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Chemical Constituents Isolated from the Moss-derived Fungus *Talaromyces* sp.

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Abstract All plants in natural ecosystems are living in symbiosis with endophytes. Recently, there has been an increasing interest in endophytes since these organisms can interact with the hosts and produce various structurally or biologically interesting molecules. This study aimed to identify these molecules from endophytes. Chemical investigation of *Climacium dendroides*-derived fungus *Talaromyces* sp. resulted in the isolation of two diphenyl ether derivatives, purpactin A (**1**) and penicillide (**2**), and two steroids, dankasterone A (**3**) and calvasterol B (**4**). The structures of the compounds were identified via extensive spectroscopic and spectrometric methods. Four compounds did not show any antioxidative activities in the on-line antioxidant activity screening system.

Keywords bryophyte, fungi, *Talaromyces* sp., diphenyl ether derivative, steroid

Introduction

Endophytes exist ubiquitously in all plant species on our planet. These organisms reside in the plant tissues under the epidermal cell layers without causing any negative effects to the host. Recent studies have revealed that approximately 0.3 million plant species exist and are the host to one or more endophytes.[1] The population of a given species varies from several to a few hundred strains. Endophytes indirectly benefit the plant by producing various secondary metabolites that contribute to plant protection and survival.[2] In support of this idea, many researchers have reported hundreds of biologically active compounds, such as alkaloids, terpenoids, flavonoids, and steroids, from endophytes.[3] Most of the compounds have been found to possess antimicrobial or anticancer activities.[3, 4] This indicates that these organisms could be potential sources to discover leads compounds. However, only 10% or less of endophytic fungi have been described so far although it is estimated that there might be at least one million different endophyte species.[1] Considering their ecological roles and the potential of yielding metabolites, to investigate the secondary metabolites of endophytes could increase the chance to discover new potent compounds. Bryophytes are the second biggest group of plant species consisting of mosses, hornworts, and liverworts.[5] Among bryophytes, mosses usually grow in a wide variety of habitats such as soil, old tree, wetland, and wet rocks, building extensive contact with a lot of microorganisms.[5] A few studies have been reported that these microorganisms, mainly fungi consisting mostly of Ascomycota, might be important for the survival of the mosses and adaptation to extreme environments.[6] Despite the ecological importance of endophytic fungi in mosses, these fungi have re-

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ceived relatively little attention so far. Accordingly, it is necessary to investigate the function of endophytic fungi and what secondary metabolites are produced from these fungi.

As part of the project to discover secondary metabolites from endophytic fungi of mosses, we primarily collected several mosses in Korea. In particular, *Climacium dendroides*, known as tree climacium moss belonging to Climaciaceae, was selected for further studies since this species is most commonly found in Korea. Then several endophytic fungi including *Talaromyces* sp., *Penicillium* sp., and *Humicola* sp. were isolated from this moss. Preliminary chemical investigation using high-performance liquid chromatography-mass spectrometry (HPLC-MS) led to selecting *Talaromyces* sp. for further isolation and identification of chemical constituents. In this study, we describe the isolation and structural characterization of the isolated compounds.

Experimental Methods

General Experimental Procedures - Nuclear magnetic resonance (NMR) spectra were acquired on a Varian 500 MHz NMR spectrometer (USA). HPLC-MS was performed using an Agilent 1200 system equipped with an Agilent 6120 single quadrupole mass detector. On-line HPLC-2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (HPLC-ABTS) was performed using an Agilent 1200 system. A Phenomenex Luna C18(2) column (5 μm, 150 × 4.6 mm i.d.) was used for both HPLC methods. Semi-preparative HPLC was performed using a Gilson 321 HPLC system with a YMC-Pack ODS-A column (5 μm, 250 × 10 mm i.d.).

Isolation and Identification of Endophytic Fungi - *Climacium dendroides* was collected in Gangneung Institute of Natural Products of KIST in November of 2017. The collected mosses were washed with sterilized distilled water, sterilized with 70 % EtOH for 5 seconds twice, and washed again with sterilized distilled water and dried. Some of the mosses were pressed onto YME agar plates (2 g of yeast extract, 5 g of malt extract, and 2 g of dextrose, 9 g of agar powder in 500 mL of distilled water) to make sure the mosses were fully sterilized. The mosses were chopped and mixed with sterilized distilled water (1 mL) by vortexing and these were diluted 1:10 and 1:100 with sterilized distilled water, respectively. Some of the samples (100 μL) were spread out over the surface of three different agar plates (YME, YME with 0.05 % (w/w) nalidixic acid, YME with 0.05 % nalidixic acid and 0.05 % nystatin). All plates were incubated at 25 °C for 3 to 7 days. Each colony was identified using a basic local alignment search tool (BLAST) and phylogenetic analysis based on 18s rRNA gene sequences.

