Two New Fungal Metabolites from an Epiphytic Fungus *Paraphaeosphaeria* Species

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As illustrated by a number of biologically active fungal metabolites such as penicillins, cyclosporins, and lovastatin, fungi are clearly an important source of useful secondary metabolites. To date, most of the useful compounds obtained from fungi have been encountered by application of random screening methods using fungi from soil environments.[1] However, with several thousand fungal metabolites now known from these sources, screening such fungi for new bioactive compounds often leads to re-isolation of known metabolites. Therefore, as an alternative strategy to isolate new fungal metabolites, employing ecological and/or taxonomic considerations when selecting target organisms for chemical investigation has been stressed by several authors.[2] For example, endophytic fungi, which reside in the tissues of living plants, are one of chemically unexplored fungal niches, and thus considered as potential sources of novel natural products.[3-8] Similarly, fungi inhabiting plant surface (epiphytic fungi) represent another promising fungal niche because not only they are virtually unexplored from a chemical standpoint, but fungal interference competition on the phylloplane microflora has been observed.[9]

During the course of initiated studies of endophytic and epiphytic fungi as potential sources of new bioactive secondary metabolites, we investigated an isolate of *Paraphaeosphaeria* sp. collected from the needles of *Pinus densiflora* in Kwanak Mt. Chemical studies of the EtOAc extract of the filtered culture broth obtained from this fungus have led to the isolation and structure determination of two new fungal metabolites named 2,3-didehydropalitantin (**1**) and culpin-1-*β*-galactopyranoside (**2**). Details of these studies are presented here.

2,3-Didehydropalitantin (**1**) has the molecular formula $C_{14}H_{20}O_4$, as deduced from ^{13}C NMR and HRESIMS data. This formula indicated five degrees of unsaturation. The ^1H NMR and DEPT data revealed the presence of one methyl group, two methylene units (one oxygenated), six olefinic protons, and four sp^3 methine units (two oxygenated). Comparison of the DEPT results and molecular formula indicated the presence of three exchangeable protons. These data, together with the signals corresponding to a carbonyl group and three double bonds observed in the ^{13}C NMR data, indicated that compound **1** must be a monocyclic compound with three free hydroxy groups. COSY (Table 1) experiments defined the partial proton spin systems in **1** corresponding to C7-C11, C8-C13-C14, and C1-C2. The ^{13}C NMR signal position assignments were confirmed by HMQC data (Table 1), and connectivities among the subunits in **1** were deduced from HMBC correlations (Table 1). For example, correlations of H-13 and H-14 with the carbonyl carbon C-12, along with correlations of H-10 and

Table 1. 1D and 2D NMR data for **1** in CD_3OD

no	δ_C (mult)[a]	δ_H (int, mult, *J* in Hz)[b]	COSY	HMBC (H → C)
1	17.1 (q)	1.74 (3H, d, 6.8)	H-2, H-3	C-2, C-3
2	129.3 (d)	5.68 (1H, dg, 14.8, 6.8)	H-3	C-1, C-4
3	131.8 (d)	6.00-6.20 (1H, m)	–	–
4	132.6 (d)	6.00-6.20 (1H, m)	–	–
5[c]	132.0 (d)	6.00-6.20 (1H, m)	–	–
6[c]	129.7 (d)	6.00-6.20 (1H, m)	–	–
7	134.3 (d)	5.55 (1H, dd, 14.4, 9.2)	H-6	C-6, C-8, C-13
8	39.3 (d)	2.74 (1H, m)	H-7, H-9, H-13	C-7, C-9, C-12
9	36.7 (t)	1.94 (2H, m)	H-8, H-10	C-8, C-11, C-13
10	72.6 (d)	4.26 (1H, dd, 6.0, 3.2)	H-9, H-11	C-12
11	77.6 (d)	4.28 (1H, d, 3.2)	H-10	C-8[d], C-10, C-12
12	209.5 (s)	–	–	–
13	54.9 (d)	2.41 (1H, m)	H-8, H-11, H-14	C-7, C-8, C-12, C-14
14	58.1 (t)	3.68 (2H, m)	H-13	C-8, C-12, C-13

[a]Recorded at 100 MHz. Carbon multiplicities were determined by DEPT experiments. [b]Recorded at 400 MHz. [c]Assignments may be interchanged. [d]Weak four-bond correlation.

