Merck). Column chromatography used silica gel 60 (Merck) as the stationary phase. Detection was performed by UV light at wavelengths of 254 and 366 nm, and also through derivatization with *p*-anisaldehyde-H_2SO_4 reagent.

Plant material and fungal isolation. *A.reinwardtii* was collected from Purwodadi Botanical Garden, East Java, Indonesia in April 2003. The plant material was identified by Dr. Irawati at the Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia (voucher no. 710/IPH.1.02.If.8/2003). Stems of *A. reinwardtii* were surface sterilized with 70% ethanol and 75% Clorox (Bayclin^{TM}). The sterilized stems were cut aseptically and parts of the inner tissues were imprinted onto agar plates containing malt extract agar (MEA) medium with added powdered plant material (15 g/L) and chloramphenicol (0.2 g/L). Pure strains were obtained by repeated inoculation of growing fungi on agar plates with fresh MEA medium (without powdered plant material and chloramphenicol). The isolated fungus (SUB-ALE-A) was identified by Dr. Arnulf Diesel (Heinrich-Heine-Universität Düsseldorf, Germany) as *C. oxysporum* based on its ITS sequence, as described previously [5,6].

Malt extract broth (MEB) culture of *C. oxysporum*. Flasks (500 mL) containing 250 mL of MEB medium were sterilized. A small part of a fungal culture from slant agar medium was transferred under sterile conditions to the MEB medium. The fungus was grown under static conditions at room temperature (approx. 30±3 °C) for four weeks.

Extraction, isolation, and structure elucidation. The culture broth and mycelia were extracted with EtOAc. The organic layers were collected, combined, and concentrated *in vacuo* to obtain a dark brown extract (5.49 g). The dried extract (3.0 g) was subjected to column chromatography on silica gel 60, and eluted with mixtures of *n*-hexane, EtOAc, and MeOH in increasing polarity, to yield 16 fractions. Fraction 7 (186 mg) was purified two times by preparative TLC (silica gel 60 F_{254}, EtOAc:$CHCl_3$: MeOH (5:5:1), followed by $CHCl_3$:MeOH (9:1), to provide sub-fraction AR-7.2.2 (11.7 mg). The isolated compound (**1**) (4.8 mg) was obtained after further crystallization of AR-7.2.2 with methanol. The molecular structure of the isolated compound was deduced by spectroscopic methods including ^{1}H NMR, COSY, HSQC, HMBC, and HRESI-MS.

3. Results and Discussion

HR-ESIMS of **1** (positive ion mode) exhibited pseudo-molecular ions $[2M+Na]^+$ at *m/z* 511.2525 (calculated, 511.2514), $[M+NH_4]^+$ at *m/z* 262.1656 (calculated, 262.1649) and $[M+H]^+$ at *m/z* 245.1387 (calculated, 245.1384), consistent with the molecular formula $C_{12}H_{20}O_5$. Isotopic pattern analysis confirmed this molecular formula. MS fragmentation of **1** (Figure 1) produced fragment ions $[M-H_2O+H]^+$ and $[M-2H_2O+H]^+$, at *m/z* 227.1283 (calculated,227.1276) and 209.1280 (calculated,209.1276), respectively, consistent with a consecutive loss of two water moieties and indicating that **1**might contain two hydroxyl groups.

The^{1}H NMR spectrum of **1** (Table 1) showed a total of six diastereotopic methylenes (δ 1.97, 1.76, 1.41, 1.24, 1.51, 1.10, 1.24[2H], 1.67 [2H], 3.23 and 2.92), three methines (δ 4.88, 4.71 and 4.30), and a methyl group at δ 1.24 ppm.

HSQC data showed that **1** had a methyl group at δ 22.3 ppm, six methylene resonances at δ 42.7, 33.2, 30.5, 29.7, 26.5, and 19.1, and three oxymethines at δ 74.8, 76.1 and 65.4, respectively. Based on its molecular formula,**1** thus had two quaternary carbons that resided at δ 174.4 and 209.4, consistent with carbonyl groups for an ester and a ketone, respectively. As the molecular formula of **1** required three double bond equivalents, **1**was concluded to be a lactone.

The COSY experiment (Figure 2) established correlations between H-2 (δ 3.23) and H-3(δ 4.71), H-6(δ 1.97) and H-5 (δ 4.30), and also a terminal methyl H_3-12 (δ 1.24) adjacent to H-11 (δ 4.88).

In the HMBC experiment (Figure2 and Table 1), an important correlation was observed between the oxyme thine proton at δ 4.88 (H-11) and the carbonyl group at δ 174.4 (C-1), which confirmed the position of the lactone moiety.

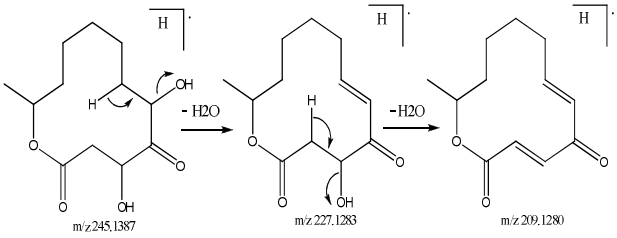

Figure 1. MS Fragmentation of 1

Table 1. Comparison of NMR Data of 1 with Pandangolide 1

Position	δ_H, mult.	δ_C, mult	COSY	HMBC (H to C)	δ_C, mult of pandangolide 1
1		174.4 qC			174.2 qC
2	A. 3.23 dd	42.7 CH_2	3, 2B	C-1, C-3, C-4	42.3 CH_2
	B. 2.92 dd		2A	C-1, C-3, C-4	
3	4.71 dd	65.4 CH	2A	C-1, C-2, C-4	65.6 CH
4		209.4 qC			210.7 qC
5	4.30 dd	76.1 CH	6	C-4, C-6, C-7	76.6 CH
6	A. 1.97 dq	30.5 CH_2	5, 6B	C-4,C-5, C-7, C-9	30.4 CH_2
	B. 1.76dddd		6A	C-7, C-9	
7	A. 1.41 dp	19.1 CH_2		C-11	19.3 CH_2
	B. 1.24 m				
8	A. 1.51 ddt	26.5 CH_2	8B	C-11	26.6 CH_2
	B. 1.10 ddt		8A	C-11, C-12	
9	1.24 m [2H]	29.7 CH_2		C-6, C-8	21.4 CH_2
10	1.67 ddt [2H]	33.2 CH_2		C-8, C-12	32.4 CH_2
11	4.88 ddq	74.8 CH	12	C-1, C-8, C-10	74.5 CH
12	1.24 m [3H]	22.3 CH_3	11	C-10	20.3 CH_3

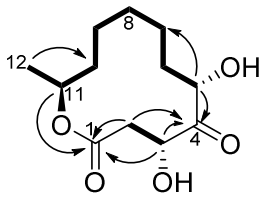

Figure 2. Important COSY (bold) and HMBC (arrows) Correlation of 1

The position of the ketone carbonyl group (C-4, δ 209.4) was established by correlation to H-2, H-6, H-3, and H-5. Based on this evidence and on comparison with previously reported NMR data [10,16], **1** was unambiguously identified as pandangolide 1.

Previously, pandangolide 1 was isolated from an unidentified fungus obtained from a sponge [16],the endophytic *Cladosporium* sp. IFB3lp-2 isolated from the mangrove plant *Rhizophora stylosa* [15], and the marine-derived *Cladosporium* sp. F14 [17].

4. Conclusion

Pandangolide 1 was isolated for the first time from the *C. oxysporum* endophyte of the terrestrial plant *A. reinwardtii*.

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