Culture, Extraction, and Isolation of Secondary metabolites - *Talaromyces* sp. was cultured in 16 L (500 mL each in a 1 L baffled Erlenmeyer flask) of YME broth using a rotary shaker (200 rpm) at 27 °C for 7 days. The supernatants were filtered and extracted with ethyl acetate (EtOAc) (2 × 16.0 L) to obtain the EtOAc layer. The pellets were extracted with acetone and methanol (MeOH) 1:1 (2 × 2.0 L) through sonication. All extracted samples were combined and evaporated under vacuum to obtain the dried extracts (2.5 g). The EtOAc extract was fractionated on silica gel by flash column chromatography (column size 30 × 1.5 cm, *n*-hexane−EtOAc−MeOH, 70:1:0 to 0:1:1) to obtain 40 fractions. Fraction 17 (17.0 mg) was subjected to semi-preparative HPLC (acetonitrile−H_2O, 16:9 in 110 min) at 250 nm to yield compounds **1** (0.6 mg, t_R 11.3 min), **3** (0.8 mg, t_R 107.1 min), and **4** (0.8 mg, t_R 109.5 min). Fraction 22 (53.5 mg) was subjected to semi-preparative HPLC (acetonitrile−H_2O, 1:9 to 1:0 in 90 min) at 270 nm to obtain compound **2** (7.5 mg, t_R 45.2 min).

Purpactin A (**1**): colorless oil; ESI-MS *m*/*z* 413.1 [M − H]$^-$ (calcd. for $C_{23}H_{25}O_7$, 413.1); ^{1}H and ^{13}C NMR data, see Table 1.

Penicillide (**2**): colorless oil; ESI-MS *m*/*z* 371.1 [M − H]$^-$ (calcd. for $C_{21}H_{23}O_6$, 371.1).

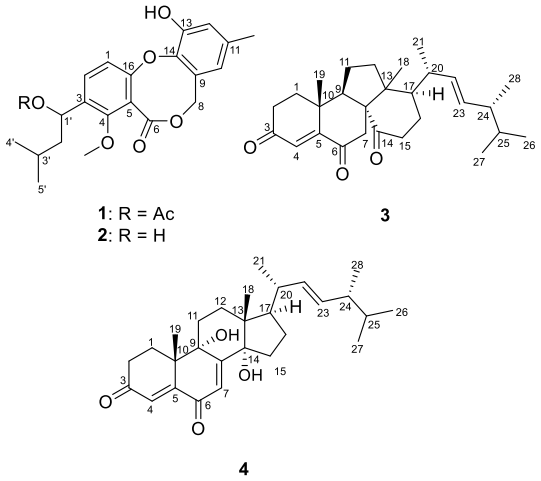

Figure 1. Structures of all isolated compounds.

Dankasterone A (**3**): colorless amorphous solid; ESI-MS *m*/*z* 425.1 $[M + H]^+$ (calcd. for $C_{28}H_{41}O_3$, 425.1); ^1H and ^{13}C NMR data, see Table 2.

Calvasterol B (**4**): white amorphous solid; ESI-MS *m*/*z* 439.1 $[M - H]^-$ (calcd. for $C_{28}H_{40}O_4$, 439.1).

Online HPLC-ABTS assay - An online HPLC-ABTS assay was carried out by referring to previous reports.[7] ABTS mixture was prepared with ABTS (0.08 mM) and potassium peroxodisulfate (0.12 mM). The chromatogram was visualized at 734 nm.