H-11 with this carbonyl carbon, enabled to establish the linkage of C-12 to C-11 and C-13 to form a cyclohexanone ring moiety in **1**. Further HMBC correlations of H-1 with the olefinic carbon at δ 131.8 (C-3) and H-2 with a carbon at δ 132.6 (C-4) allowed spectral assignments of C-3 and C-4. Although additional HMBC correlations of H-7 with an olefinic carbon at δ 129.7 (C-6) were observed, overlapping of the olefinic proton signals and lack of further HMBC correlations precluded further specific assignments of the remaining two sp^2 carbons at δ 129.7 and δ 132.0. However, since these two olefinic carbons could not be located elsewhere, the side chain in compound **1** could be completed by inserting these two olefinic carbons between C-7 and C-4. Since three exchangeable protons are present in the molecule, three hydroxyl groups must be attached to oxygenated methine carbons (C-10 and C-11), and methylene carbon (C-14), respectively, leading to assignment of the gross structure as shown in **1**.

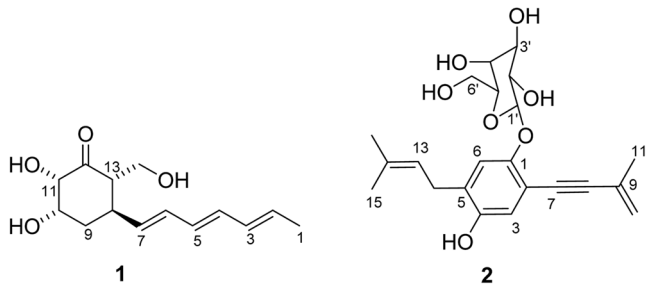
The relative stereochemistry shown for **1** was assigned on the basis of NOESY data and analysis of coupling constants. A key NOESY correlation of H-8 to H_2-14 required that these protons be on the same face of the cyclohexanone ring system. This observation thus indicated trans-axial relationship between H-8 and H-13 protons. NOESY correlation of H-13 to H-7 supported this relationship. After the axial position of H-13 was proposed by the above data, NOESY correlation of H-13 to H-11 indicated the axial deposition of H-11. Next, the *J* value (3.2 Hz) for H-10 and H-11 suggested the equatorial position of H-10, and thus the relative stereochemistry of cyclohexanone ring moiety in 2,3-didehydropalitantin (**1**) was proposed as shown. The *E* geometries of $\Delta^{2,3}$ and $\Delta^{6,7}$ double bonds were defined on the basis of coupling constants of $J_{2,3}$ = 14.8 Hz and $J_{6,7}$ = 14.4 Hz. Due to signal overlapping of other olefinic protons resonated in the region of δ 6.0-6.2, the geometry of $\Delta^{4,5}$ could not be unambiguously determined. However, structural similarities found in compound **1** and other previously reported fungal metabolites, such as palitantin[10] and penihydrone,[11] suggested the geometry of $\Delta^{4,5}$ double bond in **1** as *E*.

The molecular formula of culpin-1-β-galactopyranoside (**2**) was deduced to be $C_{22}H_{28}O_7$ on the basis of ^{13}C NMR and HRESIMS data, requiring nine degrees of unsaturation. The DEPT data indicated that 23 of the protons are bound to carbon atoms, and comparison of the DEPT results and molecular formula indicated the presence of five hydroxy groups. The ^1H, ^{13}C, and DEPT data for **2** included signals characteristic of two aromatic singlets (thus *para* to each other), 3-methyl-2-butenyl unit, a terminal olefinic unit, and an allylic methyl group. Moreover, a number of NMR signals corresponding to oxygenated methine units and methylene unit, together with a signal characteristic of anomeric carbon, strongly suggested the presence of sugar moiety in the compound.[12] Later, analysis of the 2D NMR data (COSY, HMQC, and HMBC), chemical shifts comparison, and analysis of the *J* values confirmed the presence of a galactopyranosyl group (Table 2).[13] In addition, the coupling

