Notes

Hexaketides from Phytopathogenic Fungus *Paraphaeosphaeria recurvifoliae*

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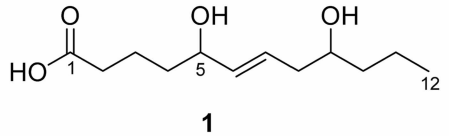
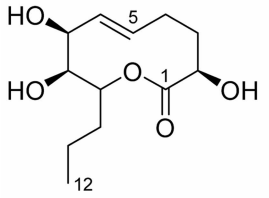
Traditionally, natural products serve as valuable agents in a variety of applications such as agrochemicals, antibiotics, immunosuppressants, antiparasitics, and anticancer agents.[1] Among all known biologically active natural products, approximately a quarter of them are obtained from fungi.[2] These fungal metabolites have been isolated after screening of 5000-7000 species, and the number represents only 5% of the total of 1.5 million fungal species proposed to exist.[3] Thus, the potential of fungi as sources of further new and useful secondary metabolites is significant.

As part of studies exploring the plant-associated fungi as potential sources of new bioactive secondary metabolites, we investigated a new phytopathogenic fungal species *Paraphaeosphaeria recurvifoliae* isolated from the leaf lesions of pendulous yucca (*Yucca recurvifolia*) in a garden located in Daejeon, Korea. Chemical investigation of the EtOAc extract of the filtered culture broth obtained from this fungus has led to the isolation of a novel hexaketide (*E*)-5,9-dihydroxydodec-6-enoic acid (**1**), along with closely related known nonenolide-type metabolites herbarumin II (**2**) and its epimer (**3**). This report describes the production, isolation, and structure elucidation of these compounds.

The crude extract of fungal culture broth was passed through a C-18 flash column and further purified by reversed-phase HPLC to obtain pure compounds **1-3**. Compound **1** has the molecular formula $C_{12}H_{22}O_4$, as deduced from ^{13}C NMR and HRESIMS data. This formula indicated two degrees of unsaturation. The ^{13}C NMR spectrum for compound **1** contained twelve resolved signals, which were deduced to be one methyl, six methylenes, four methines, and one quaternary carbon from the DEPT-135 spectrum. The ^1H NMR spectrum contained a pair of coupled olefinic resonances at δ 5.48 (J = 15.4, 6.6, 1.5 Hz) and δ 5.67 (J = 15.4, 7.3, 0.8 Hz), indicating *trans* geometry of the double bond in **1**. In addition, the presence of a carbonyl group and two oxygenated sp^3 methine groups were evident in the ^1H and ^{13}C NMR spectra, and thus compound **1** must be acyclic compound to account for required unsaturation number. Considering the molecular formula and the NMR data, the three remaining protons must be present as hydroxyl groups, and this consequently indicated the presence of carboxylic acid and two secondary alcohol functionalities in the molecule. An HMQC experiment established the one-bond proton to carbon connectivities, and a COSY experiment established the partially defined spin systems corresponding to C4-C9 and C11-C12 subunits of structure **1**. Overlapping of the remaining methylene NMR signals precluded further specific assignment, but further connectivities of remaining units were extended using HMBC correlations. A methylene group CH_2-10 was connected to CH-9 and CH_2-11 on the basis of HMBC correlations of H_2-8 and H_3-12 to C-9. In the HMBC spectrum, cross peak from oxymethine proton at δ 4.00 (H-5) to the methylene carbon at δ 22.7 (C-3) was observed. Accordingly, CH_2-3 was connected to CH_2-4. The remaining methylene protons (CH_2-2) correlated to C-1 and C-4 in the HMBC spectrum, and this observation, along with HMBC correlation of H_2-3 with C-1, established the location of C-1 and C-2. Finally, the remaining three hydroxyl groups were assigned to two secondary alcohols (C-5 and C-9) and carboxylic acid moiety (C-1) to complete the assignment of the structure **1**. Thus, the gross structure of **1** was assigned as



1



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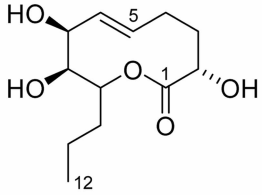

3

(*E*)-5,9-dihydroxydodec-6-enoic acid.