Results and Discussion

A chemical investigation on *Talaromyces* sp. was carried out to explore the main constituents of this fungus. Application of several chromatographic and spectroscopic techniques led to the isolation of two diphenyl ether derivatives, purpactin A (**1**)[8] and penicillide (**2**),[9] as well as two steroids, dankasterone A (**3**)[10] and calvasterol B (**4**) (Fig. 1).[11]

Compound **1** was isolated as colorless oil and its molecular formula of $C_{23}H_{26}O_7$ was confirmed by LC-MS analysis, suggesting 11 degrees of unsaturation. The ^1H NMR data (Table 1) showed four methyl signals (δ_H 2.25, 2.06, 0.95, and 0.95), one methoxy signal (δ_H 4.03), and two methylene signals (δ_H 5.12, 5.02, 1.77, and 1.50). Also, two methine signals (δ_H 6.12 and 1.64) and three aromatic signals (δ_H 7.45, 6.87, and 6.87). The ^{13}C NMR data (Table 1) assignment was carried out by analysis of 2D NMR data including heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) NMR data. These data showed 23 resonances attributable to five methyl carbons, two methylene carbons, two methine carbons, 12 aromatic carbons, and two carbonyl carbons. These 1D data were the same as those of purpactin A reported in previous literature.[8]

Table 1. NMR data of compound **1.**

	1	
position	δ_H, mult. (*J* Hz)	δ_C
1	6.87, d (8.5)	117.5, CH
2	7.45, d (8.5)	130.6, CH
3		134.4, C
4		154.6, C
5		120.1, C
6		166.8, C
8	5.12, d (14.5)	68.8, CH_2
	5.02, d (14.5)	
9		125.8, C
10	6.39, d (2.0)	120.8, CH
11		135.0, C
12	6.87, s	117.3, CH
13		147.1, C
14		141.1, C
16		151.4, C
17	2.25, s	20.7, CH_3
1′	6.12, dd (9.5, 4.5)	68.6, CH
2′	1.77, m	45.3, CH_2
	1.50, m	
3′	1.64, m	24.8, CH
4′	0.95, d (6.5)	21.8, CH_3
5′	0.95, d (6.5)	23.1, CH_3
1″		170.1, C
2″	2.06, s	21.1, CH_3
4-OCH_3	4.03, s	62.6, CH_3

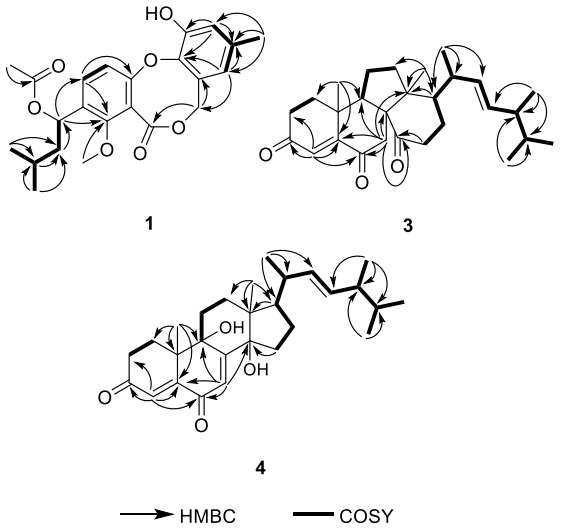

Figure 2. COSY and HMBC correlations of compounds **1**, **3** and **4**.

To confirm the exact structure, a detailed analysis of the 2D NMR data including HSQC, HMBC, and ^{1}H-^{1}H correlation spectroscopy (COSY) data was carried out (Fig. 2). The HMBC cross-peaks of H-1/C-5 (δ_C 120.1) and H-2/C-16 (δ_C 151.4) indicated an aromatic ring is attached at C-5 and C-6, and those of H-8/C-9 (δ_C 125.8), H-10/C-8 (δ_C 68.8) suggested another aromatic ring is attached at C-9 and C-14. The downfield shift of the ^{13}C NMR data at C-3 (δ_C 134.4), C-11 (δ_C 135.0), and C-1′ (δ_C 68.6) suggested that additional functional groups were attached at these positions. Furthermore, the HMBC cross-peaks of H_3-17/C-11 (δ_C 135.0), H-1′/C-2 (δ_C 130.6), and H-1′/C-1″ (δ_C 170.1) demonstrated the exact positions of 4-methylpentan-2-ol, aromatic methyl, and acetoxy group at C-3, C-11, and C-1′, respectively. Therefore, compound **1** was determined to be the same as purpactin A.