Table 2. 1D and 2D NMR data for **2** in CD_3OD

no	δ_C (mult)[a]	δ_H (int, mult, *J* in Hz)[b]	COSY	HMBC (H → C)
1	151.0 (s)	–	–	–
2	111.8 (s)	–	–	–
3	117.9 (d)	6.70 (1H, s)	–	C-1, C-5, C-7
4	150.1 (s)	–	–	–
5	130.6 (s)	–	–	–
6	118.7 (d)	6.93 (1H, s)	–	C-2, C-4, C-12
7	84.9 (s)	–	–	–
8	93.5 (s)	–	–	–
9	127.4 (s)	–	–	–
10	120.6 (t)	5.33 (1H, m) 5.24 (1H, m)	H-11	C-8, C-9, C-11
11	22.5 (q)	1.94 (3H, br s)	H-10	C-8, C-9, C-10
12	28.2 (t)	3.24 (2H, br d, 6.8)	H-13	C-4, C-5, C-6, C-13, C-14
13	122.0 (d)	5.28 (1H, m)	H-12, H-15, H-16	C-15, C-16
14	132.6 (s)	–	–	–
15	16.7 (q)	1.69 (3H, br s)	H-13	C-13, C-14
16	24.8 (q)	1.73 (3H, br s)	H-13	C-13, C-14
1	102.8 (d)	4.76 (1H, d, 7.6)	H-2	C-1
2	71.4 (d)	3.78 (1H, dd, 9.6, 7.6)	H-1, H-3	C-1, C-3
3	73.8 (d)	3.54 (1H, dd, 9.6, 3.4)	H-2, H-4	C-2
4	68.9 (d)	3.87 (1H, d, 3.4)	H-3	C-2, C-3
5	75.6 (d)	3.57 (1H, br d, 6.0)	H-6	C-4, C-6
6	60.9 (t)	3.71 (1H, dd, 11.6, 6.0) 3.74 (1H, br d, 11.6)	H-5	C-4, C-5

[a]Recorded at 100 MHz. Carbon multiplicities were determined by DEPT experiments. [b]Recorded at 400 MHz.



Compounds 1-2

constant for the anomeric proton H-1' (7.6 Hz) indicated that this moiety is connected to the aglycone moiety in the molecule *via* a β-linkage. The remainder of the structure of the molecule was proposed by analysis of COSY, HMQC, and HMBC data (Table 2). The presence of 3-methyl-2-butenyl unit was indeed confirmed by analysis of 2D NMR data, and the methylene carbon (C-12) in the unit was connected to C-5 on the basis of HMBC correlation of H-12 to C-5 and C-6 as well as H-6 to C-12. Specific assignments of aromatic signals were then obtained by chemical shift considerations and HMBC correlations observed from H-3, H-6, and H-12 (Table 2). At this point, HMBC correlations of H-1' with C-1 provided an evidence for the connection of the galactopyranosyl moiety to C-1 *via* a glycoside linkage. This assignment then indicated that the only remaining free hydroxyl group must be attached to aromatic carbon C-4 to account its chemical shift (δ 150.1). As a next step, the terminal olefinic group (C-9-C-10) was connected to the quaternary carbon C-8 and the methyl group (C-11) on the basis of HMBC cross-peaks from H-10 to C-8, C-9, and C-11. Another remaining quaternary carbon C-7 was connected to C-2 by HMBC correlations of H-3 with C-7. Finally, the formation of acetylenic bond between C-7 and C-8 was made on the basis of corresponding ^{13}C NMR shifts (δ 84.9 and 93.5) and the final degree of unsaturation required by the molecular formula, and this enabled to assign the gross structure of **2**. Compound **2** is structurally close to the known antibiotic culpin, which is a hydroquinone type fungal metabolite isolated from *Preussia* sp.[14]