The signal systems of ^{1}H and ^{13}C NMR data for compound **2** resembled those observed for compound **1**, suggesting structural similarity between **1** and **2**. The signals for two aliphatic methylene units in **1** were replaced by the signals for two new oxygenated methine units in the spectra of **2**. Another evident difference was that one of the oxymethine signals (δ 5.14) in **1** was shifted downfield by 1.57 ppm relative to that of compound **1**, and this suggested that the oxygenated methine was involved in an ester linkage to carbonyl carbon resonated at δ 175.5. The molecular formula for **2** was established as $C_{12}H_{20}O_5$ on the basis of LRESIMS and NMR data. Ultimately, taking into account these data, the structure of **2** was identified as herbarumin II by comparison of the NMR data to literature values.[4a] Herbarumin II (**2**) has been isolated from a culture broth of the fungus *Phoma herbarum* with phytotoxic effect possibly by inhibiting calmodulin-dependent cyclic nucleotide phosphodiesterase (cAMP).[4]

The NMR data for compound **3** were very similar to those of **2** except for the difference in chemical shift values for H-2 (δ 3.84 *vs.* 4.24, Table 2). LRESIMS and NMR data revealed that compound **3** has the same molecular formula as that of **2**. These data suggested a diastereomeric relationship between **2** and **3**. Once the stereochemistry of herbarumin II (**2**) was readily assigned by comparison of $[\alpha]_D$ values, chemical shifts, and *J* values to literature values,[4a] the stereochemistry of **3** was proposed by comparison of the chemical shifts and *J* values with those of **2**. Especially, the $J_{H2\text{-}H3}$ couplings in compound **2** (10.6 Hz and 3.3 Hz) and **3** (5.8 Hz and 1.8 Hz) were markedly different. Considering established conformation of nonenolide, this observation suggested pseudoaxial disposition of the hydroxyl group at C-2 in structure **3** in contrast to pseudoequatorial disposition in structure **2**. Thus the structure of compound **3** was assigned as C-2 epimer of **2**. The C-2 epimer of herbarumin II (**3**) has been prepared in the course of the synthesis of pinolidoxin, which is a phytotoxic nonenolide with pseudoaxially oriented 2-*O*-hexadienoyl group.[4a] Although direct comparison of the NMR data with those for the synthetic material was impossible due to the absence of spectroscopic data for the synthetic material, comparison of the coupling constants at the stereogenic centers at C-2, C-7, C-8, and C-9 in **3** with those of pinolidoxin was fully consistent with the proposed stereochemistry as shown in **3**. To our best knowledge, this is the first case to report the isolation of C-2 epimer of herbarumin II from natural sources.

All three compounds were tested for inhibitory activity against murine tyrosinase partially purified from melanoma B-16 cells at a concentration of 1.3 mg/mL, and compounds **1-3** showed weak activity displaying inhibitory percentage of 30%, 17%, and 12%, respectively.[5] Kojic acid was used as a positive control, and showed inhibitory percentage of 85% at the same concentration.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin Elmer 341 digital polarimeter. ESIMS data were obtained at the Korean Basic Science Institute, Daejeon, Korea. NMR spectra (1D and 2D) were recorded in CD_3OD using a JEOL JNM ECP-400 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 experiments 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

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	179.1 (s)	–	–	–
2	36.1 (t)	2.25 (2H, t, 7.3)	–[c]	C-1, C-3, C-4
3	22.7 (t)	1.65 (1H, m) 1.57 (1H, m)	–[c]	C-1, C-4
4	37.9 (t)	1.51 (1H, m) 1.47 (1H, m)	–[c]	C-5, C-6
5[c]	73.3 (d)	4.00 (1H, dd, 13.2, 6.6)	H-4, H-6	C-3, C-4, C-6, C-7
6[c]	136.6 (d)	5.48 (1H, ddt, 15.4, 6.6, 1.5)	H-5, H-7	C-5, C-7, C-8
7	128.8 (d)	5.67 (1H, dtd, 15.4, 7.3, 0.8)	H-6, H-8	C-6, C-8, C-13
8	41.3 (t)	2.18 (2H, dd, 14.3, 7.3)	H-7, H-9	C-6, C-7, C-9, C-10
9	72.0 (d)	3.58 (1H, m)	H-8	C-11
10	40.0 (t)	1.44 (1H, m) 1.36 (1H, m)	–[c]	–[c]
11	19.9 (t)	1.47 (1H, m) 1.34 (1H, m)	–[c]	C-10
12	14.4 (q)	0.92 (1H, t, 6.6)	H-11	C-10, C-11

[a]Recorded at 100 MHz. Carbon multiplicities were determined by DEPT experiments. [b]Recorded at 400 MHz. [c]Correlations were ambiguous due to signal overlapping.