Compound **2** had the molecular formula of $C_{21}H_{24}O_6$, which suggested this compound was deacetyl form of compound **1**. The 1D NMR data were superimposable to those of compound **1**. The disappearance of the acetyl group was evidenced by the upfield shift of ^{13}C NMR data at C-1′ (δ_C 66.6). The complete structure was additionally assigned by the interpretation of the 2D NMR data. Thus, compound **2** was concluded to be penicillide.[9]

Table 2. NMR data of compound **3.**

	3	
position	δ_H, mult. (*J* Hz)	δ_C
1	2.08, dd (13.5, 5.0)	38.8, CH_2
	2.04, m	
2	2.45, m	34.3, CH_2
	2.53, m	
3		199.0, C
4	6.36, s	126.5, CH
5		156.0, C
6		200.0, C
7	2.67, m	40.8, CH_2
	2.50, m	
8		62.1, C
9	2.81, t (9.0)	49.3, CH
10		36.0, C
11	2.02, m	25.1, CH_2
	1.85, m	
12	1.77, m	38.3, CH_2
	1.73, m	
13		53.9, C
14		214.8, C
15	2.48, m	37.9, CH_2
16	1.90, m	23.2, CH_2
	1.69, m	
17	1.48, m	49.3, CH
18	0.98, s	17.0, CH_3
19	1.26, s	24.0, CH_3
20	2.41, m	37.2, CH
21	1.09, d (7.0)	23.6, CH_3
22	5.27, m	132.3, CH
23	5.27, m	135.1, CH
24	1.88, m	43.2, CH
25	1.46, m	33.0, CH
26	0.82, d (6.5)	19.6, CH_3
27	0.82, d (6.5)	20.0, CH_3
28	0.91, d (7.0)	17.6, CH_3

Compound **3** was isolated as colorless amorphous solid and its molecular formula of $C_{28}H_{40}O_3$ was determined by LC-MS analysis. The ^{1}H NMR data (Table 2) displayed six methyl signals (δ_H 1.26, 1.09, 0.98, 0.91, 0.82, and 0.82) and seven methylene signals (δ_H 2.67, 2.53, 2.50, 2.48, 2.45, 2.08, 2.04, 2.02,

1.90, 1.85, 1.77, 1.73, and 1.69), five methine signals (δ_H 2.81, 2.41, 1.88, 1.48, and 1.46), and three olefinic signals (δ_H 6.36, 5.27, and 5.27). The ^{13}C NMR data (Table 2) assignment was determined by interpreting the 2D NMR data. The data displayed 28 resonances assigned to six methyl carbons, seven methylene carbons, five methine carbons, four olefinic carbons, three carbonyl carbons, and three quaternary carbons. These data were the same as those of dankasterone A,[10] which consisted of four rings. The connections of ring systems and the functional groups were determined by the HSQC, HMBC, and COSY data (Fig. 2). The relative configuration was determined to be the same as that of dankasterone A by comparing the NMR data with previous literature. Therefore, compound **3** was determined as dankasterone A.

Compound **4** had the molecular formula of $C_{28}H_{40}O_4$, which suggested compound **4** could be a steroid derivative. The 1D NMR data was the same as those of calvasterol B,[11] which was typically composed of three six-member cyclohexane ring and one five-member cyclopentane ring. The additional functional group was attached at C-17 as evidenced by the HMBC cross-peak of H_3-21/C-17 (δ_C 50.0). Also, the positions of the remaining functional groups were determined by interpreting the HMBC and COSY data. The relative configuration was deduced to be the same as that of calvasterol B based on NMR data. Therefore, compound **4** was concluded to be calvasterol B.

All isolated compounds were evaluated for their antioxidative effects using an on-line HPLC-ABTS system. The chromatogram was recorded at 734 nm as a negative peak to identify free radical scavengers. However, all compounds did not show any antioxidative effects.

In conclusion, we isolated and characterized four constituents from the culture media of *Talaromyces* sp. So far, various types of compounds including steroids, peptides, diphenyl ethers, and azaphilones were identified from the endophytic fungus *Talaromyces* sp.[12, 13] Although it has been previously reported that purpactin A (**1**), penicillide (**2**), and dankasterone A (**3**) were isolated from *Talaromyces* sp., this study is the first report that calvasterol B (**4**) was isolated from *Talaromyces* sp. Even though all isolated compounds did not show any antioxidative activities in our system, finding the biological activities of these compounds could be worthwhile because some steroids including calvasterol B showed moderate cytotoxicity against several cancer cell lines.[14] Accordingly, further study is needed to determine the use of these compounds.

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