Experimental Section

General Experimental Procedures. Optical rotation was recorded on JASCO P-1020 polarimeter. UV spectra were recorded on Amersham Ultrospec 3300 UV/Visible spectrophotometer. ESIMS data were obtained at the Korean Basic Science Institute, Taejeon, Korea. NMR spectra (1D and 2D) were recorded in CD_3OD using a JEOL 400 MHz spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to the corresponding residual solvents signals (CD_3OD: δ 3.30/49.0). HMQC and HMBC data were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively. Solvents for extractions and open column chromatography were reagent grade and used without further purification. Solvents used for HPLC were analytical grade. Flash column chromatography was carried out using Aldrich octadecyl-functionalized silica gel (C_{18}). HPLC separations were performed on an Shiseido Capcell Pak C18 column (10 × 250 mm; 5-μm particle size) with a flow rate of 2 mL/min. Compounds were detected by UV absorption at 254 nm.

Characterization and Fermentation of the Organism. A fungal strain of *Paraphaeosphaeria* sp. was isolated from the needles of *Pinus densiflora* collected in Kwanak Mt. using a dilution plating method. The strain was then plated onto a selective medium, yeast-malt extract agar (YMA; yeast-malt extract 3 g L^{-1}, tryptone 5 g L^{-1}, glucose 10 g L^{-1}, agar 15 g L^{-1}, pH 3.7) and incubated at 25 °C. The fungal isolate was identified as a new species of *Paraphaeosphaeria* based on morphology of pycnidia and pycnidiospores, and ITS rDNA sequence data (data not shown). Subcultures of the fungus were deposited at Seoul Nat'l University. Five flasks, each containing 200 mL of potato dextrose broth that had been sterilized at 120 °C for 15 min and then cooled to room temperature, were individually inoculated with 1-cm^2 agar plugs taken from stock cultures of *Paraphaeosphaeria* sp. maintained on potato dextrose agar. Flask cultures were inoculated at 25-28 °C and aerated by agitation on an orbital shaker at 150 ppm for 7 days.

Isolation and Characterization of Compounds 1 and 2. Solvent partitioning of the filtered fermentation broth with EtOAc (5 × 200 mL) provided an organic phase, which was then concentrated using a rotary evaporator to yield 690 mg of crude extract. The crude extract was subjected to C_{18} functionalized silica gel flush column chromatography (3 × 15 cm), eluting with a stepwise gradient of 10%, 20%, 40%, 50%, 60%, 70%, 90%, and 100% (v/v) MeOH in H_2O (200 mL each, 8 fractions). The fraction eluted at 50% MeOH (305 mg) was reapplied to C_{18} functionalized silica gel flush column chromatography, eluting with a stepwise gradient consisting of MeOH in water (30% to 100% MeOH). The fraction (45.8 mg) eluted with 60% MeOH in water was then subjected to semi-preparative reversed-phase HPLC using a gradient from 20 to 48% CH_3CN in H_2O (0.1% formic acid) over 40 min to yield **1** (2.8 mg; V_R = 63.2-65.2 mL) and **2** (3.9 mg; V_R = 67.7-69.0 mL).

2,3-Didehydropalitantin (1): yellow gum; $[\alpha]_D^{25}$ −3.6° (*c* 0.1, MeOH); UV (CH_3OH) 267 (ε 11640); ^{1}H, ^{13}C, and 2D NMR data, Table 1; LRESIMS *m/z* 275 [(M+Na)$^+$; rel int 100)]; HRESIMS *m/z* 275.1264 (M + Na)$^+$ (calc. for $C_{14}H_{20}O_4Na$, 275.1253).

Culpin-1-β-galactopyranoside (2): yellow gum; $[\alpha]_D^{25}$ −27.3° (*c* 0.1, MeOH); UV (CH_3OH) 207 (ε 20808), 255 (9889), 281 (7132), 315 (4944); ^{1}H, ^{13}C, and 2D NMR data, Table 2; LRESIMS *m/z* 427 [(M+Na)$^+$; rel int 100)]; HRESIMS *m/z* 427.1732 (M + Na)$^+$ (calc. for $C_{22}H_{28}O_7Na$, 427.1726).

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