Table 2. ^{1}H and ^{13}C NMR data for **2** and **3** in CD_3OD

no	2		3	
	δ_C (mult)[a]	δ_H (int, mult, J in Hz)[b]	δ_C (mult)[a]	δ_H (int, mult, J in Hz)[b]
1	176.9 (s)	–	176.5 (s)	–
2	73.6 (t)	3.84 (1H, dd, 10.6, 3.3)	70.7 (t)	4.24 (1H, dd, 5.8, 1.8)
3	34.0 (t)	1.89 (1H, m)	33.2 (t)	1.95 (1H, m)
		1.79 (1H, m)		1.85 (1H, m)
4	29.7 (t)	2.29 (1H, dm, 13.6)	27.7 (t)	2.42 (1H, dm, 13.6)
		2.09 (1H, m)		2.05 (1H, m)
5	123.5 (d)	5.49 (1H, dddd, 15.8, 9.2, 3.7, 1.5)	123.6 (d)	5.48 (1H, dddd, 15.4, 11.4, 4.0, 2.2)
6	134.1 (d)	5.56 (1H, br dd, 15.8, 2.2)	135.0 (d)	5.71 (1H, br d, 15.4)
7	73.9 (d)	4.34 (1H, quint, 1.8)	74.2 (d)	4.39 (1H, quint, 2.2)
8	74.1 (d)	3.51 (1H, dd, 9.9, 2.2)	74.1 (d)	3.58 (1H, dd, 9.5, 2.6)
9	71.9 (d)	5.15 (1H, td, 9.2, 2.6)	72.5 (d)	5.22 (1H, td, 9.2, 2.6)
10	35.2 (t)	1.82 (1H, m)	35.1 (t)	1.85 (1H, m)
		1.52 (1H, m)		1.55 (1H, m)
11	18.6 (t)	1.41 (1H, m)	19.0 (t)	1.37 (1H, m)
		1.31 (1H, m)		1.28 (1H, m)
12	14.4 (q)	0.92 (1H, t, 7.3)	14.5 (q)	0.91 (1H, t, 7.3)

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

Aldrich octadecyl-functionalized silica gel (C_{18}). HPLC separations were performed on a 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 210 nm.

Characterization and Fermentation of the Organism. A fungal strain, EML-PL001 was isolated from the leaf spots and necrosis lesion of pendulous yucca (*Yucca recurvifolia*) collected in a house garden of Korea. The strain was then plated onto a selective medium, yeast-malt extract agar (YMA; yeast-malt extract 3 g/L, tryptone 5 g/L, glucose 10 g/L, agar 15 g/L, pH 3.7) and incubated at 25 °C. The fungal isolate has been recently identified as a new species of *Paraphaeosphaeria recurvifoliae* based on the morphology of pycnidia and pycnidiospores, and ITS rDNA sequence data.[6] Subcultures of the fungus were deposited at Environmental Microbiology Lab, Chonnam Natl' 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 the fungus 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 14 days.

Isolation and Characterization of Compounds 1-3. 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 1.1 g of crude extract. The crude extract was subjected to C_{18} functionalized silica gel flash column chromatography (5 × 15 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (300 mL each, 5 fractions). The fraction eluted at 60% MeOH (232 mg) was reapplied to silica gel flash column chromatography, eluting with a stepwise gradient consisting of CH_2Cl_2 in MeOH (5% 600 mL, 10% 600 mL, and 100% MeOH 200 mL). Altogether 58 fractions were collected (25 mL each), and pooled based on TLC behavior to afford 9 major fractions. Fraction (57.1 mg) eluted with 100% MeOH (between 1325 mL and 1400 mL) was then fractionated through a Sephadex LH-20 column (2.5 × 30 cm) using a 20% MeOH in acetone, collecting a total of 20 fractions (20 mL each). Fractions were pooled by TLC profile to afford compound **1** (10.3 mg, fractions 6 to 11). Fraction (65.5 mg) eluted with 10% CH_2Cl_2 in MeOH (between 1325 mL to 1400 mL) from silica gel column was processed by semi-preparative reversed-phase HPLC using a gradient from 10 to 30% CH_3CN in H_2O (0.1% formic acid) over 50 min to yield **2** (13.5 mg; V_R = 68-72 mL) and **3** (11.0 mg; V_R = 52-56 mL).

(*E*)-5,9-Dihydroxydodec-6-enoic Acid (1): white oil; $[\alpha]_D^{25}$ + 3 (*c* 0.5, MeOH); ^{1}H, ^{13}C, and 2D NMR data, Table 1; HRESIMS *m/z* 253.1405 $(M + Na)^+$ (calc. for $C_{12}H_{22}O_4Na$, 253.1416). Copies of the original spectra are obtainable from the author of correspondence.

Herbarumin II (2): white solid; $[\alpha]_D^{25}$ +27 (*c* 0.45, MeOH); ^{1}H, ^{13}C, and 2D NMR data, Table 2; LRESIMS *m/z* 243 $(M-H)^-$.

C-2 Epimer of Herbarumin II (3): yellow gum; $[\alpha]_D^{25}$ + 15 (*c* 0.66, MeOH); ^{1}H, ^{13}C, and 2D NMR data, Table 2; LRESIMS *m/z* 243 $(M-H)^-$.

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